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Sulfated fucan as support for antibiotic immobilization

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Abstract

Xylofucoglucuronan from *Spatoglossum schröederi* algae was tested as a support for antibiotic immobilization. The polysaccharide (20 mg in 6 ml) was first activated using carbodiimide, 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide methiodide (20 mg in 2 ml), under stirring for 1 h at 25ºC and pH from 4.5 to 5.0. After adjusting the pH to 8.0, either gentamicin or amikacin (62.5 mg in 1.25 ml) was then immobilized on this chemically modified polysaccharide with shaking for 24 h in a cold room. Infrared spectra of the activated carbodiimide xylofucoglucuronan showed two bands to carbonyl (C = O at 1647.9 and 1700.7 cm^{-1}) and to amide ($C^{\Uparrow}$-NH_2) groups (1662.8 and 1714.0 cm^{-1}). Microbial characterization of the derivatives was carried out by the disk diffusion method using *Staphylococcus aureus* or *Klebsiella pneumoniae* incorporated in Müller Hinton medium. Inhibition halos of bacterial growth were observed for the antibiotics immobilized on this sulfated heteropolysaccharide before and after dialysis. However, the halos resulting from the samples after dialysis were much smaller, suggesting that dialysis removed either non-covalently bound antibiotic or other small molecules. In contrast, bacterial growth was not inhibited by either xylofucoglucuronan or its activated form or by gentamicin or amikacin after dialysis. An additional experiment was carried out which demonstrated that the sulfated heteropolysaccharide was hydrolyzed by the microorganism. Therefore, the antibiotic immobilized on xylofucoglucuronan can be proposed as a controlled drug delivery system. Furthermore, this sulfated heteropolysaccharide can be extracted easily from sea algae *Spatoglossum schröederi*.

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- Gentamicin
- Amikacin

Fucans are sulfated polysaccharides present in *Phaeophyceae* algae and L-fucose is the main sugar in these polymers (1,2). A sulfated heteropolysaccharide from the marine algae *Spatoglossum schröederi* showed a molecular mass of 19 kDa determined by high performance liquid chromatography (3). This polymer was characterized by acid and enzymatic hydrolysis, infrared spectroscopy, and nuclear magnetic resonance. Its structure was proposed as a xylofucoglucuronan, with the central core of the polymer consisting of polyglucuronate with ß-(1→4) linkage, lateral chains of sulfated fucose with α-(1→2), α-(1→3), and xylose ß-(1→4). There has been increasing interest in sulfated fucans

because they present several pharmacological properties such as anticoagulant (4-7), anti-tumor (8-10), anti-viral (11), and anti-adhesive (12) activities.

Xylofucoglucuronan from *S. schröederi* algae is proposed here as a support for antibiotic immobilization. This polymer contains functional groups available to bind biologically active molecules. Also it shows characteristics similar to those of hyaluronic acid that has already been studied as a drug carrier (13). Gentamicin and amikacin and *Staphylococcus aureus* and *Klebsiella pneumoniae* were used as antibiotic and microorganism models, respectively. This class of antibiotics has free NH_2 groups that can be covalently linked to the xylofucoglucuronan molecule.

The *S. schröederi* xylofucoglucuronan (20 mg) was kindly provided by Dr. Edda Leite, Departamento de Bioquímica, Universidade Federal do Rio Grande do Norte, Natal, RN, Brazil, and its chemical modification was monitored by its infrared spectrum using a Bruker IFF66 spectrophotometer (Karlsruhe, Baden-Wurttenberg, Germany) from 4000 to 400 cm^{-1}. A small band at 2075.6 cm^{-1} was found which corresponds to the N = C = N groups typical of carbodiimide. Two bands were also observed, a strong one at 1847.9 cm^{-1} and a slight one at 1700.7 cm^{-1} which correspond to the C = O, possibly from ester groups, suggesting the carboxyl group activation of the xylofucoglucuronan. The gentamicin spectrum presented a band at 3417.3 cm^{-1} relative to either the OH or NH_2 and another stronger band at 1124.3 cm^{-1} typical of C-N. The infrared spectrum of gentamicin immobilized on xylofucoglucuronan before dialysis was similar to the spectrum of just the antibiotic. However, the derivative spectrum obtained after dialysis presented two small bands, 1662.8 and 1714.0 cm^{-1}, that suggest amide linkage resulting from the attachment of the antibiotic to the polymer.

The antibiotics amikacin sulfate (Novamin) and gentamicin sulfate were purchased from Bristol-Myers Squibb, São Paulo, SP, Brazil, and Paisley, Scotland, respectively. Carbodiimide, 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide methiodide, was purchased from Sigma, St. Louis, MO, USA. All other reagents were of analytical grade.

Xylofucoglucuronan (20 mg) was dissolved in 6 ml distilled water and the pH was adjusted to 4.7 with 0.1 M hydrochloric acid. The carbodiimide (10 mg/ml - 2 ml) was added under stirring for 1 h with pH control from 4.5 to 5.0. The pH was then adjusted to 8.0 with 0.1 M sodium hydroxide. An antibiotic solution (50 mg/ml - 1.25 ml) was added to the xylofucoglucuronan-carbodiimide activated solution and the mixture was maintained with shaking for 24 h in a cold room. After immobilization, the derivatives obtained were dialyzed for 24 h against water, with water changes every 4 h to remove excess antibiotic and other undesirable unbound compounds which remained after xylofucoglucuronan activation.

The bacterial sensitivity assay used was disc diffusion according to the method of Bauer et al. (14). *S. aureus* DAUFPE 01 and *K. pneumoniae* DAUFPE 396 were from the culture collection of the Departamento de Antibióticos, Universidade Federal de Pernambuco, Recife, PE, Brazil.

The sensitivity of *K. pneumoniae* to the derivatives obtained from gentamicin immobilized on xylofucoglucuronan is shown in Table 1. Standard gentamicin showed an inhibition halo 23 mm in diameter, whereas dialyzed gentamicin did not present an inhibition halo, suggesting that the antibiotic was removed during dialysis. Gentamicin immobilized on xylofucoglucuronan presented an inhibition halo 19 mm in diameter (82.6%) before dialysis and 12 mm in diameter (52.2%) after dialysis, showing that a reduction of about 30% in the microorganism growth inhibition occurred after dialysis. This means that either all the antibiotic

was not bound to xylofucoglucuronan or small derivative molecules were removed during dialysis. Xylofucoglucuronan and activated xylofucoglucuronan did not present a halo of microorganism growth inhibition. The sensitivity of *S. aureus* to the derivatives obtained from gentamicin immobilization on xylofucoglucuronan is also shown in Table 1. Gentamicin presented an inhibition halo 27 mm in diameter and dialyzed gentamicin did not show any inhibition halo, showing once again that gentamicin was removed by dialysis. The effect of gentamicin immobilized on xylofucoglucuronan observed from the results of the sensitivity assay for *S. aureus* differed little from that recorded for *K. pneumoniae*. The inhibition halo obtained with the antibiotic immobilized before dialysis was 25 mm in diameter (92.6% inhibition) and was then reduced to 18 mm in diameter (66. 7% inhibition), showing a 26% reduction of microorganism growth inhibition after derivative dialysis. This reduction could be attributed to the fact that the antibiotic was not bound to the polysaccharide or to the removal of small derivative molecules after dialysis. Also, xylofucoglucuronan and activated xylofucoglucuronan did not present inhibition of microorganism growth.

The assay of bacterial sensitivity to amikacin immobilized on xylofucoglucuronan is shown in Table 2. First, standard amikacin presented a halo of *K. pneumoniae* inhibition measuring 29 mm in diameter. The dialyzed amikacin sample did not present an inhibition halo. Therefore, this result also suggests that the antibiotic not immobilized on the polymer was completely removed during the dialysis process. Amikacin immobilized on xylofucoglucuronan presented an inhibition halo 29 mm in diameter before dialysis (100% inhibition). On the other hand, amikacin immobilized on the polymer presented an inhibition halo 17 mm in diameter after dialysis, that corresponded to 58.6% inhibition. These data also show that there was a 41.4% reduction in microorganism growth inhibition, which suggests that the unbound antibiotic and/or the small derivative molecules were removed by dialysis. The assay of *S. aureus* sensitivity to standard amikacin (Table 2) showed a microorganism growth inhibition halo measuring 22 mm in diameter, whereas dialyzed amikacin did not show any inhibition halo. Furthermore, amikacin immobilized on xylofucoglucuronan presented an inhibition halo 22 mm in diameter before dialysis (100% inhibition), which was reduced to 17 mm in diameter (77.3%

Table 1. Sensitivity of *Klebsiella pneumoniae* and *Staphylococcus aureus* to gentamicin immobilized on xylofucoglucuronan.

Preparation	*Klebsiella pneumoniae*		*Staphylococcus aureus*	
	Inhibition halo diameter (mm)	Inhibition (%)	Inhibition halo diameter (mm)	Inhibition (%)
Gentamicin[1]	23	100	27	100
Dialyzed gentamicin[1]	0	0	0	0
Xylofucoglucuronan[2]	0	0	0	0
Xylofucoglucuronan activated with carbodiimide[2]	0	0	0	0
Gentamicin immobilized on xylofucoglucuronan before dialysis[1]	19	82.6	25	92.6
Gentamicin immobilized on xylofucoglucuronan after dialysis[3]	12	52.2	18	66.7

[1]Ten microliters gentamicin (100 µg) was applied to the disk. [2]Thirty microliters xylofucoglucuronan (100 µg) was applied to the disk. [3]The amount of linked antibiotic was not determined.

Table 2. Sensitivity of *Klebsiella pneumoniae and Staphylococcus aureus* to amikacin immobilized on xylofucoglucuronan.

Preparation	*Klebsiella pneumoniae*		*Staphylococcus aureus*	
	Inhibition halo diameter (mm)	Inhibition (%)	Inhibition halo diameter (mm)	Inhibition (%)
Amikacin[1]	29	100	22	100
Dialyzed amikacin[1]	0	0	0	0
Fucan[2]	0	0	0	0
Xylofucoglucuronan activated with carbodiimide[2]	0	0	0	0
Amikacin immobilized on xylofucoglucuronan before dialysis[1]	29	100	22	100
Amikacin immobilized on xylofucoglucuronan after dialysis[3]	17	58.6	17	77.3

[1]Ten microliters gentamicin (100 µg) was applied to the disk. [2]Thirty microliters xylofucoglucuronan (100 µg) was applied to the disk. [3]The amount of linked antibiotic was not determined.

inhibition) after dialysis, suggesting that there was a 22.7% reduction of microorganism growth inhibition by the derivative after dialysis. Finally, xylofucoglucuronan and activated xylofucoglucuronan standards did not present a halo of *S. aureus* growth inhibition.

An additional experiment was performed to demonstrate that the microorganism could hydrolyze this sulfated heteropolysaccharide. Thus, xylofucoglucuronan (50 mg) was incubated with an *S. aureus* suspension (5 ml containing 6 x 10^9 cells/ml) in the fermentation medium and the products released were monitored by the method of Miller (15). An increase in reducing power (0.2 x 10^{-3} absorbance/min) was observed, as opposed to no increase for the control. Therefore, one may assume that xylofucoglucuronan is hydrolyzed by extracellular hydrolases produced by the microorganism.

On the basis of the results shown here we conclude that xylofucoglucuronan from *S. schröederi* algae can be used as a water-soluble support for gentamicin and amikacin immobilization and that these derivatives can be proposed for controlled drug release systems.

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