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3'- Sialyllactose-decorated Silver nanoparticles: lectin binding and bactericidal properties

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ABSTRACT

Simple technique of preparation of sialolectin-specific nanosilver-labeled colloidal neoglycoconjugates, based on maleic anhydride copolymers and β-N-glycyl-3'-sialyllactose (SL-AgNPs), was developed. Their physicochemical properties were investigated using TEM, UV-vis-, and ^{1}H-NMR spectroscopy. Interaction of SL-AgNPs with sialic acid specific lectin from *Maakia amurensis* was studied. Minimum inhibitory concentrations of SL-AgNPs for *Staphylococcus aureus* ATCC 29213 and *Enterococcus faecalis* ATCC 29212 were also determined. For poly(ethylene-*alt*-maleic anhydride) based nanocomposite, an additive influence of nanosilver and 3'-sialyllactose on antibacterial activity against *Enterococcus faecalis* was found.

Key words: *antibacterial activity, lectins, silver nanoparticles, 3'-sialyllactose, copolymers of maleic acid.*

1. INTRODUCTION

Owing to their antiviral, fungicidal and antibacterial properties [1, 2], silver nanoparticles (AgNPs) are extensively used for the production of different consumer goods, and especially for medical purposes. AgNPs were shown to increase the efficacy of antibiotics, inhibit HIV and other viruses, and exhibit wound healing activity [1]. AgNPs are incorporated into implants, catheters, wound dressings, and other medicinal items [3].

Recently, we have reported the preparation of AgNPs stabilized with maleic acid copolymers, and demonstrated their antibacterial properties. Colloidal solutions of these AgNPs were shown to possess strong antifungal activity against yeasts, and antimicrobial activity against a number of Gram-negative and Gram-positive bacteria [4].

Glycocalix – the carbohydrate coat covering cell surfaces of animal cells, containing the repertoire of glycoproteins, glycolipids and proteoglycans is an essential target for microorganisms. Most bacteria, fungi, and viruses contain lectins, or adhesins, – carbohydrate binding proteins which provide an interaction with cellular glycans, thus enabling easier host colonization. Sialic acids, members of 9-carbon backbone monosaccharide family called nonulosonic acids, found mostly on a terminal, nonreducing position of glycans, are an important target for a number of pathogens, including *Staphylococcus aureus*, *Parainfluenza virus 5*, and many others [5]. The ampicillin-loaded glyco-NPs were found to induce aggregation of *Staphylococcus aureus* and *Escherichia coli*, and resulted in antibacterial activity [6]. 3'-Sialyllactose, important bacteriostatic component of mammalian milk, modulates the structure of cellular glycans, thus being a convenient model for pathogen adhesion studies [7]. It was found that some glycoconjugates are potential receptors for enteropathogenic *Eschirichia coli*, and may inhibit their attachment to eukaryotic cells [8]. S-Linked sialyloligosaccharides bearing liposomes and micelles were also shown to be influenza virus inhibitors [9].

In this paper a new type of AgNPs, decorated with 3'-sialyllactose bound to maleic acid copolymer, was described. Antibacterial properties of the resulted SL-AgNPs were studied.

2. EXPERIMENTAL SECTION

2.1. Materials. Poly(ethylene-*alt*-maleic anhidride) (EM) with an average molecular weight M=25000 was purchased from Monsanto (USA), poly(N-vinyl-pyrrolidone-*alt*-maleic anhidride) (VM, M=40000) was prepared earlier [10]. $AgNO_3$, $NaBH_4$, and *Maackia amurensis lectin* (MAA) were Sigma-Aldrich products. β-N-Glycyl-3'-sialyllactose (Gly-3'-SL) was synthesized earlier [11]. All other reagents were of analytical grade and used without further purification. Milli-Q-purified water was used.

2.2. Instrumentation. pH values were determined using Fisher Scientific 300 403.1 pH-meter (USA). UV-visible absorption spectra were obtained on UVIKON-922 spectrophotometer (Germany). Transmission electron microscopy (TEM) micrographs were performed with LEO 912 AB microscope (Omega, Karl Zeiss; Germany) operated at an accelerating voltage 100 kV. For TEM observations, a drop of colloid solution was placed onto Formvar-coated copper grid, and then evaporated. The particle size distribution was obtained from a count of 200-300 individual particles. ^{1}H NMR spectra at 500 MHz were recorded in D_2O on a Bruker DRX500 SF=500.13 MHz instrument at T=299K. IR spectra (KBr) were recorded on Fourier- spectrometer Magna IR-720 (Nicolet, USA).

2.3. Methods.

2.3.1. Synthesis of neoglycoconjugates (GCs) and glyconanoparticles (GNPs).

2.3.1.1. Synthesis of GCs, general procedure. For activation of the polymer-stabilizer EM (VM), a powdered sample was heated

over P_2O_5 (3 h, 110^{0}C) *in vacuo*. To the activated polymer (0.17 g in 3 mL DMF), N-Gly-3'-SL (0.16 g in 10 mL H_2O), and 2 mL of 0.1 M $NaHCO_3$ were added with vigorous stirring. After stirring (24 h, ~20^{0}C), the solution was threefold diluted with water (20 mL), concentrated by ultrafiltration, and freeze-dried. Degree of substitution (elemental analysis, or NMR) was 15% (for EM-Gly-3'-SL**),** or 14% (for VM-Gly-3'-SL) (weight), respectively.

2.3.1.2. Synthesis of GNPs (EM-Gly-3'-SL/Ag^0 and VM-Gly-3'-SL/Ag^0). Colloidal solutions of nano-sized silver GNPs were obtained by the borohydride reduction of the metal salt at ~20^{0}C in the presence of polymeric neoglycoconjugates (EM-Gly-3'-SL and VM-Gly-3'-SL) [12]. Breafly, a proper amount (for the required ratio of reagents) of freshly prepared solution of $AgNO_3$ (0.1 M), was added to the freshly prepared solution of GC (0.01M, here, the molar concentration of copolymer refers to the monomeric maleic acid units) in water at pH 7 (titration with 5% aqueous NaOH) with vigorous stirring. After 5-10 min, a freshly prepared aqueous solution of $NaBH_4$ (0.1 M, 2-fold molar excess with respect to silver ions) was added to the polymeric salt with vigorous stirring. The reaction mixture was allowed to stand (24 h,~20^{0}C). Dried samples of polymeric GNPs were obtained after ultrafiltration and freeze-drying.

2.3.2. In vitro interaction of GNPs with MAA. Optical spectra of GNPs were registered at concentration 0.1 mg/mL (0.01M PBS, pH 6.5). Spectra changes were registered after addition of 5-40 μL of the lectin (2 mg/mL, 0.02M TBS, pH 7.4, 25^{o}C).

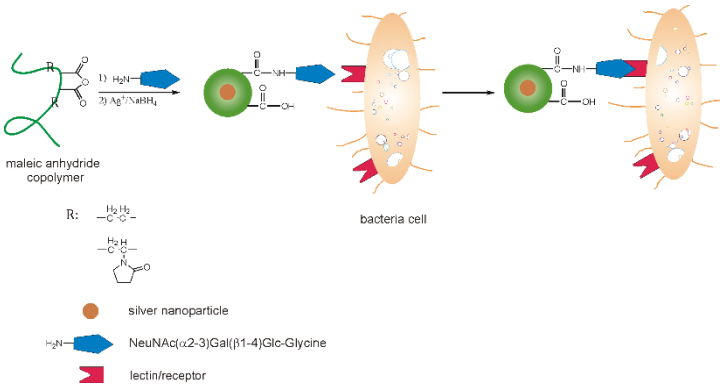
2.3.3. Determination of the sensitivity of microorganisms to GNPs. Determination of the minimum inhibitory concentration (MIC) of the GNPs, against to the strains studied, was carried out by serial microdilution method in nutritive broth on a plate according to a standard procedure ("Methods for Dilution Antimicrobial Susceptibility Tests for Bacteria that Grow Aerobically, Approved Standard", 9th ed., CLSI document M07-A9. Clinical and Laboratory Standards Institute, 950 West Valley Road, Suite 2500, Wayne, Pennsylvania 19087, USA, 2012.]). The test was carried out in 0.2 ml volume using a sterile 96-well plate for immunological studies.

The final concentration of the investigated microorganism was 10^5 CFU/mL. Trypticase-soy broth (0.1 mL) was poured into each well of the plate. The number of wells was determined by the necessary dilution range, the last well was left for setting negative control.

The maximum concentration of silver nanoparticles working solution was 512 μg/mL. 0.1 mL of the working solution was pipetted into the first well containing 0.1 mL of broth. After mixing, 0.1 mL of the mixture was pipetted to the second well, initially containing 0.1 mL of broth. The procedure was repeated until a sufficient number of dilutions were made. For inoculation, a microbial suspension of test microorganisms prepared on a nutrient broth was used. 0.1 mL of the inoculum was introduced into a well containing 0.1 mL of the appropriate dilution of the GNPs preparation, as well as in the last well with a nutrient broth without the test preparation (positive control). Taking into account the dilutions performed and the inoculum added, the range of concentrations studied was from 1 to 512 μg/mL. The plates covered with a sterile film were incubated at 37° C for 24 hours. The culture growth in the presence of GNPs was compared with the one in a reference cell without GNP (positive control) under visual control (wells in plates were viewed in transmitted light). The minimum inhibitory concentration was determined as a lowest concentration of GNPs in the well suppressing the growth of the microorganism tested.

3. RESULTS SECTION

Glyconanoparticles (SL-AgNPs), based on β-N-glycyl-3'-sialyllactose and maleic acid copolymer stabilized silver nanoparticles, were prepared. We investigated their physicochemical properties, and evaluated their bactericidal potential. Colloidal GNPs were prepared in two stages: i) synthesis of polymeric neoglycoconjugates (GCs), and ii) nano-metal introduction into GCs (Scheme 1).



Scheme 1. Preparation of sialyllactose-polymer conjugates containing silver nanoparticles for the investigation of antibacterial activity.

The main advantages of maleic anhydride copolymers are as follows:

- structural feature (strict alternation of co-monomer units),
- commercial availability or, at least, possibility of synthesis according to known procedures,
- ready regulation of hydrophobic-hydrophilic balance inside the family of maleic anhydride copolymers,
- simple preparation of polymer derivatives,
- solubility in water in form of maleic acid form,
- nontoxicity.

Maleic anhydride groups of the copolymers easily reacted in water with primary glycine amino group of N-Gly-3'-sialyllactose in one stage via formation of amide bond between glycyl-spacered carbohydrate and copolymer of maleic anhydride, without any condensing agents. Resulted GCs contained 14-15% (mol) of carbohydrates, and the glycan epitopic density was about 1 carbohydrate unit per 10-11 maleic anhydride residues of the copolymer. As maleic anhydride copolymers degrade into maleic acid copolymers upon storage, thermal activation was used before the introduction of the glycosynthon.

Figure 1, *1* demonstrates the IR spectrum of EM. It is characterized by the presence of the stretching vibrations of the carbonyl of the ionized carboxyl groups ν C=O 1559, 1395 cm^{-1}.

Thermal treatment of the stabilized copolymer (Figure 1, *2*) led to the appearance in the spectrum of vibration bands at 1779 and 1710 cm^{-1} corresponding to the cyclic structure of succinic anhydride. Characteristic vibrations of the amide bond at 1642 cm^{-1} (amide I), 1569 and 1403 cm^{-1} (amide II) were found in the spectrum of the GNPs (Figure 1,*3*).

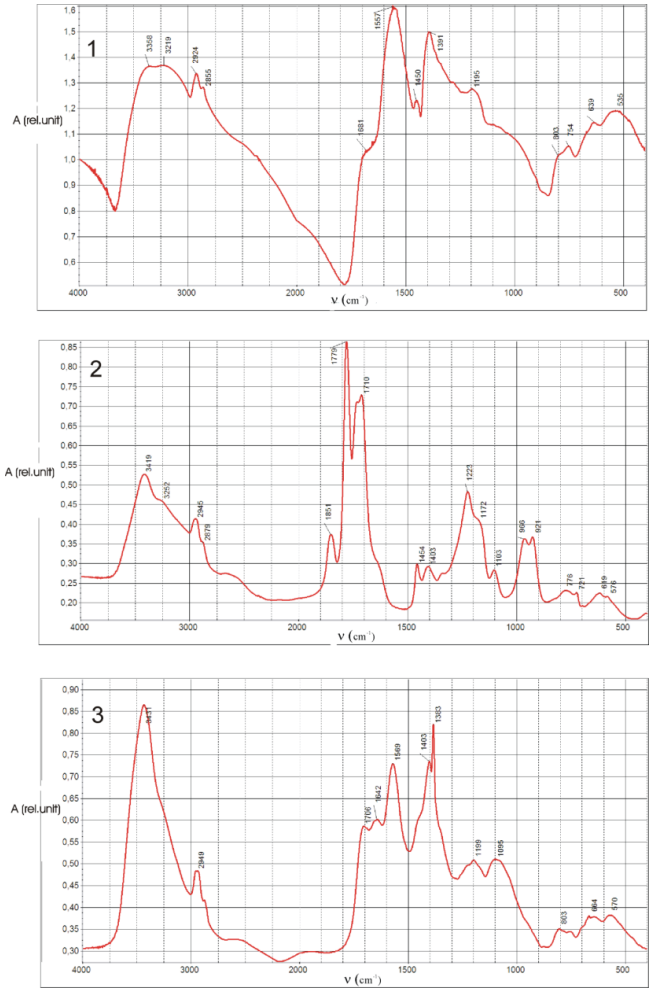


Figure 1. The IR spectra of the EM before (1) and after (2) thermal influence, as well as that of the EM-Gly-SL (3)

^{1}H-NMR spectrum of the carbohydrate ligand – N-Gly-3'-sialyllactose was considered in detail earlier [11]. ^{1}H-NMR spectrum of neoglycoconjugate EM-Gly-SL (covalent complex of poly(ethylene-*alt*-maleic acid)/NeuNAc(α2-3)Gal(β1-4)Glc-Gly) is presented in Figure 2.

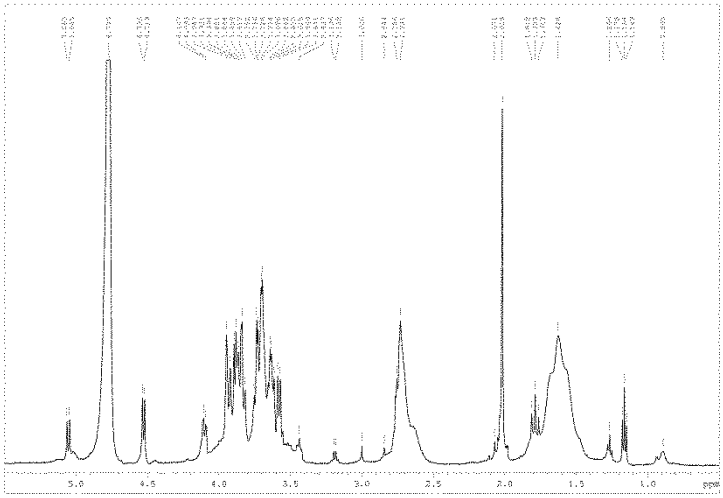


Figure 2. ^{1}H NMR spectrum of EM-Gly-SL

Chemical shifts (δ_H, ppm): 2.73 (m, w, H (methine protons of maleic acid)), 1.63 (m, w, H (ethylene protons of polymer chain)), 0.89 (w, H from CH_3 of polymer chain). Chemical shifts of carbohydrate ligand – 1.79 (t, 1 H, H (3) Neu5Ac, 2.02 (s, 3 H, NAc); 3.44 (br.t, 1 H, H(2) Glc, 3.57—3.95 (m, 17 H); 4.09 (dd,1 H, H(3) Gal; 4.52 (d, 1 H, H(1) Gal); 5.04 (d, 1 H, H(1) Glc).

Silver nanoparticles were prepared by reduction of the silver salt with $NaBH_4$ in the presence of copolymers of maleic acid: poly(*N*-vinyl-2-pyrrolidone-*alt*-maleic acid) and poly(ethylene-*alt*-maleic acid) containing 3'-sialyllactose ligands, and were named VM-Gly-SL/Ag^0 and EM-Gly-SL/Ag^0, respectively. The morphology of the particles obtained: spherical nanoparticles 1-3 nm (transmission electron microscopy data – Figure 3)

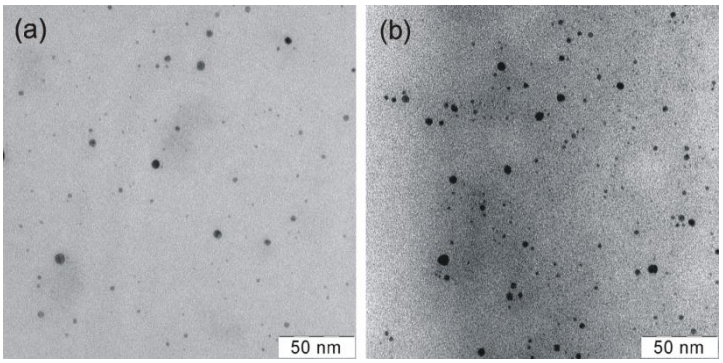


Figure 3. The TEM images of silver nanoparticles in the samples:EM-Gly-SL/Ag^0 (a) and VM-Gly-SL/Ag^0 (b)

Characteristic optical spectra of the colloids prepared had absorption band centered at 400-410 nm due to the surface plasmon resonance of silver NPs (Figure 4).

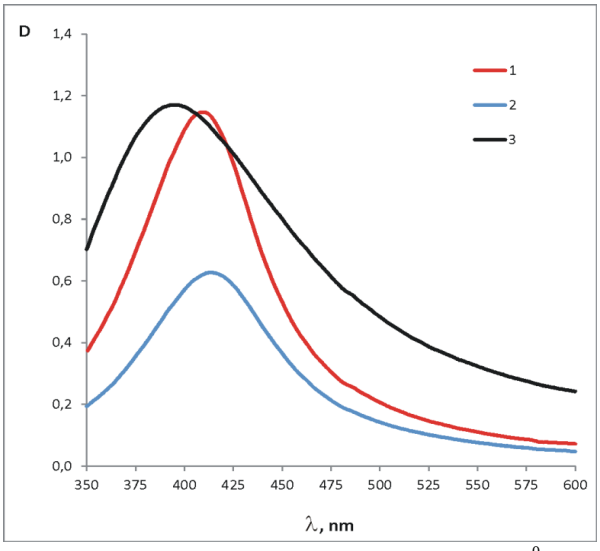


Figure 4. Absorption spectra of the samples: EM-Gly-SL/Ag^0 (1),VM-Gly-SL/Ag^0(2), and VM-Gly-SL/Ag^0 after addition of 50 μL lectin (3)

Interaction of 3'-sialic acid specific lectin (MAA) with SL-AgNPs was monitored using UV-visible absorbance measurements, exploiting unique optical properties of AgNPs, and their sensitivity to polymeric shell environment. Appearance of the specific complex upon binding of the lectin with SL-AgNPs caused changes in optical properties of the sols, and can be illustrated by changes of the spectra of the initial metal-containing GNPs (Figure 4, *2*,*3*). Red shift of the spectra maximum occurred. Absorbance spectra were recorded 24h after mixing of metal-containing GNPs with the lectin. Concentration of the SL-AgNPs

used was measured in accordance with an extinction coefficient of AgNPs (1.1 x 10^4 $dm^3mol^{-1}cm^{-1}$). The shift was revealed for VM-Gly-SL/Ag^0–MAA pair (Figure 4, *2,3*). For EM-Gly-SL/Ag^0–MAA pair, the shift was less pronounced, comparing to VM-Gly-SL/Ag^0–MAA. Most probably, in case of poly(N-vinyl-pyrrolidone-*alt*-maleic acid) as glyconanoparticles shell, N-vinyl-pyrrolidone residues may enhance binding through hydrogen bonds formation of GNPs with the protein. Relatively insignificant shift of plasmon resonance maximum for system nanometal NPs/lectin – glycoprotein was also observed earlier [13]. Red shift in spectra is apparently under significant influence of durability of the stabilizing cover, and the size of metal nanoparticles. In our case, stabilizing cover of the nanoparticles was carbon-chain polymer, in contrast with the more popular low molecular weight stabilizers. Nanoparticles, stabilized by low molecular weight cover, seemed to subject agglomeration caused by the external influence (adding of massive linker) apparently more effectively than polymer-stabilized one. What is more, in our case, nanoparticles size was significantly less, comparing the ones prepared using citric acid, and so metal NPs were more stable in solution (the smaller nanoparticles size, the larger active contacting area). Also, the shift of plasmon resonance maximum for system GNPs–lectin most probably is connected both with lectin structure: microenvironment of the binding sites, molecular mass, etc., together with the nature of polymeric shell in GNP (degree of hydrophobicity of polymer), and association degree of polymeric GNP in solution.

An action of the nanocomposites on conditionally pathogenic microorganisms was carried out with a serial microdilution method. Dilution methods are the most appropriate ones for the determination of the minimum inhibitory concentration (MIC) values, since they offer the possibility to estimate the concentration of the antimicrobial agent tested in broth medium (microdilution). Broth microdilution method is one of the most basic antimicrobial susceptibility testing methods. This method may be used for *in vitro* quantitative measurement of the antibacterial and fungicidal activivites. MIC value recorded is defined as the lowest concentration of the assayed antimicrobial agent that inhibits the visible growth of the microorganism tested in microdilution wells as detected by the unaided eye. MIC values of the SL-AgNPs for two bacterial strains were obtained, and Fractional Inhibitory Concentration Indexes (FIC) were calculated (Table 1).

Table 1. Antimicrobial activities of the nanocomposites.

Sample	Components content (%, weight)		MIC(mg/L)		FIC	
	Ag^0	SL	Sample	Ag^0	Sample	Ag^0
Staphylococcus aureus ATCC 29213						
EM/Ag^0	40		46	18.4		
EM-Gly-SL/Ag^0	25	15	90	22.5	1.95	1.22
VM/Ag^0	32		84	27		
VM-Gly-SL/Ag^0	25	14	168	42	2.0	1.5
Enterococcus faecalis ATCC 29212						
EM/Ag^0	40		93	37.2		
EM-Gly-SL/Ag^0	25	15	90	22.5	0.97	0.60
VM/Ag^0	32		84	27.7		
VM-Gly-SL/Ag^0	25	14	168	25.2	2.0	0.9

FIC can be calculated using the equation:

$$FIC\ A = MIC\ A_{\text{in combination}}/MIC\ A_{\text{alone}}.$$

For FIC Ag^0 : MIC A_{alone} = MIC Ag^0 in EM(VM)/Ag^0 and

MIC $A_{\text{in combination}}$ = MIC Ag^0 in (EM)VM-Gly-SL/Ag^0.

For FIC Sample : MIC A_{alone} = MIC Sample in EM(VM)/Ag^0 and

MIC $A_{\text{in combination}}$ =MIC Sample in (EM)VM-Gly-SL/Ag^0.

The FIC between 0.5 and 1 was interpreted as “addition”, and between 1 and 4 as “indifference”. For sample, based on poly(ethylene-*alt*-maleic anhydride) and β-N-glycyl-3’-sialyllactose, it was discovered the additive effect of nanosilver and carbohydrate ligand on the antimicrobial properties with respect to the strain *Enterococcus faecalis* (FIC Ag^0 – 0.6 and 0.9; FIC Sample (EM-Gly-SL/Ag^0) – 0.97.

4. CONCLUSIONS

Simple and convenient technique for preparation of nanosilver-labeled colloidal neoglycoconjugates, based on maleic anhydride copolymers, and β-N-glycyl-3’-sialyllactose – potential receptor for microorganisms, was developed. Colloidal solutions of such GNPs had specific optical properties due to surface plasmon resonance. GNP nanoparticle size was in the range 1-3 nm. The carbohydrate content in GNPs was 14-15 %.

The SL-AgNPs obtained had antibacterial action against microorganisms tested with MICs = 50-170 mg/L. Introduction of the oligosaccharide with bacteriostatic effect into nanocomposition may result in appearance of the additive effect for the entire system. This was demonstrated for poly(ethylene-*alt*-maleic anhydride)/β-N-glycyl-3’-sialyllactose nanocomposition, showing the additive effect of nanosilver and carbohydrate ligand on the antimicrobial properties against to the strain *Enterococcus faecalis.*

This approach may be used for construction of the antimicrobials containing specific carbohydrate ligands with affinity to bacterial adhesins.

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