VANCOMYCIN AND CHITOSAN MODIFIED MAGNETIC PARTICLES FOR ANTIBACTERIAL PROPHYLAXIS IN ORTHOPAEDICS

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Abstract

Multiresistant bacterial strains are a very serious clinical problem in a hospital environment. Solving these types of nosocomial infections requires new and unique approaches. It is known that vancomycin is an antibiotic used for the treatment of serious infections such as S. aureus strains. Nanomedicine using new nanotechnologies brings new options like application of surface coating modifications for the targeting treatment. Superparamagnetic iron oxide nanoparticles (SPION) particles were created by a physicochemical method of synthesis. For the purpose of this testing, SPION particles have been modified by chitosan (300 rpm, 25 ℃, 24 h), then purified on the magnet. SPION/Chito particles were modified by vancomycin (300 rpm, 25 ℃, 6 h). The completed particles were washed with a PBS buffer (pH 7). The concentration of vancomycin (after the modifications) in SPION/Chito/VANCO particles was 100 μg/ml. The stability of finalized particles was tested in PBS (pH 7). The concentration of vancomycin was determined by HPLC. After that, the antibacterial activity was determined by measurement of growth curves (methicillin sensitive and resistant S. aureus) at 450 nm/540 nm for 24 hours (25 °C, 300 rpm in LB medium). The results were evaluated as AUC growth curves. Bacterial cultures were grown reproducibly in pure medium (AUCs were considered baseline). The study provided initial information about the effect of newly prepared types of nanoparticles on S. aureus species.

Keywords: Chitosan, iron nanoparticles, antibacterial activity, antibiotics, orthopaedic implant, HPLC

1. INTRODUCTION

Intensive use of antibiotics exerts evolutionary pressure on microorganisms, leading to the emergence of resistant strains. In hospital environments, these strains pose a serious threat to patients, resulting in severe complications and even death. Additionally, those patients are susceptible to pathophysiological and

pharmacokinetic changes [1], which cause insufficient concentrations of antibiotics. On specialized units, higher inhibition concentrations are often observed in cultivated bacterial strains. Also, it is important to realize, that another apparatus like extracorporeal circulation to support hemodynamic during respiratory, or renal failure evokes pharmacokinetic changes [1]. Methicillin-resistant *S. aureus* (MRSA) is a common pathogen, that is colonizing healthy individuals [2]. For those reasons, it is important to gather new possibilities for overcoming emerging resistance. New compounds and substances are isolated from plants and animals [3]. One of the most researched antibacterial substances is polysaccharide chitosan containing a positive charge [4]. Chitosan enables the creation of a polymeric net, on which other molecules, including antibiotics, can bind [5]. Selected groups of antibiotics (vancomycin, gentamycin) are carefully monitored, and their clinical usage is bound to suppression of very serious infections [6]. Many nanomaterials were designed with good biological effects to maximally suppress serious bacterial infections. Superparamagnetic iron oxides can be prepared as composition particles [7]. These nanoparticles could be useful within orthopaedical operations following bone damage [4]. It has been shown, that those particles should not have direct toxicity to the bone cells, but could significantly reduce the bacterial infections caused by pathogens [6]. The goal of this work was an optimization of the conditions to monitor the antibacterial effect of chitosan and vancomycin modified SPION [8].

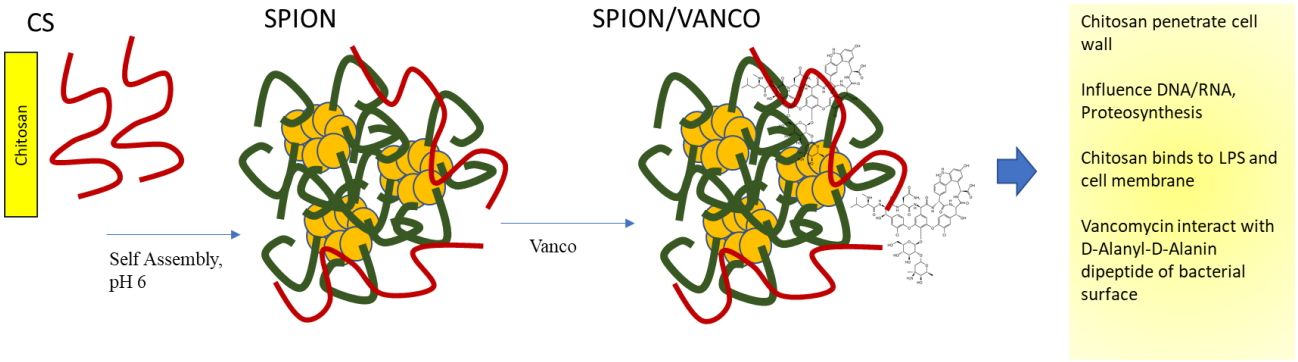

Figure 1 Scheme of the biological action of chitosan on a bacterial cell. Chitosan binds to LPS membranes. It penetrates the cell and affects DNA/RNA and thus proteosynthesis. Vancomycin binds to dipeptides on the surface of the bacterial cell. The prepared SPION nanoparticles can be used directly in the treatment of serious infections.

2. MATERIAL AND METHODS

Chemicals

Methanol, NaCl, and other chemicals (KCl, NaCl, $AgNO_3$, $NaHPO_4$, NaH_2PO_4, etc.) used were purchased from Merck (Darmstadt, Germany) with a purity of 99%. For LB media preparation, 10 g/L tryptone, 5 g/L yeast extract (Merck, Darmstadt, Germany), and 10 g/L NaCl (Merck, Darmstadt, Germany) were used. Deionized water was prepared using reverse osmosis equipment Aqual 25 (AQUAL s.r.o., Brno, Czech Republic) and subsequently treated to an 18 MΩ purity by an ELGA deionizer from Purlab Flex (London, UK). Conductivity and pH were measured with a MU 6100L multimeter from VWR (Radnor, PA, USA). The pH-electrode (662-1161 Phenomenex pH electrode pH 0–14/3M KCl, Torrance, USA) was regularly calibrated with two-point calibration (VWR buffers, at 25 °C).

Synthesis of SPIONs

Nanoparticles were synthesized by a chemical route according to an abbreviated protocol: 1.3 g of $Fe(NO_3)_3 \cdot 9H_2O$ was dissolved in 80 mL of water (18 MΩ). A quantity of 1.4 mL 25% NH_3 was diluted in 8.6 mL water and 0.2 g $NaBH_4$ was dissolved in this mixture and, subsequently, the mixture was stirred (300 rpm, 10 min at 22 °C). The solution colour turned to dark brown. The mixture was heated to 100 °C for 2 h and stirred

(300 rpm, stirring overnight at room temperature). The magnetic particles were separated from the solution via a magnet and washed several times in water. Later they have been lyophilized 48 h at -80 °C and converted into solution using sonication (60 min, 40 W). Characterized by spectral analysis (Single-beam spectrophotometer VWR, USA) and by hydrodynamic light scattering (Zetasizer Nano ZS ZEN3600 from Malvern Instruments UK).

Chemical analysis

Total protein was determined by the biuret method using a single-beam spectrophotometer (VWR, USA). The biuret method is a test used for detecting the presence of peptide bonds. In the presence of peptides, a copper (II) ion forms a violet-coloured complex in an alkaline solution. A 200 µL volume of biuret reagent (100 mM potassium sodium tartrate, 100 mM sodium hydroxide, 15 mM potassium iodide and 6 mM copper sulphate) was pipetted into a plastic cuvette with the subsequent addition of 4 µL of the sample. Absorbance was measured at λ = 546 nm after 10 minutes of incubation. The resulting value was calculated from the absorbance value of the pure biuret reagent and from the absorbance value after 10 minutes of incubation with the sample.

Metallothioneins

Electrochemical measurements were performed with an AUTOLAB Analyser (Metrohm, Herisau, Switzerland) connected to VA-Stand 663 (Metrohm, Herisau, Switzerland), using a standard cell with three electrodes. The working electrode was a hanging mercury drop electrode (HMDE) with a drop area of 0.4 mm^2. The reference electrode was an Ag/AgCl/3M KCl electrode, and the auxiliary electrode was a graphite electrode. The supporting electrolyte was prepared by mixing buffer components. The samples analyzed by differential pulse voltammetry (DPV) were deoxygenated prior to measurements by purging with argon (99.999%) saturated with water for 20 s. In our studies, the Brdicka supporting electrolyte contained 1 mM $Co(NH_3)_6Cl_3$, 1 M NH_3(aq), and 1 M NH_4Cl, pH = 9.6; a surface-active agent was not added. The DPV Brdicka reaction parameters were as follows: an initial potential of –0.6 V, an end potential of –1.85 V, a modulation time of 0.057 s, an interval of 0.2 s, a step potential of 1.05 mV/s, a modulation amplitude of 250 mV, and accumulation time of 240 s [9, 10].

HPLC analysis of vancomycin

It was analyzed by HPLC. A gradient elution was used on a Zorbax 5 µm, 4.6 x 250 mm column. The mobile phase was acetonitrile: water 95: 5. Detection took place on a UV/Vis detector at 210 nm, injection 50 µl flow rate: 1.5 ml/min. The concentration was evaluated using the calibration curve method. The vancomycin signal was at RT: 5.2 min. and LOD 78 ng/mL, LOQ 234 ng/mL with RSD 2.8%.

Measurement of growth curves

Biological samples of MRSA (SA1, SA3) and MSSA (SA2) strains were maintained by a passage in a liquid medium (Biosan, 300 rpm, 37 °C). Infinite F50 (TECAN, Switzerland) was used to measure growth curves for 24 h. The growth curves obtained were subsequently evaluated as AUC areas. Each analysis was performed in three independent experiments and 8 replicates (n = 24). They were evaluated in the Qinslab system (EcoNanoLife).

Statistical Data Analysis

Available experimental data was processed and evaluated mathematically and statistically directly in the Qinslab database. The exclusion of extreme values for data sets was performed by calculation in the Grubbs test. Experimental work was performed in at least three independent experiments (n_1). Each sample in the experiments was analyzed at least five times (n_2). The data in this work are presented as average values. LOD (limit of detection) values were determined according to the work of Hubaux and Vos at a significance level of 95% [11]. Data visualization was performed using the Qinslab database.

3. RESULTS AND DISCUSSION

In our previous experimental studies, we tested the effects of nanoparticles on the behaviour of bacterial strains. In this study, we focused on a detailed study of the biological behaviour of MRSA and MSSA strains in the presence of individual components of modified nanoparticles. First, the sensitivity of MRSA and MSSA strains to vancomycin was tested by monitoring the growth curves (AUC). We found that all strains showed fairly significant resistance to vancomycin. Concentrations below 100 µg/L resulted in growth inhibition and below 400 µg/L resulted in bacterial elimination. SPIONs were modified with chitosan followed by vancomycin (**Figure 1**). In some studies, SPION particles alone have been reported to induce bacterial inhibition [7]. The effect is mainly based on the generation of oxygen radicals, which damage cellular structures. In our study, the prepared SPION nanoparticles (applied at a concentration of 1 mg/ml) without further modification did not show any inhibitory effects on the tested MRSA and MSSA strains (**Figure 2**).

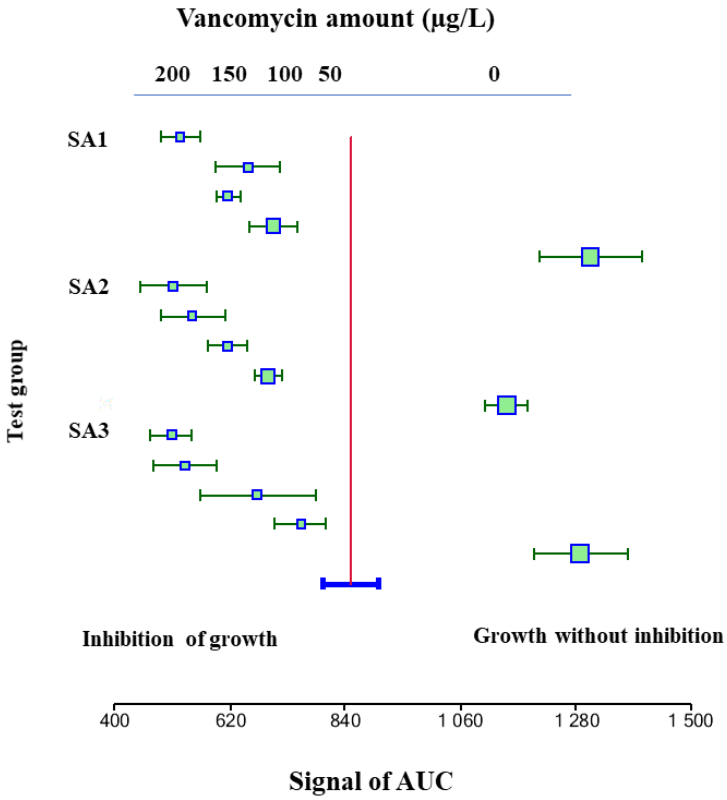

Figure 2 The effect of SPION (1 mg/mL) in combination with vancomycin was studied. A constant concentration of SPION was added to all monitored samples. Measurement 24 h and 40 h, 450 nm and 540 nm, 1 min, shaking low 5 s, thermo plate, 350 µl LB medium, 10 µl growing bacterial culture (passaging in LB medium after 160 h and cultivation at 37 ℃, 120 rpm) temperature cultivation 25 ℃. The obtained growth curves were evaluated as the total area under the curve (AUC) by the subtraction method without correction (0). Data were evaluated using the Forest plot method. SA sensitive (SA1 and SA3) and methicillin resistant (SA2) were tested. Applied final concentration of vancomycin (0, 50, 100, 150 and 200 µg/L).

In other experiments, the effect of chitosan was tested. As described in the work of No et al., the activity of chitosan is dependent on its molecular weight and the concentration of the solvent used (acetic acid and ethanol). 1% acetic acid and 2 M ethanol have been reported to produce inhibitory effects. The preparation of chitosan was optimized so that the inhibitory effect of acetic acid and ethanol did not occur. We monitored the reduction of the concentration of acetic acid and ethanol so that the growth of the tested bacterial strains was not inhibited. We have shown that such a concentration is 120 mM acetic acid and 160 mM ethanol. Concentrations of acetic acid and ethanol were chosen for the experiment, which induced a very weak

inhibitory change. In such an environment, it was possible to observe the inhibitory effects of chitosan (1; 0.5; 0.25; 0.125 and 0.025 g/l). Even a concentration of 0.125 g/l chitosan caused 90-95% growth inhibition in all tested strains, and 25 mg/l chitosan caused 70-80% growth inhibition. Subsequently, we prepared SPION nanoparticles modified with chitosan and vancomycin. We monitored the presence of vancomycin on the surface of the nanoparticles using a biuret reaction. The method shows very good sensitivity to vancomycin and allowed us to determine the relative yield of the modification around 80-85%, (**Figure 3**).

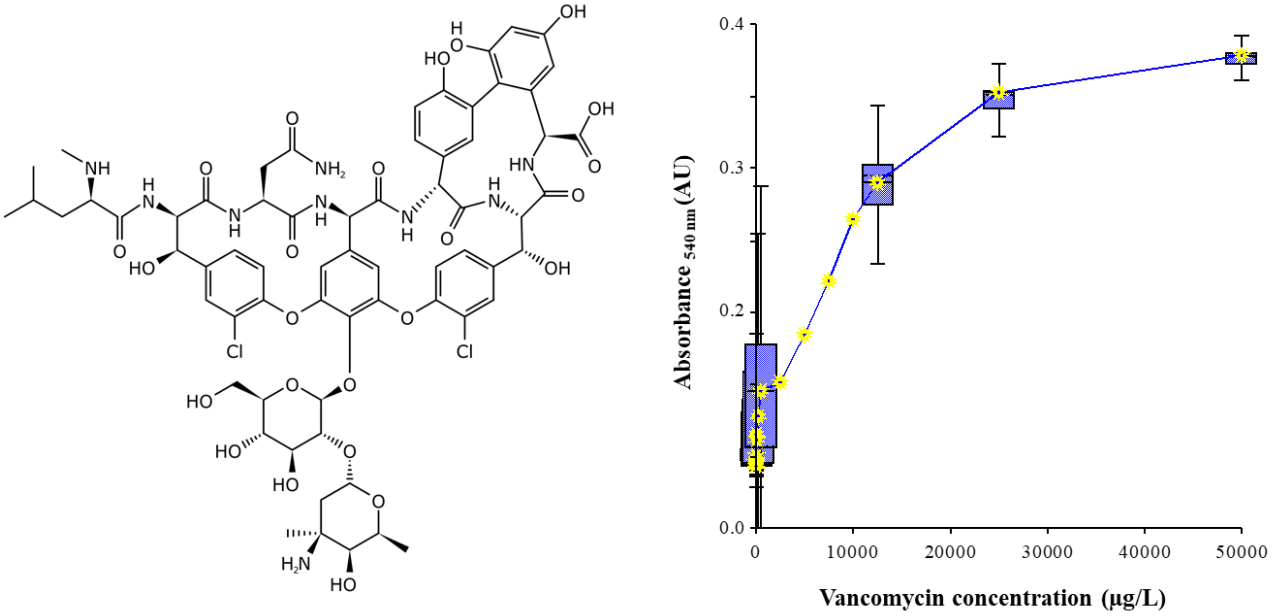

Figure 3 On the UV/VIS spectrum, we see the formation of a vancomycin complex with copper ions to form a coloured biuret. On the dependence of vancomycin on concentration and absorbance A540 nm, we obtained a linear dependence in the range (0 – 250 mg/L, r 0.9937; QC 13.62; LOD 17; LOQ 51 mg/L). SPION nanoparticles were modified with 5 mg/mL vancomycin (rotator 24 h, 360 rpm, 25 ℃, 5 s).

We monitored chitosan covered nanoparticles by reacting with ninhydrin. Here, the coverage of nanoparticles was around 15-20%. The release of vancomycin from the nanoparticle surface was observed using HPLC. SPION/Chito/Vanco was placed in PBS, pH 7 at 25 ℃ and rotator 300 rpm. Individual samples were taken every 6 hours for 24 hours. From the curves of the cumulative release of vancomycin, it was found that 90% of it was released in the first 500 min. SPION/Chito/Vanco showed inhibitory effects of 50–60% at SPION concentrations of 100 mg/ml. When applying 150 and 200 mg/ml, this inhibitory effect was between 80-90%. In addition to the assessment of growth curves, some selected parameters were monitored. MT level was analyzed by Brdicka electrochemical analysis of catalytic signals. In general, the MT amount depend on concentration of nanoparticles and cells stress reaction.

4. CONCLUSION

The inhibitory effects of selected molecules on the inhibitory effects on MSSA and MRSA strains of SPION nanoparticles modified with chitosan and vancomycin were monitored. Appropriate inhibitory concentrations were sought for the bacterial strains used by us. Thanks to the modification of SPION nanoparticles, even at relatively low concentrations of individual antibacterial components, it was possible to achieve a significant antibacterial effect of around 90%. In further research, additional modifications with metal nanoparticles will be used to strengthen this effect. Moreover, these particles will be directed for their application potential in orthopaedic applications.

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REFERENCES

[1] RUIZ-RAMOS, J.; GRAS-MARTIN, L.; RAMIREZ, P. Antimicrobial Pharmacokinetics and Pharmacodynamics in Critical Care: Adjusting the Dose in Extracorporeal Circulation and to Prevent the Genesis of Multiresistant Bacteria. *Antibiotics-Basel.* 2023, vol. 12, no. 3, pp. 475

[2] HOLM, M. K. A.; JORGENSEN, K. M.; BAGGE, K.; WORNING, P.; PEDERSEN, M.; WESTH, H.; MONK, J. M.; BARTELS, M. D. Estimated Roles of the Carrier and the Bacterial Strain When Methicillin-Resistant Staphylococcus aureus Decolonization Fails: a Case-Control Study. *Microbiol. Spec..* 2022, vol. 10, no. 5, pp. 1-12.

[3] AHMAD, S.; MUNIR, S.; ZEB, N.; ULLAH, A.; KHAN, B.; ALI, J.; BILAL, M.; OMER, M.; ALAMZEB, A.; SALMAN, S. M.; ALI, S. Green nanotechnology: a review on green synthesis of silver nanoparticles - an ecofriendly approach. *Int. J. Nanomed.* 2019, vol. 14, no. pp. 21.

[4] DJOSIC, M.; JANKOVIC, A.; STEVANOVIC, M.; STOJANOVIC, J.; VUKASINOVIC-SEKULIC, M.; KOJIC, V.; MISKOVIC-STANKOVIC, V. Hydroxyapatite/poly(vinyl alcohol)/chitosan coating with gentamicin for orthopedic implants. *Mater. Chem. Phys.* 2023, vol. 303, no. 127766, pp.1-12.

[5] QI, L. F.; XU, Z. R.; JIANG, X.; HU, C. H.; ZOU, X. F. Preparation and antibacterial activity of chitosan nanoparticles. *Carbohydr. Res.* 2004, vol. 339, no. 16, pp. 2693-2700.

[6] KAFALI, M.; SAHINOGLU, O. B.; TUFAN, Y.; ORSEL, Z. C.; AYGUN, E.; ALYUZ, B.; SARITAS, E. U.; ERDEM, E. Y.; ERCAN, B. Antibacterial properties and osteoblast interactions of microfluidically synthesized chitosan - SPION composite nanoparticles. *J. Biomed. Mater. Res. Part A.* 2023, pp. 1-16.

[7] TAYLOR, E. N.; WEBSTER, T. J. The use of superparamagnetic nanoparticles for prosthetic biofilm prevention. *Int. J. Nanomed.* 2009, vol. 4, pp. 145-152.

[8] RASHID, M.; RABBI, M. A.; ARA, T.; HOSSAIN, M. M.; ISLAM, M. S.; ELAISSARI, A.; AHMAD, H.;RAHMAN, M. M. Vancomycin conjugated iron oxide nanoparticles for magnetic targeting and efficient capture of Gram-positive and Gram-negative bacteria. *Rsc Advances.* 2021, vol. 11, no. 57, pp. 36319-36328.

[9] PETRLOVA, J.; POTESIL, D.; MIKELOVA, R.; BLASTIK, O.; ADAM, V.; TRNKOVA, L.; JELEN, F.; PRUSA, R.; KUKACKA, J.; KIZEK, R. Attomole voltammetric determination of metallothionein. *Electrochim. Acta.* 2006, vol. 51, no. 24, pp. 5112-5119.

[10] MELICH, L.; FORTOVA, M.; HOSNEDLOVA, B.; PODHAJSKY, J.; RYCHLY, O.; WERLE, J.; BURESOVA, K.; DUNOVSKA, K.; VYSLOUZILOVA, L.; KLAPKOVA, E.; KOTASKA, K.; CEPOVA, J.; JEDLICKOVA, B.; STEPANKOVA, O.; PRUSA, R.; KIZEK, R. Studium hladiny metalothioneinu v séru pacientů se zhoubným nádorem. *Chem. Listy.* 2023, vol. 117, no. 9, pp. 573-580.

[11] HUBAUX, A.; VOS, G. Decision and detection limits for linerar calibration curves. *Anal. Chem.* 1970, vol. 42, no. 8, pp. 849-851.