ANTIMICROBIAL PROPERTIES AND STRUCTURE OF AG- AND CU-CHITOSAN NANOCOMPOSITES SYNTHESIZED IN SUPERCRITICAL CARBON DIOXIDE

Ernest Said-Galiev*[1], Arif Gamzazade[1], Marat Gallyamov[2], Timofei Grigoriev[1], Alexei Khokhlov[1], Natali Bakuleva[3], Irina Lyutova[3] , Vladimir Volkov[4] , Eleonora Shtykova[4], Cyril Dembo[4]

[1]State Institution of Russian Academy of Sciences A.N. Nesmeyanov Institute of Organoelement Compounds RAS, Vavilova Str. 28, Moscow 119991, Russia

[2]Physical department of the Moscow State University, Vorobievy Gory, Moscow 119899, Russia

[3]State Institution A.N. Bakulev Centre of cardiovascular surgery of Russian Academy of Medical Sciences, Leninsky prosp. 8, Moscow 117931, Russia

[4]State Institution of Russian Academy of Sciences A.V. Shubnikov Institute of Crystallography RAS, Leninsky prosp. 59, Moscow 1193331, Russia

*Corresponding author FAX: +7(495) 135-50-85, e-mail: ernest@ineos.ac.ru

INTRODUCTION

Chitosan attracts attention of researchers last years as a biologically active, biocompatible and biodegradable material possessing many positive properties [1-5]: sorption, bactericide, fungicide, antitumor, immunity enhancing effect, selective activity to pathogenic microflora, stimulation of epithelium growth. One believe that the mechanism of antimicrobial action of chitosan nanoparticles deals with chelation of cell food elements or with formation of polyelectrolyte complex between positive charged chitosan and negative charged cell chemical structure that prevents to food substances to penetrate through cell membrane and gives result to death of a cell. An increase of chitosan antimicrobial activity is reached by immobilization on it of bactericide metals nanoparticles such as Cu^{++}. This operation is simply to carry out by addition of $Cu^{++}X$ salt aqueous solution to aqueous acid chitosan solution and then precipitation of the formed complex Cu^{++} - chitosan into NaOH aqueous solution. Chitosan strongly holds cation Cu^{++} due to Cu^{++} - chitosan complex formation where Cu^{++} coordinates with NH_2 and OH groups of chitosan. Cu^{++} interacts with negative charged cell membrane sites that gives rise to increase a penetration rate of chitosan nanoparticles through cell membrane, then fistula formation and outlet of intracellular cytoplasm. After a time the cell decomposes to separate fragments. A presence of charged metal nanopartical in chitosan very increases its antimicrobial activity. The dependence of E-coli antimicrobial activity of chitosan and its metal-complexes on particles size and charge is given in the table 1[2].

Concentration	Chitosan	CNP	CNP- Cu^{++}	Doxicyclin
MIC	8	1/16	1/32	1
MBS	64	1	1	4

MIC- minimum growth inhibitory concentration, MBS- minimum bactericidal concentration, CNP- chitosan nanoparticles, CNP- Cu^{++} - charged chitosan complex.

Analysis of the table data brings to conclusion that particles size plays a key role in antimicrobial activity of chitosan.

In spite of intensive investigation of chitosan metal-complexes some questions meanwhile are not understood up to now. The task of the work is checking and comparison of antimicrobial

activity of metal-chitosan complexes with charged and zero-valency nanoparticles of bactericide metals. Another task is to determine the possibilities and the concrete role of fluid technology in development of effective antimicrobial chiotosan materials.

MATERIALS AND METHODS

***Materials*:** Chitosan **(**«Sonat» Ltd. Corp.)**,** Mn~ 80 KDa with 98% deacetylation degree. Metal precursors: 1,5-(cyclooctadiene)(hexafluoroacetylacetonate)silver(I) (COD Ag[hfacac]) and copper (II) (hexafluoroacetylacetonate) hydrate (Cu(hfacac) x H_2O, x<1, were received from Aldrich and used without additional cleaning. CO_2 (99,997v. %) was purchased from MPPG-company. H_2 (H_2O = 0.02 g/cm^3, Ar + O_2 = 0.01 v. %).

***Experimental set*:** The set is standard and described elsewhere [6]. We used a tubular reactor from stainless-steel with internal volume 10 $cm^3$and heating magnetic stirrer.

Methods of chitosan metal-composite - and metal - complexes synthesis: We used two technological two-stage schemes (Fig.1):

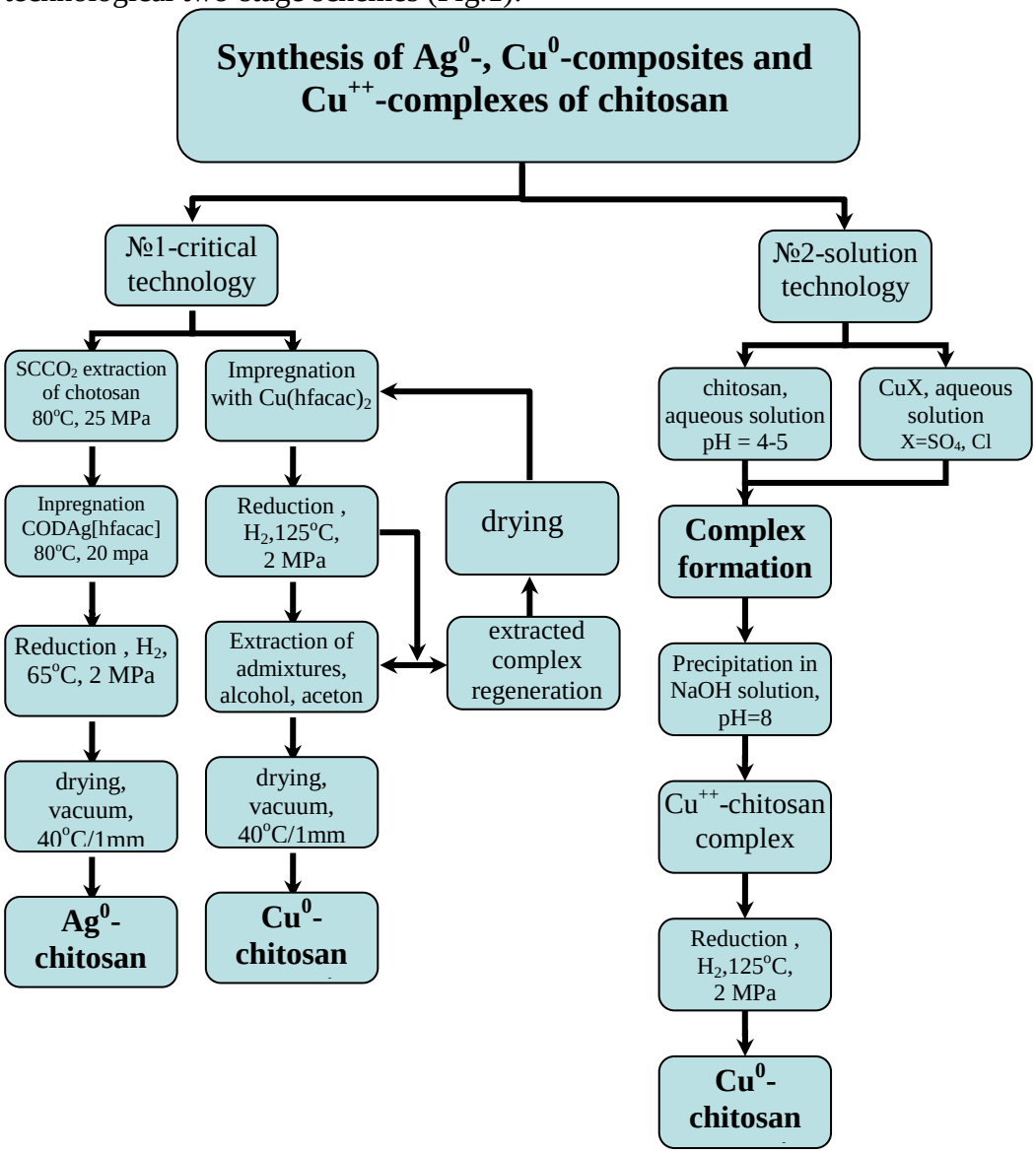

Fig. 1

The scheme №1 – impregnation of Ag- and Cu- complexes from its SC CO_2 solutions and following reduction of a metal with H_2. The schemes for silver and copper are divided by inclusion of previous treatment of chitosan powder with SC CO_2 for creation of microporous structure in a case of Ag and washing with organic solvents after every synthesis stage in a case of Cu for extraction of unimpregnated and unreduced complex and second products of the ligand transformatios at metal reduction. The products were controlled at every stage by elemental analysis (RFA for a metal and spectrophotometry for fluorine. SAXS method was used for composite structure investigation.
The scheme №2: Cu-complex of chitosan was synthesized by interaction of its and Cu-salt aqueous solutions and was precipitated in 0.1 N sodium hydroxide. Then it was reduced with H_2. The conditions of every stage are shown on the scheme. A swelling of chitosan in SC CO_2 was studied by gravimetric method using the electronic balance to 10^{-5} g "OHAUS AB-250"and its software. Chitosan dissolution in SC CO_2 was studied in view cell from "Sitec" with sapphire windows and internal volume of 3.3 cm^3 .The software was homemade. Biological tests were carried out at the Bakulev Centre of cardiovascular surgery. Their principal scheme: dissolution of metal- chitosan composites and complexes in acid water – making of Stafillococ epidermidis, E-coli and B. cereus clinical cultures-addition of them to chitosan solutions – keeping for 30 min and 60 min-deposition on Petri dishes – exposition at 37^{o}C for 24 hours and then results observation.

RESULTS

Our attempts to dissolve chitosan and its 15 different derivatives have turned to failure, although there are some publications that inform about its dissolution in SC CO_2 but the concrete conditions are not pointed. At the same time the equilibrium swelling degree of chitosan and its derivatives are located in the range of 3-12 w. % (by our data). This property gives a possibility to impregnate in chitosan different functional additives, for example, metal –organic complexes from their SC CO_2 solutions. Though a solubility of Ag-complex in SC CO_2 is very pour (~ 10^{-5} mol / l by authors data) and one of the Cu-complex is 0.13 mol/l [7], substantial their concentration (~ 6 w. % for Ag and 4.7 w. % for Cu) manage to create in polymer matrix due to high value of partition coefficient $K = K_1/K_2$, where K_1 is the equilibrium complex concentration in chitosan powder, K_2 is its concentration in SC CO_2 solution. K in our experiments was equal to 7 for Cu -complex impregnation.
Some conclusions on metal-chitosan composites synthesis are next:

- The silver complexes are easy reduced with H_2 at 65^{o}C while the copper ones demand of higher temperature (125^{o}C).
- A content of reduced Ag in the complexes reaches ~ 6 w. %. A difference with theoretical value is 5-8 %.
- A part of impregnated Cu-complex is 76 w.% from loaded one while a common composite mass losses after reduction approximately corresponds to a part of the ligand in total weight chitosan + complex.
- The end value of Cu in complexes reaches 4.6 w. %.
- Fluorine is not eliminated completely after washing and vacuum drying. Therefore it is recommended to use complexes giving at a reduction and decomposition volatile products and not containing fluorine because of its high reactivity.

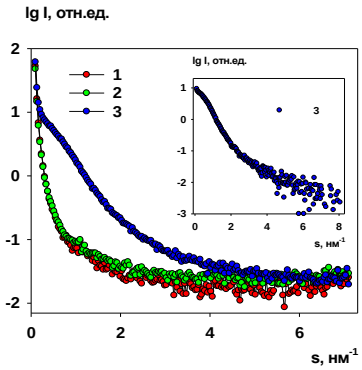

Fig. 2

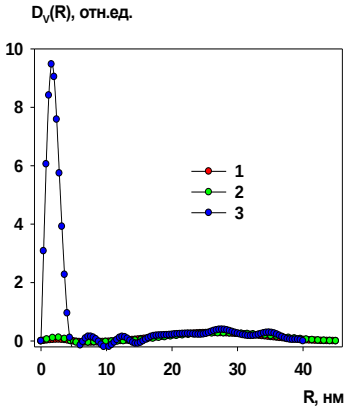

Fig. 3

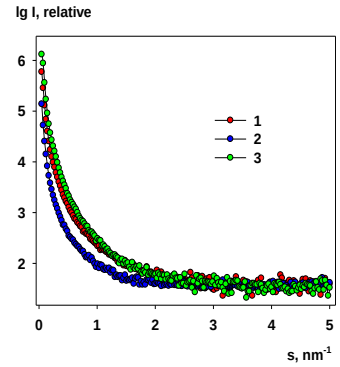

Fig. 4

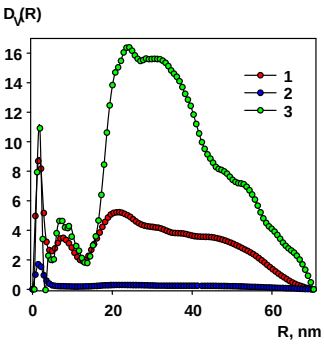

Fig. 5

The structure of metal-chitosan composites was investigated by SAXS- method. Ag-chitosan composites: all experimental curves are characterized with scattering in smallest angles (Fig.2). It is scattering with pores for polymer matrix (curve 1), central scattering is one from the matrix and impregnated complex (curve 2) and the matrix with reduced metal nanoparticles (curve 3). The polymer matrix scattering was subtracted to have scattering only from metal nanoparticles. Differential curve is presented on on inset. Functions of volume distribution on size were calculated with a help of "GNOM' program and are given on the Fig.3. Distribution curves (1,2) for initial and impregnated matrices are characterized by nanoformations practically all sizes from small up to 50 nm with two fractions of middle radius 2-3 nm and 25-30 nm with predominance of large scale formations. The same fractions are present also in metal nanoparticles distribution by size (curve 3) but in this case particles with radius 2.5- 3 nm predominate. Amplitude of the distribution by size in the area of finest nanoparticles is an order higher for reduced metal as compared with two remaining samples. This is evidence of real metal reduction. At the same time the appeared metal nanoparticles are limited in their growth with internal geometry of polymer matrix and have breakup as the initial matrix.

Cu - chitosan composites: all experimental curves are characterized by scattering in finest angles (Fig.4).

It is scattering with pores for polymer matrix (curve 1), with matrix and impregnated complex (2), with matrix and metal nanoparticles (3). One can see that scattering amplitude of the complex is smaller than ones for the matrix and reduced metal nanoparticles. Rational explanation is decrease of contrast between a pore and a matrix due infill of pores with complex or organic products of the ligand decomposition as a result of metal reduction already at impregnation stage. Calculated volumetric function of distribution by size $D_v(R)$ for all three samples confirm this assumption. Amplitude $D_v(R)$ function of sample 2 are substantially lower than ones of two another samples as is seen from Fig.5.

The distribution for all three samples has a complicated character and includes a fraction of small particles with radius of 2-3 nm and also a wide spectrum of dispersive objects with a radius up to 70 nm. There are two marked maximums with middle radii 10 and 30 nm. At that the amplitude of distribution function of the sample with reduced metal nanoparticles is substantially higher than one of another two functions that confirms the metal reduction.

Besides the $D_v(R)$ form curve repeats one for initial matrix that is for pores in the polymer. So it is possible to maintain that impregnation of the complexes and their following reduction

have occurred exactly in matrix pores but not at their surface, i.e. appeared metal nanoparticles were limited in their growth with internal geometry of polymer matrix and have the same fractional composition as the initial matrix. Calculated gyration radii of dispersive heterogeneities are given in tables 2 and 3.

Table 2. Radii of gyration for Ag-complex impregnation and reduction.

A sample	R_g, nm
Polymer matrix (chitosan powder)	20 ± 0.3
Matrix impregnated with complex COD Ag[hfacac]	22 ± 0.4
Matrix with reduced metal	2.2 ± 0.03

Table 3. Radii of gyration for Cu - complex impregnation and reduction

A sample	Rg, nm
Polymer matrix (chitosan powder)	37.9 ± 0.6
Matrix impregnated with $Cu(hfacac)_2$	38.1 ± 0.5
Matrix with reduced metal-	38.3 ± 0.9

The values of middle gyration radii R_g confirm a predominance of small nanoparticles in the sample with reduced silver that is explained as we believe with large input of small nanopores formed due preliminary treatment of chitosan with SC CO_2. Middle radii of gyration for dispersive heterogeneities R_g in Cu-chitosan composite (table 3) are practically in close agreement for all studied samples i.e. the conclusion about formation of metal nanoparticles in the polymer pores to where the metal complex was impregnated is confirmed once again.

The samples of nanometal-chitosan composites and Cu^{++} - chitosan complexes were tested for antimicrobial activity. We used next materials at the first stage of investigation: sample N1 - Cu^{++} - cuccinylchitosan – aqueous solution, N2 - Cu^{++} - N- sulfocuccinylchitosan – aqueous solution, N3 - Cu^{++} - O- sulfocuccinylchitosan – aqueous solution, N5 – Cu^0 – chitosan synthesized by critical technology, 1% PEG solution,

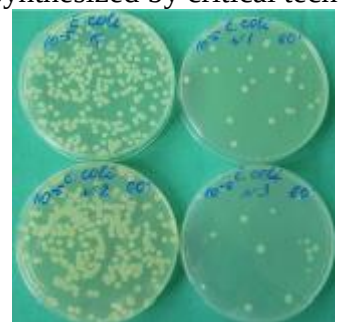

Fig. 6

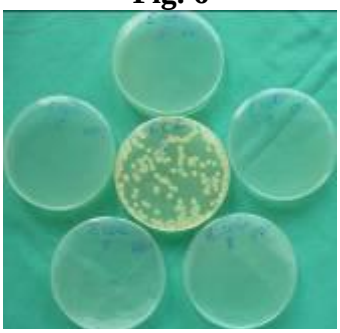

Fig. 7

N6 – Cu^0 –chitosan synthesized in solution but reduced with H_2, N7 – Ag^0 - chitosan synthesized by critical technology, N8 – Cu^{++} - carboximethylchitosan – aqueous solution, N9 – carboximethylchitosan, 2% PEG solution.

Clinical cultures S.epidermidis and E-coli. Food media: tripticaso-bean broth, Chinton-Muller agar, 0.9% NaCl solution, 1N hydrochloric acid. All samples in different degree have demonstrated antimicrobial activity. It was not very strong for samples 1-3. Bacteria growth decreased as compared with control K sample but was not suppressed completely (Fig.6). The samples 5-9 have shown excellent antimicrobial activity (a growth was suppressed completely (Fig.7).

A testing was complicated at second stage of investigation with addition of spore form bacteria B. cerius to S. epidermidis. Next result were obtained: a growth of S. epidermidis culture was absent in deposition zones of 6 and 7 samples. Repeated growth of S. epidermidis was observed in deposition zones of 5, 8 and 9 samples.

<u>Resume on biological tests:</u>

- all investigated Ag- and Cu-derivatives of chitosan display antimicrobial action with respect to S.epidermidis, E-coli and B. cereus clinical cultures
- degree of antimicrobial action depends on a structure of microorganism cell membrane, bacteria cells concentration, aggregate state of microorganisms culture.

- The sample 6, Cu^{++}-chitosan synthesized in solution but reduced with H_2 into Cu^0-valence and 7, Ag^0- chitosan synthesized by fluid technology are most perspective with respect to investigation of antimicrobial activity
- The sample 5, Cu^0 - chitosan synthesized by fluid technology did not come in the group of most active samples apparently because of size effect manifestation (contains metal particles more than 10 nm that is critical size of penetration through cell membrane for metal nanoparticles [8].

CONCLUSION

- The structural-modifying role of fluid technology consisting in growth stimulation of the least size(less than 10 nm) nanopores fraction in polymer matrix determinative finally a preferred size of reduced metal nanoparticles (smaller than critical size indispensable for their penetration through a cell membrane) has shown by the example of chitosan.
- Nanometal-polymer composites of chitosan containing immobilized metal nanoparticles in zero valency state display a larger antimicrobial activity than the same metals in ionic form.

Acknowledgement

The authors thank the Russian Academy of Sciences (DCMS Program №6"Synthesis and investigation of supramolecular structures properties", Presidium RAS Program №27, №11, Innovation Project №30179) and RFBR (grants №08-03-12152, 08-03-13540-ofi-ts, 09-03-91227-ST, 09-03-12144-ofi-m,) for financial support.

REFERENCES:

[1] Musarelly, R.A.A. Chitin /R.A.A. Muzarelli. Oxford: Pergamon Press, 1977.-309p.
[2] Lifeng Qi; Zirong Xu; Xia Jang; Calhong Hu; Xiangfei Zou. Carbohydrate Research, Vol. 339, **2004**, p. 2693
[3] Zhnyong Guo; Hongying Liu; Xialin Chen, Xia Ji; Pengcheng Li. Bioorganic and Medical Chemistry Lett., Vol.16, **2006**, p. 6348
[4] Lifeng Qi; Zirong Xu. Ibid., **2006**, p. 4243
[5] Lifeng Qi; Zirong Xu; Xia Yang; Yan . Li; Mingi Wang. Ibid., Vol.15, **2005**, p. 1397
[6] Said-Galiev, E.E.; Vygodskii, Y.S.; Nikitin, L.N.; Vinocur, R.A.; Khokhlov, A.R.; Pototskaya, I.V.; Kireev, V.V.; Schaumburg, K. Polym. Sci., Vol. 46, **A 2004, p.** 634
[7] Fedotov, A.N.; Simonov, A.P.; Popov, V.K.; Bagratashvili, V.N. J.Phys.Chem., Vol. 101, **B 1997,** p**.** 2929
[8] Krutyakov, Yu.A.; Kudrinskii, A.A.; Olenin, A.Yu.; Lisichkin, G.V. Chem. Rev.)(Russian), Vol. 77, **2008**, p. 242.