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ANTIBACTERIAL ACTIVITY OF COMBINED NANODIAMONDS AND SNAIL FRACTIONS WITH BIOCOMPOUNDS WITH MW BELOW 10 KDA AND ABOVE 30 KDA

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Abstract

The use of nanomaterials for enhancement of the antibacterial activity of different compounds is a new approach contributing to the efforts of overcoming the emerging antibiotics resistance. This study explores the combined antibacterial activity of nanodiamonds and snail peptides against *Brevibacillus laterosporus BT271*. Two peptide/protein fractions from *Cornu aspersum* mucus were used – with molecular weight (MW) below 10 kDa and with MW above 30 kDa. Four types of nanodiamonds (ND) were tested (positively charged, single digit and two types of detonation ND). Peptide fractions were characterized by using MALDI-TOF. The fraction with MW below 10 kDa consisted of many different peptides, while the fraction with proteins above 30 kDa showed proteins at m/z 26879.2 Da, 33903.11 Da, 43583.81 Da, 53986.81 Da, 79091.82

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Da, 100650.42 Da, and 141 550.82 Da. When mixture peptides+ND was applied directly on the bacterial culture, the nanodiamonds showed enhanced antibacterial effect from 8% up to 42%. The combination of nanodiamonds and the mucus fraction with MW above 30 kDa had strongest bactericidal effect (1593.04 mm^2/mgP*µL). The results demonstrated that ND have potential to enhance the antibacterial activity of the snail peptides but also highlighted that their effects should be carefully studied.

Key words: nanodiamonds, *Cornu aspersum*, peptides, antibacterial activity, *Brevibacillus laterosporus*

Introduction. It is widely known that the phenomenon of antibiotic resistance poses serious threats on many aspects of human life [1]. Antimicrobial peptides (AMP) are considered to be novel drug candidates counteracting this resistance [2]. In mollusks AMP have high antibacterial potential because these organisms lack adaptive immunity and depend solely on physical barriers and the innate immune system [3].

Nanotechnologies revolutionized many fields of science and everyday life. They also give new means to overcome the antibiotic resistance and enhance the activity of diverse antibacterial compounds [4]. Nanodiamonds are carbon nanoparticles which are actively used in different biomedical applications. They are studied as carriers for anticancer therapeutics [5]. Also they possess high potential for enhancement of the gene delivery and in the replacement of the viral vectors [6]. Several articles showed that nanodiamonds have bactericidal activity with or without antibiotics. For example nanodiamonds (ND) with lysozyme were shown to have disruptive effect on *Escherichia coli* cells [7]. A very interesting example of clinical application of nanodiamonds as antibacterial agents was presented by Lee et al. [8]. Beranová et al. [9] demonstrated antibacterial activity of commercially produced nanodiamonds against *Escherichia coli.* Iyer et al. [10] showed antibacterial activity of the same type of nanodiamonds when applied on *E. coli* infected bladder cells.

The aim of this study was to explore the antibacterial activity of the combined application of snail peptides and nanodiamonds on model highly resilient bacterial strain with potential to degrade diverse xenobiotics.

Materials and methods. In the present study the modulation activity of ND on antibacterial properties of peptides isolated from *Cornu aspersum* was investigated. Two protein fractions were tested – one peptides with MW (molecular weight) below 10 kDa and one proteins with MW above 30 kDa. *Brevibacillus laterosporus BT271* were used as model Gram "+" bacteria since they are active biodegraders of more than 30 cyclic xenobiotics (similarly to many antibiotics) and are highly resilient in unfavourable environment.

Materials. *Brevibacillus laterosporus BT271* – these bacteria are Gram positive, rod-shaped, endospore-forming. The strain is isolated from contaminated soils and has high proven potential for biodegradation of xenobiotics with cyclic

chemical structure. The strain possesses high potential to adapt for biodegradation of 30 different xenobiotics with aryl-containing component in their molecules. This presupposes that the strain will be highly adaptive to resistance towards the antibiotics with aromatic structure [11].

Cultivation media – for seeding material 18-hours bacterial culture of *B. laterosporus BT271* was prepared in Nutrient media (HiMedia, India). In-depth and surface inoculation were performed on Nutrient agar (HiMedia, India).

Nanodiamonds – four types of nanodiamonds were used in the experiments: positively charged ND (PlasmaChem, GmbH), single digit ND (PlasmaChem, GmbH), detonation nanodiamonds with size of 2–10 nm (ATM Istanbul, Turkey) and detonation nanodiamonds with size of 1 nm (ATM Istanbul, Turkey).

Mucus – it was collected and purified from *Cornu aspersum* snails, grown in Bulgarian farms using patented technology without causing suffering to any snail [12]. After several steps of homogenization and purification also subject of patent, including filtration and centrifugation for removal of rough particles, the crude mucus extract was purified. The obtained mucus extract was separated by ultrafiltration using Millipore filters (1, 10, and 30 kDa) into several fractions: peptide fraction 1 containing compounds with MW between 1–10 kDa, and protein fraction 2 (with MW above 30 kDa).

Methods. Mass spectrometry analysis – the molecular masses of isolated fractions were measured by an Autoflex™III, High-Performance MALDI-TOF & TOF/TOF System (Bruker Daltonics) which uses a 200 Hz frequency-tripled Nd–YAG laser operating at a wavelength of 355 nm. Some 50 pmol of the peptide fractions were dissolved in 0.1% (v/v) TFA and applied to the target. Analysis was carried out using α-cyano-4-hydroxycinnamic acid as a matrix. Two µL of the sample was mixed with 2.0 µL of matrix solution (7 mg/mL of α-cyano-4-hydroxycinnamic acid (CHCA) in 50% ACN containing 0.1% TFA) and only 1.0 µL of the mixture was spotted on a stainless steel 192-well target plate. They were dried at room temperature and subjected to mass analysis. A total of 3500 shots were acquired in the MS mode and collision energy of 4200 was applied. The mass spectrometer was externally calibrated with a mixture of angiotensin I, Glu-fibrino-peptide B, ACTH (1–17), and ACTH.

Nanodiamonds and peptides were added to the inoculated bacteria simultaneously. The four types of nanoparticles were used in concentration 100 mg/L. The proposed complexes were made by incubating the nanodiamonds with the peptides fraction for 30 min at room temperature.

The concentration of the peptides was determined by the micro-biuret method.

Surface inoculation – *B. laterosporus BT271* was cultivated on Nutrient agar. The seeding material was inoculated and spread on the top of the agar layer. The bacterial suspension contained $1.8{-}2.0 \times 10^7$ cells per petri dish. The mixture of nanodiamonds and snail peptides was put as drop on the agar with the bacteria. The petri dishes were incubated for 48 h. The results were calculated on the base

of the clear area with no bacterial growth.

In-depth inoculation – the bacteria were suspended in agar layer, while the mixture ND+peptides were inoculated in wells with diameter of 7–8 mm. In each petri dish 15 mL of the media were poured. It contained definite number of cells – $1.8{-}2.0 \times 10^7$ cells per petri dish. In the wells in the agar 50 µL of mixture of ND+pepdides was inoculated. The petri dishes were incubated for 48 h. The results were calculated on the base of the clear area with no bacterial growth around the wells.

The results are represented as area without bacterial growth per 1 mg of protein per 1 µL of the sample and were calculated by the following formula:

$$AB\ (\mathrm{mm}^2/\mathrm{mgP{*}\mu L}) = a\ (\mathrm{mm}^2)\ /\ P\ (\mathrm{mg}){*}V\ (\mu\mathrm{L}),$$

where AB is the antibacterial effect, a is the area with no microbial growth, P is the concentration of proteins, V is the inoculated volume of the sample.

Results and discussion. It is known that nanodiamonds are non-toxic, bio-compatible nanoparticles. The results obtained from the investigation of the antibacterial properties of these carbon nanoparticles once more confirmed their suitability for biological applications – nanodiamonds, applied alone, did not show any inhibiting effect on the bacterial growth in surface or in-depth inoculation.

Peptides and complexes of peptide-ND. MALDI-TOF-MS analyses of isolated fractions from mucus of garden snail *Cornu aspersum* show peptides with different molecular masses (Fig. 1).

The electrophoresis (Fig. 2a) revealed several compounds in a region 26–33 kDa, at ∼ 42 kDa, between 45–50 kDa, at ∼80 kDa, and between 100–250

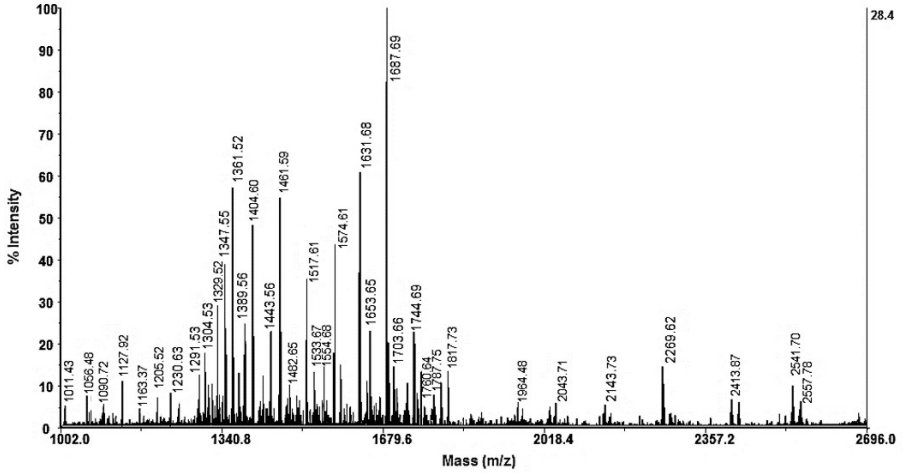
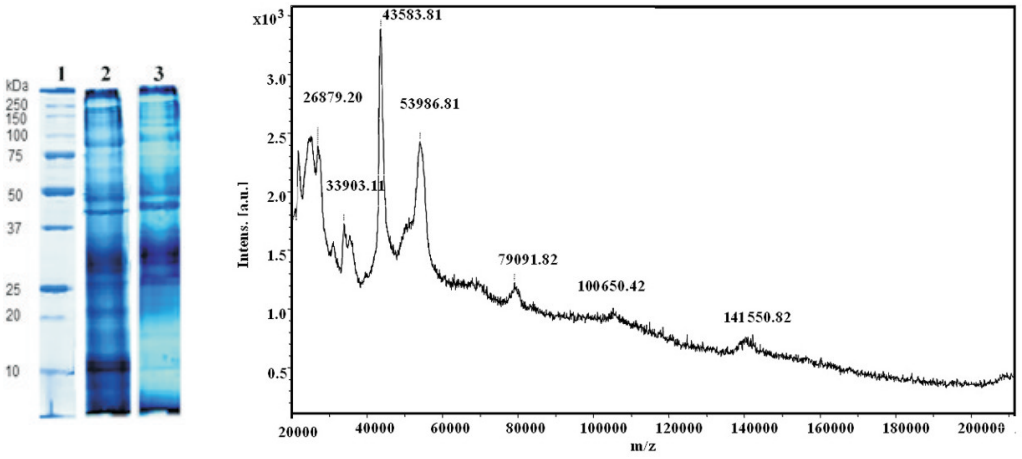


Fig. 1. Snail mucus fraction below 10 kDa – MALDI-TOF-MS spectrum of peptides in supernatant

kDa. Using MALDI-TOF/MS assays, accurate protein masses in active fraction above 30 kDa were determined. As shown in Fig. 2b, the presence of proteins with masses in region 25–150 kDa was confirmed. Some of these proteins are analogous to proteins in the mucus that have been identified through previous researches in the mucus of *C. aspersum* [13] and *A. fulica* [14]. The presented data (Fig. 2b) confirmed the presence of protein with mass 53986.81 Da. Furthermore, the protein determined in a region ∼80 kDa (detected at m/z 79091.82 Da) probably corresponds to protein with MW of 83.67 kDa (achacin) which is active against *Streptococcus mutans* and *Actinobacillus actinomycetemcomitans* isolated from mucus of another terrestrial snail *A. fulica* [14].



a) b)

Fig. 2. Snail mucus fraction with MW > 30 kDa: a) 12.5% SDS-PAGE of protein fractions in the mucus (**position 1** – standard; **position 2** – sample crude mucus extract; **position 3** – mucus fraction above 30 kDa); b) MALDI-TOF-MS spectrum in the range 20–200 kDa

Proteins in a region above 100 kDa could have corresponded to mucoproteins (mucins) detected in mucus from *Helix aspersa*, *Helix pomatia* [15], and mucus secretions obtained from of *Achatina fulica* and *Archachatina marginata*, with antibacterial activity [16]. The in vitro investigations showed that water extracts of mucus from *A. marginata* and *A. fulica* have high antibacterial potency on bacterial isolates (including *Staphylococcus* sp., *Pseudomonas* sp. and *Streptococcus* sp.) from wounds of 28 patients [16].

The results presented in Fig. 2 are in agreement on the antimicrobial properties of the mucus from *H. aspersa, C. aspersum* and *A. fulica* [13,14,17]. Detected proteins at m/z 26879.2 Da, 33903.11 Da, 43583.81 Da, 53986.81 Da, 79091.82 Da, 100650.42 Da and 141 550.82 Da are specific for mucus of garden snail *C. aspersum* grown in Bulgarian farms and probably are responsible for its antibacterial activity.

Antimicrobial activity of peptides and ND. The surface application of the peptides had 7 times stronger antibacterial effect compared with in-depth inoculation. This effect was further increased by combining the peptides with ND (Table 1). These effects could have clinical importance because ND are non-toxic and increase the bactericidal activity of the peptides when applied directly.

T a b l e 1

Antibacterial effect of the two protein fractions with and without ND

Protein fraction	*Less than 10 kDa*		*More than 30 kDa*	
Culturing method	In-depth inoculation	Surface inoculation	In-depth inoculation	Surface inoculation
Effect of the protein (without ND)	156.96 mm^2/mgP*µL	1145.24 mm^2/mgP*µL	184.53 mm^2/mgP*µL	1329.30 mm^2/mgP*µL
Effect of the protein with ND (mean value for the four tested ND)	129.91 mm^2/mgP*µL	1404.72 mm^2/mgP*µL	167.28 mm^2/mgP*µL	1593.04 mm^2/mgP*µL
Effect of the positively charged ND (compared to peptide fractions only)	83.80%	115.69%	102.72%	110.60%
Effect of the single digit ND (compared to peptide fractions only)	83.90%	124.31%	77.44%	118.68%
Effect of the ND (size 2–10 nm) (compared to peptide fractions only)	81.68%	119.88%	89.10%	141.68%
Effect of the ND (size 1 nm) (compared to peptide fractions only)	81.68%	130.75%	93.34%	108.40%

The highest antibacterial effect was reached when nanodiamonds were attached to the proteins in the fraction of molecules with MW above 30 kDa (1593.04 mm^2/mgP*µL) (Table 1). The fraction with bigger MW probably contained more complex molecules. Likely this contributed to the strongest effect. The antibacterial effects of the different nanodiamonds in combination with peptides with MW below 10 kDa were very similar among the different combinations (Fig. 3a, c). This indicates that the registered effects are more related to the presence of the nanoparticles than to their structural properties.

The mixture of nanodiamonds and peptides with MW above 30 kDa showed higher antibacterial activity in the in-depth inoculation (with 37.37 mm^2/mgP*µL) and in the surface inoculation (with 188.32 mm^2/mgP*µL) compared to the complexes with peptides from < 10 kDa fraction (Fig. 3b, d). The higher antibacterial activity found in the experiments with the protein fraction with MW above 30 kDa is probably related with the fact that the active compounds are with bigger, more complex molecules. Thus, they can have more complex action on the

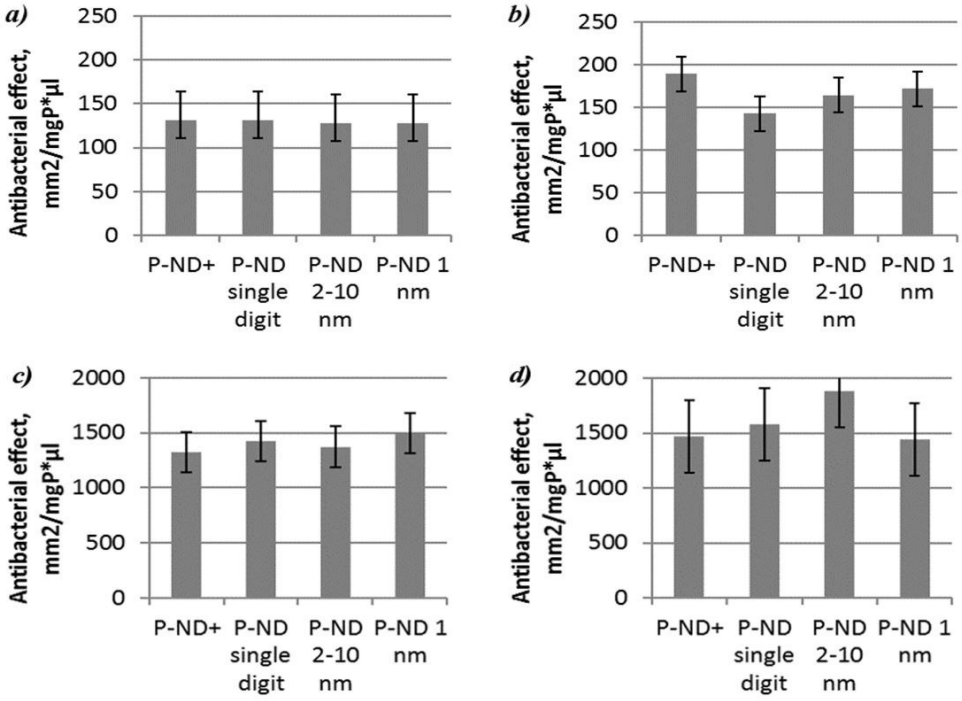


Fig. 3. Antibacterial effect in in-depth inoculation of *B. laterosporus*: a) peptide fraction with MW less than 10 kDa with four types of nanodiamonds, and b) peptide fraction with peptides with MW more than 30 kDa with four types of nanodiamonds; and in surface inoculation: c) peptide fraction with MW less than 10 kDa with four types of nanodiamonds, and d) peptide fraction with peptides with MW more than 30 kDa (*P-ND+* – peptides and positively charged nanodiamonds, *P-ND single digit* – peptides and single digit nanodiamonds, *P-ND 2–10* nm – peptides and detonation nanodiamonds with size 2–10 nm, *P-ND 1* nm – peptides and detonation nanodiamonds with size 1 nm)

microorganisms. That is probably the reason why the antibacterial effect varied in higher extent when proteins are in combination with nanodiamonds while the peptides with lower MW (< 10 kDa) had more unified activity.

Contrary to the results for in-depth inoculation, the data obtained from surface inoculation showed that nanodiamonds improved the antibacterial effect when applied directly to the microorganisms. This was true for both peptide fractions but the augmenting effect was stronger for the one with MW above 30 kDa (up to 1883.35 mm^2/mgP*µL). For both fractions strongest effect was registered for the detonation nanodiamonds which increased the peptides activity by up to 42% (Table 1). This could be related to the availability of functional groups as carboxyl- and hydroxyl- which can interact with peptides and make their transport more efficient. Single digit nanodiamonds also raised considerably (22%) the bactericide properties of the peptides (Table 1). These particles do not form large aggregates

and probably facilitate the transport of the biologically active molecules through the lipid membranes of the cells.

Conclusions. The results obtained in the present study showed that the peptide fractions with MW below 10 kDa and above 30 kDa isolated from *C. aspersum* mucus had antibacterial effect against the Gram (+) *B. laterosporus*. This effect was much stronger when they were applied directly to the microorganisms and also when they are in mixture with nanodiamonds.

The future investigations will be directed to investigate the interaction of ND and peptides in the peptide and protein fractions to elucidate the mechanisms of the antibacterial activity.

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