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# Reduction of cytotoxicity of Myramistin by adsorption on nanodiamonds

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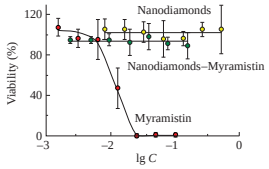
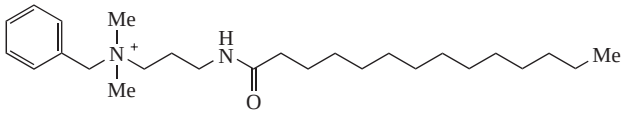
**Detonation nanodiamonds, being a good sorbent, were shown to affect considerably cytotoxicity of cationic surfactants and to possess the significant binding efficiency.**




Nanodiamonds (NDs) produced by detonation[1] reveal outstanding hardness, chemical stability, high thermal conductivity, biocompatibility, and high adsorption capacity. Possible applications of NDs in the design of drug delivery systems are the issue of numerous reviews and monographs.[2–13] High surface area of NDs and the presence of functional groups on their surface result in both a pronounced ability to influence the properties of adsorbed water[14] and high adsorption capacity in relation to surfactants[15] and biomolecules.[16]

Recently, widely used antiseptic benzyldimethyl[3-(myristoylamino)propyl]ammonium chloride monohydrate, commercially available as Myramistin®, was reported to effectively adsorb on the surface of NDs.[17] The adsorption isotherm obeyed the Langmuir law at low concentrations of the surfactant. It was also demonstrated that the adsorbed Myramistin could be removed from the adsorption layer only in the presence of serum albumin. The biological activity of Myramistin is assumed to be possibly modulated by adsorption on NDs.




Myramistin

The aim of this study was to reveal the changes in Myramistin cytotoxicity and uptake by eukaryotic cells as a result of its adsorption on NDs.

Tritium label was introduced in both Myramistin (Infamed, Russia) and NDs (Sinta, Belarus) by means of tritium thermal activation method.[17,18] Additionally, Myramistin was analyzed by HPLC after purification from the labile tritium[†] and TLC. Final specific radioactivity of NDs and Myramistin were 6.0 and 7.5 Ci $g^{-1}$, respectively. Then, Myramistin was adsorbed on the surface of NDs according to the previously described procedure.[‡,17] The surfactant adsorption was about 60 μmol $g^{-1}$. These complexes were used in the experiments with the adhesive epithelial-like MCF-7 cells.[§] The cytotoxicity was analyzed according to the published procedure.[19] Cell viability was evaluated by the MTT assay[20] and calculated as the ratio of survived cells in the test samples to the control. The cytotoxicity was estimated as a concentration of active agent in the sample corresponding to 50% of cell survival ($IC_{50}$). This value was estimated by non-linear fitting of the experimental data.

$$\text{Viability (\%)} = \frac{100\%}{1 + (IC_{50}/C)^p}, \quad (1)$$

where $C$ is the concentration of the sample, and $p$ is the parameter indicating the steepness of the dose response viability curve.

Nanodiamonds possessed positive ζ-potential in water (approximately +30 mV) and were expected to show high cytotoxicity, as far as in most cases interaction between cationic particles and negatively charged membrane components results in development of toxic effects.[21] However, resuspending NDs in DMEM resulted in the reversal of their charge, ζ-potential decreased to –10 ± 3 mV, obviously due to adsorption of anions of DMEM on NDs surface.

† NDs were purified from the labile tritium by washing with water.[18] An Agilent 1200 system was used to perform HPLC. The mobile phase with a linear gradient consisted of solution of acetonitrile and 1% aqueous trifluoroacetic acid. UV detection was carried out at 260 nm.

‡ Radioactive label was either in Myramistin or in NDs. The suspension of NDs or [$^3$H]NDs (NDs content, 0.7 mg) was mixed with [$^3$H]Myramistin (or Myramistin, 2 g $dm^{-3}$). The complex was purified from free Myramistin *via* multiple centrifugation and resuspension in the distilled water (7 times).

§ This cell line was maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% of fetal bovine serum, 50 mg $dm^{-3}$ streptomycin, and 50 units $cm^{-3}$ penicillin. The cells were grown in the incubator (NAPCO, USA) at 37 °C in atmosphere of 95% air and 5% $CO_2$. First, the cells were seeded into 96-well flat-bottomed polystyrene plates, where cell density was about $1 \times 10^4$ $cm^{-2}$ (3500 cells per well) a day before the experiment. Then the test solutions/suspensions containing Myramistin, or NDs, or their complexes (antiseptic content was about 60 μmol $g^{-1}$) were appropriately diluted in the serum-free culture medium. The cells were treated with these test samples (0.2 $cm^3$ per well) and incubated for 1 h in $CO_2$ atmosphere. The control samples were treated by the serum-free medium without additives. All experimental points were carried out in triplicate. After incubation, the test solutions were replaced with the medium containing 10% serum, and the cells were maintained for additional 3 days under standard conditions.

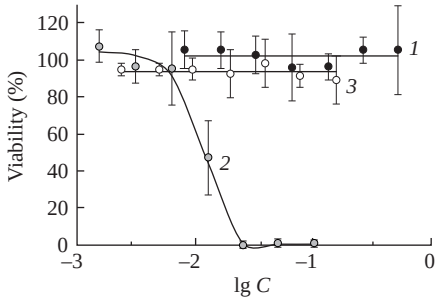



**Figure 1** Viability of MCF7/R cells as a function of the active agent concentration: (*1*) NDs, (*2*) Myramistin and (*3*) Myramistin adsorbed on NDs.

Adsorption of Myramistin on the surface of NDs also resulted in the considerable broadening in ζ-potential distribution, but the mean value remained practically unchanged (Figure S1, Online Supplementary Materials). This result points to non-uniform character of Myramistin binding to NDs. Importantly, the mean diameter of nanoparticles remained nearly unchanged upon Myramistin adsorption indicating that the formation of very thin monomolecular layer occurred on NDs surface.

Negative charge of NDs dispersed in culture medium results in their negligible cytotoxicity (Figure 1, curve *1*), NDs did not exhibit any toxic effects up to concentration of 520 mg dm$^{-3}$. These result is in agreement with published data,[3] where NDs with different surface groups displayed low toxicity toward different types of cells.

In contrast, Myramistin exhibited very high cytotoxicity, $IC_{50}$ value estimated according to equation (1) was 27 ± 0.9 μmol dm$^{-3}$ (12.5 ± 0.4 mg dm$^{-3}$). The maximal tolerable concentrations of Myramistin were about 13 μmol dm$^{-3}$ (6 mg dm$^{-3}$), which was lower than critical micelle concentration (6 mmol dm$^{-3}$).[23]

Moreover, adsorption of Myramistin on the surface of NDs resulted in a significant decrease in its cytotoxicity. Thus, we can conclude that (i) Myramistin–NDs complex is stable under the experimental conditions and (ii) despite of the broadening of ζ-potential distribution after Myramistin adsorption, the mechanism of surfactant binding is both hydrophobic and electrostatic in nature, otherwise Myramistin–NDs complex would demonstrate cytotoxicity.

To determine binding efficiency of the test compounds with cells,¶ we suspended [$^3$H]NDs, [$^3$H]Myramistin, [$^3$H]NDs–Myramistin, and [$^3$H]Myramistin–NDs in DMEM containing glutamine (4 mmol dm$^{-3}$) and sonicated the mixture for 2 h. Final concentrations and specific radioactivity are summarized in Table 1.

Represented as the dependence of the relative amount of active agent bound to MCF7/R on the amount of cells in the sample, binding isotherms were linear and intersected the *y*-axis at the value above zero (Figure S2) that is determined by some unspecific binding of active agent. Similar and relatively large sections on the *y*-axis were distant from zero for all samples containing NDs due to considerable contribution of NDs sedimentation to the observed values. At the same time, adsorption of Myramistin on NDs particles did not affect their sedimentation behavior. In contrast to NDs, binding isotherm of Myramistin had nearly zero sections on the *y*-axis that is consistent with hydrophilic nature of this antiseptic.

**Table 1** Concentrations and specific radioactivities of NDs and Myramistin in the suspensions.

| Suspension composition | NDs concentration/ mg dm$^{-3}$ | Myramistin concentration/ mg dm$^{-3}$ | Specific radioactivity/ mCi dm$^{-3}$ |
|---|---|---|---|
| [$^3$H]Myramistin–NDs | 37.9 | 0.95 | 1.92 |
| [$^3$H]NDs–Myramistin | 51.0 | 1.28 | 2.70 |
| [$^3$H]Myramistin | – | 0.42 | 1.06 |
| [$^3$H]NDs | 29.1 | – | 1.48 |

An increase in the cell monolayer density gradually led to the elevated amount of bound active agent, however, to a different extent for each active agent. The slopes of binding isotherms reflected the ability of the active agents to bind to cells, which is referred to as binding efficiency (BE) below.

The obtained results indicate that NDs exhibit very high BE values (BE = 0.035 ± 0.007 per million cells), two orders of magnitude higher than those previously observed for amphiphilic copolymers of polypropylene oxide and hyperbranched polyglycerol[19] and Pluronics[22] under similar conditions. It seems likely that extremely high BE of NDs is a consequence of their deposition and concentrating at the cell monolayer during incubation.

Adsorption of Myramistin on these particles slightly diminished their binding to cells efficiency (BE = 0.017 ± 0.003 per million cells) indicating that the formation of the adsorption layer on the surface of NDs did not favor the uptake by cells. However, by tracking the [$^3$H]Myramistin in the complex with NDs, the considerable accumulation of the complex in cells (BE = 0.127 ± 0.007 per million cells) was revealed, implying that uptake of the particles covered with large amounts of Myramistin may effectively deliver the latter to the cells. BE value of ND–[$^3$H]Myramistin complex was even higher than that of [$^3$H]Myramistin (BE = 0.102 ± 0.004 per million cells) and reflected high efficiency of uptake of the complex. However, interaction with cells did not result in a considerable release of Myramistin from its complex with NDs, which may be concluded from very low toxic effect observed in this case.

Thus, the obtained results demonstrate that detonation NDs can be successfully used as carriers for cationic amphiphilic compounds, despite the fact that no release of the active substance from these complexes may be expected. The observed phenomenon should be taken into account when considering NDs as drug carries.

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*Online Supplementary Materials*

Supplementary data associated with this article can be found in the online version at doi: 10.1016/j.mencom.2017.07.036.

¶ The suspensions (0.9 cm$^3$) were added to MCF7/R cells in 12-well plates, and the amount of cells was varied from 0 (control sample) to 0.68×10$^6$. The wells were incubated at 37 °C in $CO_2$ atmosphere for 1 h. Then, the cell monolayers were carefully washed with PBS (5 × 1 cm$^3$) and lysed in 1 N NaOH (0.25 cm$^3$) overnight. The lysates (about 0.10 cm$^3$) were used for estimation of protein content by the Lowry method. The amount of cells in each sample was calculated according to those values and the calibration curve, which was plotted in the course of the main experiment.[19] The amount of bound active agent was estimated by measuring the radioactivity of the lysates and normalizing the values obtained to the amount of cells.

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