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International Journal

Research Article

Nano diamonds as Effective Vectors for Micro RNA-Based Drug Delivery

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Received: May 10, 2018; **Published:** May 16, 2018

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Abstract

Micro RNAs are short non-coding RNA molecules, which are important regulators of cell functions. Deregulation of certain micro RNA level is contributing factor to tumor formation and progression. Possible gene therapy by modulation of the micro RNA level is currently considered. Recently it has been proved that nano diamonds coated with PEI serve as efficient transfection and monitoring system. The aim of our work was to deliver micro RNA inhibitor and micro RNA mimic sequences into specialized mammalian cells using fluorescent nano diamond particles and to test the nano complex uptake and cell compatibility during primary *in vivo* application. Short RNA molecules were bound to the PEI coated nano diamonds, transported into the colon cancer cell line in vitro and observed in a confocal microscope. The miRNA mimic coated nano diamonds were used for complex uptake and toxicity study in peritoneal macrophages and splenocytes after *ex vivo* stimulation or *in vivo* administration. Due to the possibility of continuous monitoring of carrier internalization, its transfection efficacy, and cell compatibility, the fluorescent nano diamonds represent a suitable RNA carrier with a potential use in gene therapy.

Keywords: Micro-RNA; RNA interference; Nano Diamond; Drug Delivery; Gene Therapy

Abbreviations: miRNA: micro RNA; ND: nano diamond; PEI: polyethylene-imine; PBS: Phosphate-Buffered Saline; FBS: fetal bovine sera; miR1: miRNA-1 mimic; antimiR21: antisense RNA targeting miRNA-21

Introduction

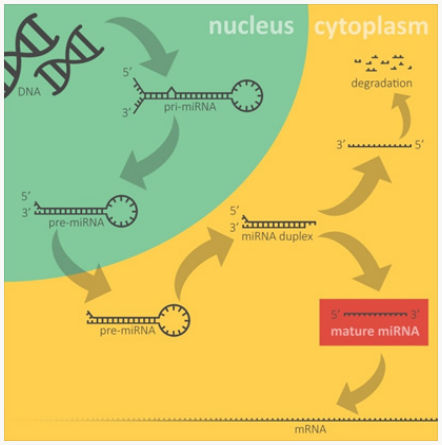

Figure 1: microRNA processing and maturation in mammalian cells.

Micro RNA (miRNA) molecules are short (21bp), single stranded non-coding RNAs that have been identified in many eukaryotic organisms, from the simplest ones to humans. They represent an important regulatory mechanism of cells because they control the level of gene expression at the post transcriptional level. The micro-RISC (miRISC) complex containing matured micro RNA interacts with the complementary mRNA sequences. miRISC complex bounded to complementary mRNA sequence will either inhibit translation or induce mRNA cleavage [1]. Figure 1 shows processing and maturation of precursor micro RNA transcribed from intergene regions of mammalian DNA. The precursor micro RNA undergoes transport into cytoplasm and splicing resulting in matured miRNA capable to regulate target mRNAs.

Due to its regulatory role, alteration of micro RNAs level results in development of pathological stage. Nowadays, alterations such

as low/high expressions or promoter methylations of most micro RNAs have been already associated with specific diseases [2-7]. The prominent regulatory roles of micro RNAs also pointed out micro RNAs as potential target of gene therapy [8]. There are plenty of studies describing potential use of particular micro RNA in various therapeutically approaches including cancer therapy [9]. Regarding cancer development we can divide micro RNAs into two major groups:

I. Micro RNAs promoting cancer cell growth that are often up-regulated in cancer cells and

II. Micro RNAs that is down-regulated in cancers due to their ability to induce apoptosis or inhibit cell proliferation.

Some micro RNAs such as microRNA-21 are of particular interest also due to its great property- circulating in bodily fluids. The microRNA-21 produced by tumor cells in order to increase their proliferation, invasion, and survival is also released into plasma and the situation in tumor reflects the values of circulating microRNA-21 [10-11]. Thus this micro RNA can also serve as a diagnostic marker for cancer development or even maybe a marker of therapy efficacy. Figure 2 shows the urine and plasma levels of microRNA-21 in animals (Balb/c mouse strain) bearing colon carcinoma (derived from CT26 colon cancer cells). The tumor-bearing animals exhibit significantly higher values of microRNA-21 not only in plasma but also in urine that may also offer the possibility of non-invasive tumor diagnostics.

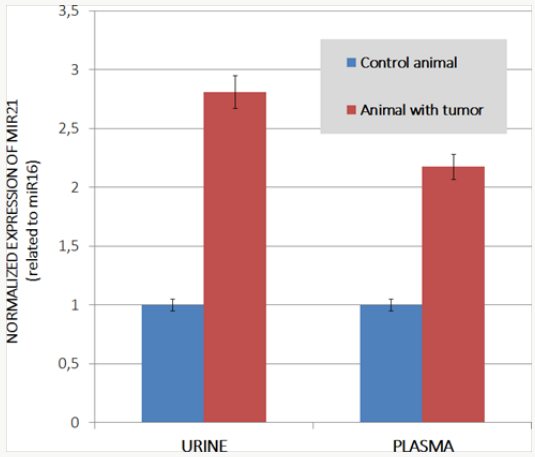

Figure 2: graph showing level of miR-21 after tumor development.

During microRNA-based drug design, the first group of microRNAs, also called oncogenic microRNAs due to their up-regulation in tumors, is targeted by an inhibitory sequence. This sequence-antimiR - is complementary to particular microRNA. On the other hand, in order to replace down-regulated microRNA (antioncogenic microRNA) we design microRNA mimic-a sequence that is identical to the microRNA of interest [12]. One of the main obstacles in gene therapy is the was of transportation of such antimiR or miRNA mimic into specialized cancer cells. The delivery system has to be non-toxic, effective in entering plasma membrane, and capable to release cargo into cytoplasm [13-14]. In this study we present 35nm nano diamond (ND) particles as convenient delivery system for short RNA (antimiR as well as miRNA mimic) transportation into mammalian cells. The nano diamond particles used as carriers offer additional benefit of intrinsic fluorescence that enables their real-time trafficking and monitoring RNA transportation without using any additional fluorophores that could impair the affectivity of RNA delivery and function. Finally we discuss the nano particle uptake and toxicity on primary cells such as peritoneal macrophages and splenocytes during *in vivo* application.

Materials and Methods

ND-PEI-RNA Complex Preparation and Zeta Potential Measuring

The high pressure high temperature synthesized fluorescent nano diamonds of sizes 35nm and 100nm were kindly obtained from Dr. Petráková (the Faculty of Biomedical Engineering, Czech Technical University in Prague). Nanodiamonds with concentration 1mg/ml were coated with 0.9mg/ml PEI 800 and then labelled with 0.5nmol 20bp short RNA (antimiR21 or miRNA1 mimic; ThermoFisher Scientific). Zeta-potential of raw and coated NDs was measured on Zetasizer Nano Z (Malvern).

Cells Stimulation

CT26.WT murine colon cancer (ATCC® CRL-2638™) and IC21 murine macrophage (ATCC® TIB-186™) cell lines were maintained in Gibco™ RPMI media (Thermo Fisher Scientific), supplemented with 10% Gibco™ FBS (Thermo Fisher Scientific) and 44mg/L Gentamicin (Sandoz) in a humid atmosphere with 5% of CO_2. 24 hours before an experiment, cells were seeded in 70-80% confluence in starvation media (without FBS). Final concentration of NDs was 25μg/mL.

Nanodiamonds Uptake Imaging

Cells were seeded on plates with glass bottom and incubated with ND complexes. For visualization purposes the antimiR21 has been labeled with AlexaFluor-488. Cell nuclei were stained with Hoechst 33342 (1μg/ml, Invitrogen) and F-actin fibers were stained using CytoPainter F-actin staining kit (Abcam) according to the manufacturer protocol at the end of the incubation period. The images were recorded with an Olympus Fluo View™ FV 1000 confocal microscope (objective 40X, NA 0.95) and they were analyzed with Olympus Fluo View 2.0 software. Excitation/emission settings on confocal microscope were following: Hoechst (nuclei) 405nm/461nm; AlexaFluor-488 (antimiR21) 488 nm/525 nm; F-actin 550nm/575nm; NDs 559nm/655-755nm.

Isolation of Primary Cells from Balb/C Animals

Balb/c animals (females, age 8-10 weeks) were euthanized according to the protocol approved by Czech Veterinary Committee

and policy for handling with the experimental animals. The peritoneal macrophages were obtained via lavage of peritoneal cavity with PBS (total volume 5ml) and splenocytes were isolated from dissected spleen homogenized via nylon mesh. Obtained cells were washed twice with PBS and re suspended in culture media RPMI 1640 supplemented with 1% FBS.

Cytotoxicity Assays after *Ex Vivo* Application of Nanodiamonds

Peritoneal macrophages and splenocytes isolated from untreated healthy Balb/c animals were seeded on flat bottom 96-well plates in 1% FBS growth media ($2x10^4$ cells/well) and stimulated with NDs and ND-PEI-miR1 complexes for 24 hours. Supernatants were then processed using LDH Cytotoxicity Detection Kit PLUS (Roche) according to the manufacturer protocol and the LDH release was measured using Infinite 200 PRO multimode reader (Tecan). Control transfection was performed using the same RNA concentration and a commercial transfection reagent X-treme GENE HP DNA (Sigma Aldrich) according to the manufacturer protocol.

In vivo ND and ND-PEI-miR1 Application

Three groups of Balb/c animals (3 females per group, age 8-10 weeks) were injected twice with either PBS, NDs, or ND-PEI-miR1 complexes 24 hours and 30 minutes prior the cells withdrawal. 30 µg of NDs in PBS per one dose (with appropriate amount of linked miR1 in the third group) were injected intraperitoneally (i.p.). At the end of incubation time, splenocytes and peritoneal macrophages were harvested as described earlier and tested for nanoparticle uptake and viability.

Cytotoxicity Assay After *In Vivo* Application of Nanodiamonds

Peritoneal macrophages and splenocytes isolated from Balb/c animals were stained by Propidium Iodide and analyzed by the BD LSR II flow cytometer in order to reveal viable (Propidium Iodide negative) cells. Forward scatter (FCS) parameter analyzed by the flow cytometer was used to evaluate changes in cell appearance after nanoparticles uptake.

Results and Discussion

In order to successfully deliver RNA molecules into specialized cells, the RNA molecules had to be effectively coated onto Nanodiamond particles. The advantage of the dual coating system (PEI800 and RNA) enables to stabilize the nanoparticle complexes and link negatively charged RNA to negatively charged particle. Moreover the bond between PEI800 and RNA is pH sensitive that subsequently enables to release cargo in endosomal vesicles [15-16]. The use of PEI800 and sharp edged nanodiamonds also promotes destabilization of the endosomal membrane and release of RNA into cell cytoplasm [16-17]. The successful PEI surface coating and RNA linkage can be evaluated by measurements of zeta-potential of the solutions in each step during complex preparation. Figure 3 shows zeta-potential of uncoated NDs and NDs coated with PEI800 or PEI800 and RNA (miRNA1 mimic or antimiR21). For bare NDs the value of zeta-potential is around -30mV. When the surface is treated with PEI 800, value of zeta-potential shifts to values over +30mV. After RNA links to the ND-PEI surface, zeta-potential again shifts to values around -30mV.

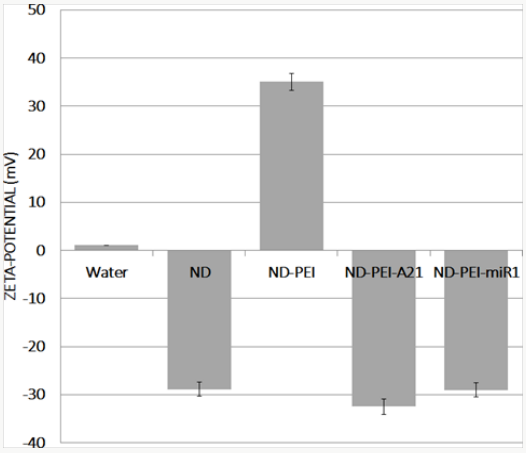

Figure 3: graph showing zeta-potential values of ND, ND-PEI, ND-PEI-A21, and ND-PEI-miR1.

The efficient uptake of RNA carrier is essential for RNA delivery. First we have tested bright 100 nm nanodiamond particles that enable easy detection and characterization of internalized particles. In order to internalize such a big particles we used phagocytic cells that are naturally engulfing bigger particles from their surroundings. Thus IC21 mouse immortalized macrophages were used to visualize 100 nm fluorescent NDs of concentration of 1mg/ml. Figure 4 shows internalized nanodiamonds excited with 559 nm laser, emission in far red spectrum (655-755nm) is very strong. We compared fluorescence of coated and uncoated NDs: first row (A) shows cells stimulated with bare NDs, second row (B) shows stimulation with ND-PEI, third row (C) shows cells which were stimulated with ND-PEI- miR1 complex. The 100 nm particles are very clearly visible however we aimed to deliver RNA into cells such as cancer cells that lack the engulfing capacity of macrophages and smaller particles are internalized much easier. Also the 100 nm particles with coating might be too big to be administered *in vivo* and reach high rate of accumulation within the tumor via enhanced permeability effect [18].

To ensure the high internalization rate into cancer cells we have used smaller (35nm) particles. Staining of CT26.WT cells with Phalloidin was performed to visualize any potential structural changes in F-actin fibers of the cells stimulated with ND-PEI-antimiR21. AntimiR21 linked on ND-PEI was labelled by fluorescent probe (AlexaFluor-488) because ND and Phalloidin emission spectra interfere and therefore they could not be distinguished from each other. Figure 5 shows results from confocal microscopy, first picture is an overlay of detected fluorescence from all measured channels - signals from Hoechst (nuclei), Phalloidin (F-actin), and

AlexaFluor- 488 (ND-PEI-antimiR21). Here we showed that the 35 nm particles coated with PEI and antimiR21 internalize in high rate into colon cancer cells. The antimiR21 linked to the nanodiamond particles has been efficiently released into cytoplasm, restored expression of miRNA21 target tumor suppressor genes, and lead to the inhibition of cancer cell proliferation and invasion and to the induction of apoptosis [16]. In order to target tumor cells *in vivo* the successful RNA carrier has to exhibit (next to its effective internalization into tumor cells) no adverse effect on immune cells or hematopoietic organs like spleen (in mouse models). Since the nano complexes can be internalized by macrophages and potentially they could accumulate in organs such as spleen [19] we performed primary *ex vivo* and *in vivo* studies to reveal the possible toxic effect and rate of internalization. We chose primary cells that are i) the first cell population encountering nanodiamond complexes in multicellular organism after i.p. administration - the peritoneal macrophages and ii) the cells that will encounter nanodiamond complexes possibly accumulated in spleen. Figure 6 shows results from *ex vivo* experiment, cells isolated from Balb/c animal were stimulated with ND-PEI-miR1 complexes for 24 hours. Confocal microscopy was performed to find out if it is possible to detect internalized ND complexes and their distribution. LDH assay was done on the same sample to exclude potential ND-PEI-miR1 toxicity. Positive ND signal was detected in peritoneal macrophages (Figure 6D) that were stimulated with ND-PEI-miR1. There are no positive ND specific signals in splenocytes (Figure 6E). LDH assay showed no significant toxicity of ND-PEI-miR1 on neither peritoneal macrophages nor splenocytes. We also delivered miRNA1 mimic via commercial transfection reagent (HP-miRNA1 samples) and there was no difference in toxicity when compared to RNA delivered by nanodiamonds or untreated cells.

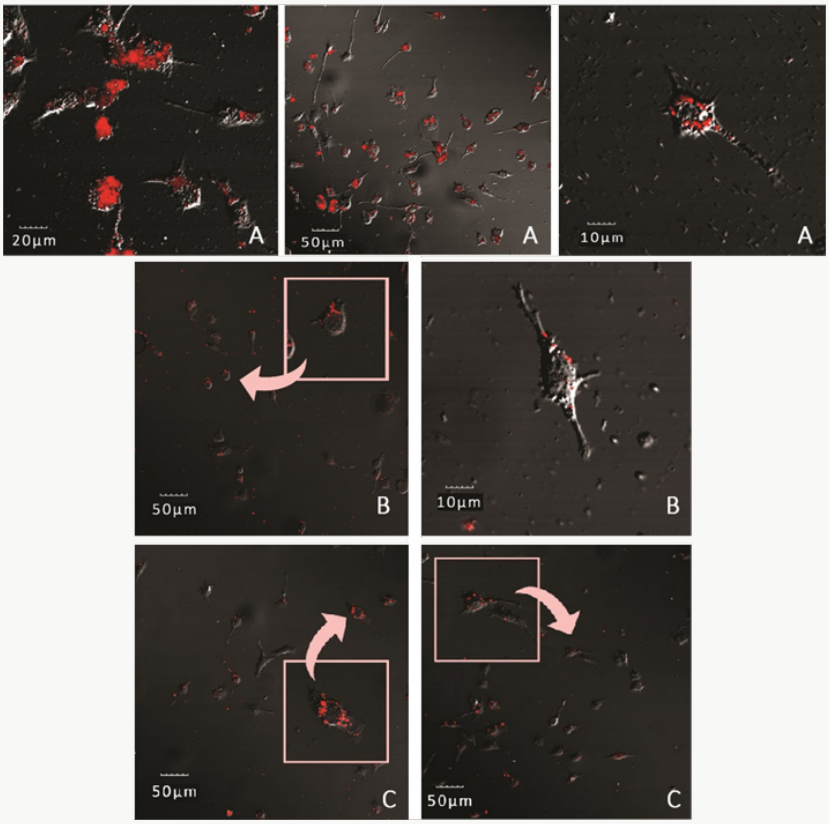

Figure 4: Detection of 100nm nanodiamonds inside IC21(macrophage) cells; A)ND, B)ND-PEI, C)ND-PEI-miR1.

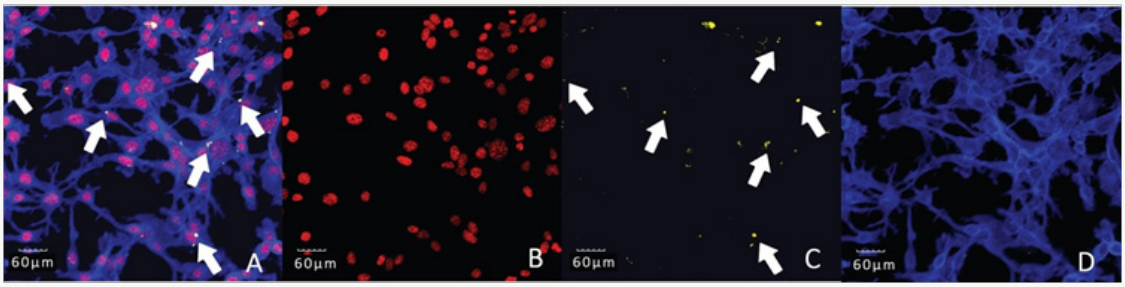

Figure 5: Detection of ND-PEI-antimiR21 in CT26 (colon cancer cells); A)merged, B)nuclei, C)35nm nanodiamonds-PEI-antimiR21, D) F-actin.

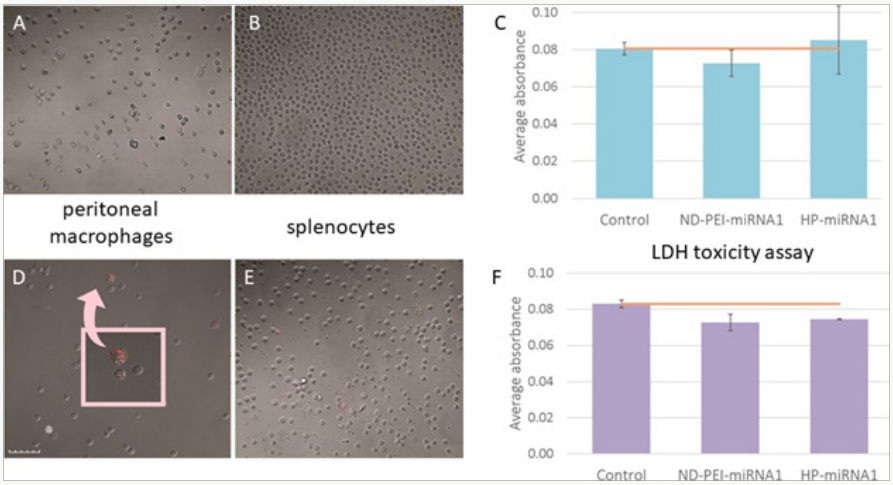

Figure 6: ex vivo uptake of ND-PEI-miR1 by primary cells isolated from Balb/c mouse strain and LDH toxicity assay; A-B) negative control - no diamonds applied, D-E)stimulation with ND-PEI-miR-1 for 24 hours. Scale bar represents 100 µm; LDH assay measured on: C)peritoneal macrophages, F) splenocytes.

Figure 7 shows *in vivo* experiment that included 3 groups of Balb/c animals treated i.p. with two consecutive doses of PBS, ND, or ND-PEI-miR1. Cells isolated from animals 24 hours after the first administration were visualized by confocal microscopy and analyzed by flow cytometry. Positive ND signal was detected in peritoneal macrophages (Figure 6C) from animals injected with ND-PEI-miR1. There were no positive ND signals in splenocytes (Figure 6D) isolated from animals treated with ND- PEI-miR1. Flow cytometry of isolated cells stained with Propidium Iodide showed no significant toxicity of ND-PEI-miR1 on neither peritoneal macrophages nor splenocytes (Figure 7E). There was a significant shift in forward scatter parameter (Figure 7E) in population of peritoneal macrophages isolated from animals that received NDs alone and in lesser extent there was a shift in cells isolated from animals that received ND-PEI-miR1 complex. This can reflect the fact that the NDs without coating can interfere with biological membranes and adhere to the membrane before the internalization [17]. The NDs shielded via polymer PEI800 and RNA coating might adhere in a lesser extent or they are faster internalized than the uncoated NDs.

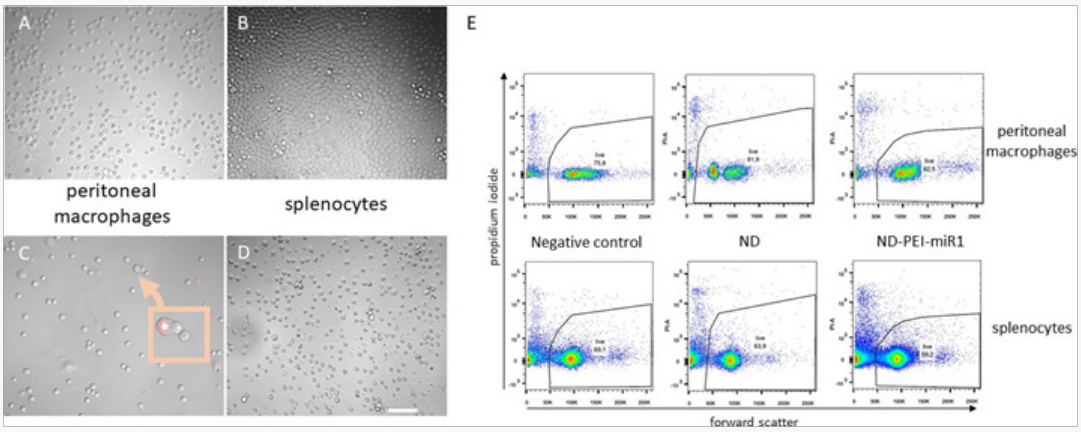

Figure 7: *in vivo* uptake of ND-PEI-miR1 injected i.p. into Balb/c mouse strain; A-B)negative control–saline buffer (PBS) applied, C-D)i.p. application of ND-PEI-miR-1. Viability assay of primary cells after i.p. nanocomplex application (E). Scale bar represents 100µm.

Conclusion

The fluorescent nanodiamonds showed great properties as carriers of short RNAs. The surface of nano diamond particles can be effectively coated with antimiR as well as with miRNA mimic molecules. Due to intrinsic long-term fluorescence of nano carrier the transfection complex can be monitored as it enters the target cells cytoplasm. The nano construct containing nano diamond, PEI, and short RNA is not toxic to the primary cells that are first encounters when the complex is applied into multicellular organism. It is possible to detect the nanocomplex internalized into phagocytizing cells and distinguish specific cell morphology changes via conventional assay on flow cytometer.

Acknowledgement

The authors thank to Dr. Vlaďka Petráková (Czech Technical University, FBME, Kladno) for providing us with nano diamond particles and to Dr. Ivan Rehor (Institute of Organic Chemistry

and Biochemistry, CAS, Prague) for assisting with zeta-potential measurements. The present study was supported by the Ministry of Health of the Czech Republic (grant no. 15-33094A).

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