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SHORT COMMUNICATION

Detonation nanodiamonds are promising nontoxic delivery system for urothelial cells

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Abstract Detonation nanodiamonds (DNDs) are carbon-based nanomaterials that are among the most promising nanoparticles available for biomedical applications so far. This is due to their biocompatibility, which could be contributed to their inert core and conformable surface nature. However, DNDs cytotoxicity for urothelial cells and the routes of their internalization remains an open question in the aspect of nanodiamond surface. We therefore analyzed four types of DNDs for cytotoxicity and internalization with normal urothelial cells and two types of cancer urothelial cell lines in vitro. Viability of any of the cell types we used was not compromised with any of four DNDs we evaluated after 24-, 48- and 72-h incubation in three different concentrations of DNDs. Transmission electron microscopy revealed that all four types of DNDs were endocytosed into all three types of urothelial cells tested here. We observed DNDs in endosomes, as well as in multivesicular bodies and multilamellar bodies. These results propose using of DNDs as a delivery system for urological applications in human nanomedicine.

Keywords Detonation nanodiamonds · Cytotoxicity · Internalization · Transmission electron microscopy · Urothelial cells · Delivery system

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Introduction

Detonation nanodiamond (DNDs) is a carbon derived nanomaterial that has become a promising candidate in biological and medical applications in recent decades (Ho 2009; Holt 2007; Kaur and Badea 2013). They are very useful, e.g., for targeted drug delivery, as markers for cell imaging, as biosensors and stable solid support for synthesis of peptides and for coating of medical implants (Chen et al. 2013; Holt 2007; Lien et al. 2012; Zhu et al. 2012). Although DNDs biocompatibility is widely accepted fact (Kaur and Badea 2013; Paget et al. 2014; Schrand et al. 2007), their risk for human health remains largely unknown. Numerous studies supported the notion that DNDs do not induce any significant cytotoxic effects on different cell lines for example renal (Yu et al. 2005), liver, kidney and intestine cell lines (Paget et al. 2014). However, they have not been tested yet on the urothelial cells of the urinary bladder and their ultrastructural localization within the cells has not been determined.

DNDs are stable nanoparticles and can be subjected to surface modifications allowing the introduction of the desired functional groups or biologically active molecules (proteins, nucleic acids, drugs) onto their surface (Kaur et al. 2012; Liu et al. 2004). DNDs, also known as ultradispersed diamonds, are synthetized relatively inexpensively on a large scale by the detonation of carbon explosives (Greiner et al. 1988). Their attractiveness for various biomedical applications is enhanced

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due to their low cost synthesis method, good biocompatibility, simplistic surface functionalization, and especially because no release of xenogenic metals producing free radicals is possible for refined DNDs and are by these differing from metal-core nanoparticles.

Here we report for the first time that DNDs entered the normal and cancer urothelial cells, but did not induce any significant cytotoxic effects. Transmission electron microscopy revealed that DNDs were internalized into the cells and ended up in different types of endocytotic compartments.

Materials and methods

Four different types of DNDs, denoted as NSFPA, NASHCl, YTM (YTM ARGE A.S., Turkey) and DND-30 (Grish, China), were prepared as described previously (Keremidarska et al. 2014; Mitev et al. 2014a). NSFPA and NASHCl (later denoted as NSHA in (Keremidarska et al. 2014; Mitev et al. 2014a) were free from metal impurities and non-diamond carbon (in case of NSFPA), while YTM and DND-30 had higher levels of Cr and Ba, Pb, respectively (Mitev et al. 2014b). The experiments were approved by the Veterinary Administration of the Slovenian Ministry of Agriculture and Forestry (permit no. 34401-1/2010/6). Normal porcine urothelial cell cultures (NPU), human papillary urothelial neoplasm (RT4) and invasive urothelial neoplasm (T24) cell lines were cultured as previously described (Resnik et al. 2015; Visnjar et al. 2012; Visnjar and Kreft 2015). Briefly, NPU cells were grown in UroM medium, while RT4 and T24 cells were grown in A-DMEM/F12 (1:1) medium. These media without DNDs were termed as control media in our experiments.

For cytotoxicity and internalization assays all three types of cells (NPU, RT4 and T24) were incubated in control medium and in control medium with added each of four types of DNDs (NSFPA, NASHCl, YTM or DND-30) at the concentrations of 5.5, 11, and 22 μg/ml for 24, 48, and 72 h. Relative cell viability was determined by CellTiter-Glo® Luminiscent Cell Viability Assay (Promega) according to the manufacturer's instructions. For internalization assessment, cells were incubated in control medium and in control medium with added each of four types of DNDs (NSFPA, NASHCl, YTM or DND-30) at the concentrations of 11 μg/ml for 24 h. For transmission electron microscopy, cells were prepared as described previously (Visnjar et al. 2012), except that ultrathin sections were not contrasted and were examined with Jeol 100CX transmission electron microscope.

For cell viability analysis, each treatment was performed in triplicates. All of the statistical comparisons between control and four different DNDs groups for each cell type were performed with Microsoft Office Excel (2010 Edition) using ANOVA and the two-tailed unpaired Student's t test, with the level of significance set at $p < 0.001$.

Results and discussion

Cell viability assay showed that normal urothelial cells (NPU) and both types of cancer cell lines, i.e. papillary urothelial neoplasm (RT4) and invasive urothelial neoplasm (T24) cells, were highly viable after 24-, 48-, and 72-h incubation in control medium and in control medium with added NSFPA, NASHCl, YTM or DND-30 detonation nanodiamonds in all three concentrations tested here (5.5, 11, and 22 μg/ml) (Fig. 1). After 72-h incubation in control medium with added DND-30, which have higher Ba and Pb surface content than other DNDs (Mitev et al.

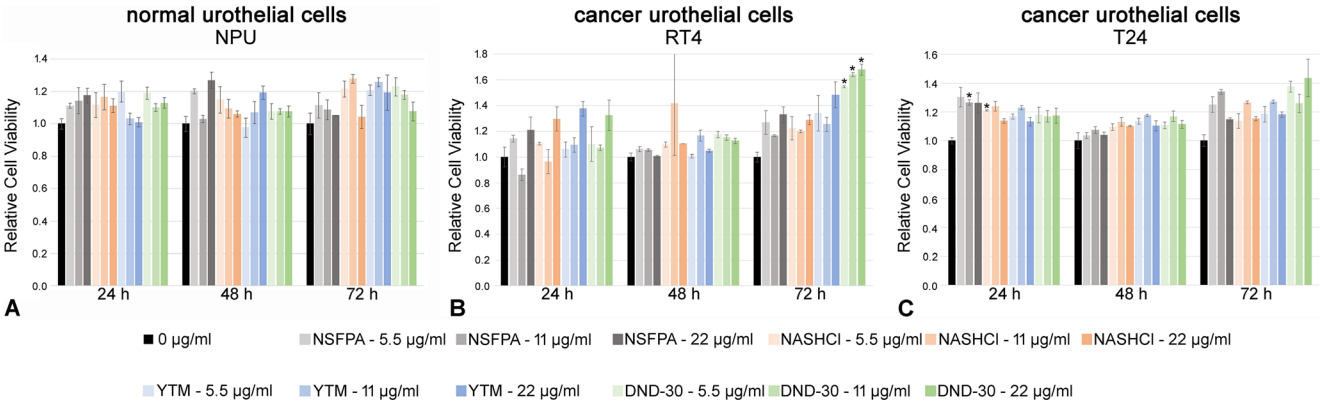

Fig. 1 Effect of DNDs on cell viability measured by CellTiter-Glo® Luminiscent Cell Viability Assay. **a** Relative cell viability of normal urothelial cells (NPU) and **b**, **c** cancer urothelial cells (RT4, T24) after 24-, 48-, and 72-h incubation with control medium and medium with added four types of DNDs (NSFPA, NASHCl, YTM or DND-30). All the comparisons of control medium (0 μg/ml) to each type of DNDs on NPU, RT4 and T24 cells showed no statistically significant differences, except DND-30 on RT4 cells at concentration of 22 μg/ml after 72-h incubation and NSFPA on T24 cells at concentration of 11 μg/ml and NASHCl at concentration of 5.5 μg/ml after 24-h incubation (*$p < 0.001$), which all showed increased amount of ATP present

2014b), the relative cell viability of RT4 cells was statistically significantly increased in comparison to control group (Fig. 1b). Additionally, the relative cell viability of T24 cells after 24-h incubation with NSFPA (11 μg/ml) and with NASHCl (5.5 μg/ml) was statistically significantly increased in comparison to control group, while after 48- and 72-h incubation no statistically significant changes were observed (Fig. 1c). We conclude that DNDs are not cytotoxic to normal and cancer urothelial cells, which is in agreement with previously published data on other cell types (Yu et al. 2005). These results support suggestions that DNDs could be widely exploited in various diseases and conditions such as different types of cancers, in the regenerative medicine as bone and dental implants or parts of implants as well as for antibacterial use.

Transmission electron microscopy revealed that all four types of DNDs were internalized in all three types of cells tested here (Fig. 2). Differently shaped clusters of DNDs with maximal caliper diameter ranging from 100 to 700 nm were observed in different endocytotic compartments within all three types of cells after 24-h incubation. In addition, the aggregate size of the used DND suspensions did not crucially influence the internalization efficiency. Among different endocytotic compartments, containing DNDs, we were able to distinguish endosomes (e.g. Fig. 2a, d, g, h, j, k),

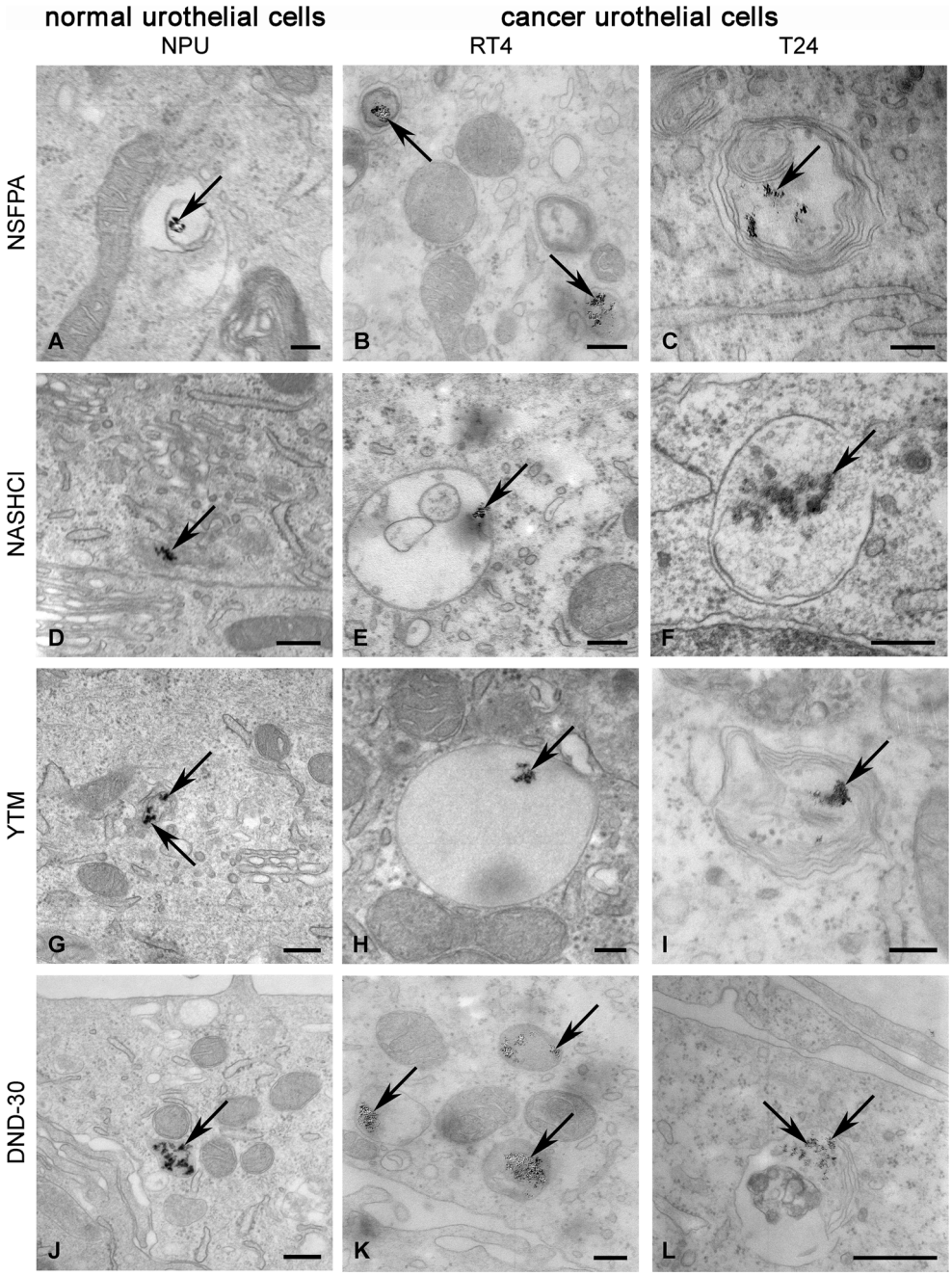

Fig. 2 Internalization of DNDs into normal and cancer urothelial cells. **a–c** Internalization of NSFPA, **d–f** NASHCl, **g–i** YTM and **j–l** DND-30 into NPU, RT4 and T24 cells. In all three types of cells, clusters of DNDs (*arrows*) were observed in different endocytotic compartments. In NPU cells, DNDs were found in early endosomes (**a**, **d**, **g**, **j**). In RT4 cells, DNDs were found in multilamellar bodies with only few lamellas (**b**), in multivesicular bodies (**e**) and in other differently shaped endosomes (**h**, **k**). In T24 cells, DNDs were observed mainly in multilamellar bodies (**c**, **i**, **l**). *Scale bar*, **b–e**, **g**, **j**, **k** 1 μm. **a**, **h**, **i**, **l** 500 nm. **f** 200 nm

multivesicular bodies (MVBs) (e.g. Fig. 2e, f) and multilamellar bodies (MLBs) (e.g. Fig. 2b, c, i). These results are in accordance with data, that normal urothelial cells commonly possess MVBs, which are a part of normal endosomal/lysosomal pathway (Guo et al. 2009) and that MLBs have been described in multiple cancer cells (Handerson and Pawelek 2003). Moreover, it was established that MLBs can also be formed via the process of autophagy (Hariri et al. 2000), which is in agreement with our observation that many MLBs in T24 cells looked like MLBs fused with autophagic vacuoles (Fig. 2c, l). All four types of DNDs were endocytosed in partially differentiated NPU cells, which provides new evidence that NPU cells at a low differentiation level can endocytose specific types of nanoparticles (DNDs). These results supplement our previous in vitro (Imani et al. 2015; Kreft et al. 2009) and in vivo (Hudoklin et al. 2013) data, showing that the endocytotic ability of highly differentiated urothelial cells in comparison to endocytotic activity of partially differentiated urothelial cells is significantly reduced.

To sum up, we showed that DNDs entered the urothelial cells, but did not induce any significant cytotoxic effects on normal and cancer urothelial cells in vitro. These results support the potential of researched DNDs as nontoxic delivery system for urological applications in human nanomedicine, especially as targeted drug delivery system in innovative urinary bladder cancer treatment strategies.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

Chen T, Lu F, Streets AM, Fei P, Quan J, Huang Y (2013) Optical imaging of non-fluorescent nanodiamonds in live cells using transient absorption microscopy. Nanoscale 5:4701–4705. doi:10.1039/c3nr00308f

Greiner NR, Phillips DS, Johnson JD, Volk F (1988) Diamonds in detonation soot. Nature 333:440–442. doi:10.1038/333440a0

Guo X et al (2009) Involvement of vps33a in the fusion of uroplakin-degrading multivesicular bodies with lysosomes. Traffic 10:1350–1361. doi:10.1111/j.1600-0854.2009.00950.x

Handerson T, Pawelek JM (2003) Beta1,6-branched oligosaccharides and coarse vesicles: a common, pervasive phenotype in melanoma and other human cancers. Cancer Res 63:5363–5369

Hariri M, Millane G, Guimond MP, Guay G, Dennis JW, Nabi IR (2000) Biogenesis of multilamellar bodies via autophagy. Mol Biol Cell 11:255–268

Ho D (2009) Beyond the sparkle: the impact of nanodiamonds as biolabeling and therapeutic agents. ACS Nano 3:3825–3829. doi:10.1021/nn9016247

Holt KB (2007) Diamond at the nanoscale: applications of diamond nanoparticles from cellular biomarkers to quantum computing. Philos Trans A Math Phys Eng Sci 365:2845–2861. doi:10.1098/rsta.2007.0005

Hudoklin S, Zupancic D, Makovec D, Kreft ME, Romih R (2013) Gold nanoparticles as physiological markers of urine internalization into urothelial cells in vivo. Int J Nanomedicine 8:3945–3953. doi:10.2147/IJN.S44363

Imani R, Veranic P, Iglic A, Kreft ME, Pazoki M, Hudoklin S (2015) Combined cytotoxic effect of UV-irradiation and TiO2 microbeads in normal urothelial cells, low-grade and high-grade urothelial cancer cells. Photochem Photobiol Sci 14:583–590. doi:10.1039/c4pp00272e

Kaur R, Badea I (2013) Nanodiamonds as novel nanomaterials for biomedical applications: drug delivery and imaging systems. Int J Nanomedicine 8:203–220. doi:10.2147/IJN.S37348

Kaur R et al (2012) Lysine-functionalized nanodiamonds: synthesis, physiochemical characterization, and nucleic acid binding studies. Int J Nanomedicine 7:3851–3866. doi:10.2147/IJN.S32877

Keremidarska M, Ganeva A, Mitev D, Hikov T, Presker R, Pramatarova L, Krasteva N (2014) Comparative study of cytotoxicity of detonation nanodiamond particles with an osteosarcoma cell line and primary mesenchymal stem cells. Biotechnol Biotechnol Equip 28:733–739. doi:10.1080/13102818.2014.947704

Kreft ME, Romih R, Kreft M, Jezernik K (2009) Endocytotic activity of bladder superficial urothelial cells is inversely related to their differentiation stage. Differentiation 77:48–59. doi:10.1016/j.diff.2008.09.011

Lien ZY, Hsu TC, Liu KK, Liao WS, Hwang KC, Chao JI (2012) Cancer cell labeling and tracking using fluorescent and magnetic nanodiamond. Biomaterials 33:6172–6185. doi:10.1016/j.biomaterials.2012.05.009

Liu Y, Gu ZN, Margrave JL, Khabashesku VN (2004) Functionalization of nanoscale diamond powder: Fluoro-, alkyl-, amino-, and amino acid-nanodiamond derivatives. Chem Mater 16:3924–3930. doi:10.1021/cm048875q

Mitev DP, Townsend AT, Paull B, Nesterenko PN (2014a) Microwave-assisted purification of detonation nanodiamond. Diam Relat Mater 48:37–46. doi:10.1016/j.diamond.2014.06.007

Mitev DP, Townsend AT, Paull B, Nesterenko PN (2014b) Screening of elemental impurities in commercial detonation nanodiamond using sector field inductively coupled plasma-mass spectrometry. J Mater Sci 49:3573–3591. doi:10.1007/s10853-014-8036-3

Paget V et al (2014) Carboxylated nanodiamonds are neither cytotoxic nor genotoxic on liver, kidney, intestine and lung human cell lines. Nanotoxicology 8(Suppl 1):46–56. doi:10.3109/17435390.2013.855828

Resnik N, Repnik U, Kreft ME, Sepcic K, Macek P, Turk B, Veranic P (2015) Highly selective anti-cancer activity of cholesterol-interacting agents methyl-beta-cyclodextrin and ostreolysin A/

pleurotolysin B protein complex on urothelial cancer cells. PLoS One 10:e0137878. doi:10.1371/journal.pone.0137878

Schrand AM, Huang H, Carlson C, Schlager JJ, Omacr Sawa E, Hussain SM, Dai L (2007) Are diamond nanoparticles cytotoxic? J Phys Chem B 111:2–7. doi:10.1021/jp066387v

Visnjar T, Kocbek P, Kreft ME (2012) Hyperplasia as a mechanism for rapid resealing urothelial injuries and maintaining high transepithelial resistance. Histochem Cell Biol 137:177–186. doi:10.1007/s00418-011-0893-0

Visnjar T, Kreft ME (2015) The complete functional recovery of chitosan-treated biomimetic hyperplastic and normoplastic urothelial models. Histochem Cell Biol 143:95–107. doi:10.1007/s00418-014-1265-3

Yu SJ, Kang MW, Chang HC, Chen KM, Yu YC (2005) Bright fluorescent nanodiamonds: no photobleaching and low cytotoxicity. J Am Chem Soc 127:17604–17605. doi:10.1021/ja0567081

Zhu Y et al (2012) Excessive sodium ions delivered into cells by nanodiamonds: implications for tumor therapy. Small 8:1771–1779. doi:10.1002/smll.201102539