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Nanodiamonds coupled with plant bioactive metabolites: A nanotech approach for cancer therapy

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ABSTRACT

Nanodiamond application in biotechnological and medical fields is nowadays in continuous progress. In fact, biocompatibility, reduced dimensions and high surface chemical interaction are specific features that make nanodiamonds perfect intracellular carriers of bioactive compounds. By confocal microscopy, we confirmed that nanodiamonds were able to penetrate in cell cytoplasm but we also demonstrated how they remained embedded in nuclear membrane just exposing some little portions into nuclear area, definitively clarifying this topic. In this work, for the first time, nanodiamonds were conjugated with plant secondary metabolites, ciproten and quercetin. Moreover, since drug-loading on nanoparticles was strongly conditioned by their chemical surface, different types of nanodiamonds (oxidized, wet chemical reduced and plasma reduced) were synthesized in this work and then functionalized with plant compounds. We found that ciproten and quercetin antiproliferative effects, on human (HeLa) and murine (B16F10) tumor cells, were improved after nanodiamond conjugation. Moreover, plant molecules highly reduced their *in vitro* pro-oxidant, cytotoxic and pro-apoptotic activity when associated with nanodiamond. We are led to suppose that natural drug-nanodiamond adducts would act at cellular level by different molecular mechanisms with respect to plant metabolite pure forms. Finally, our results showed that chemical and structural modifications of nanodiamond surfaces influenced the bioactivity of transported drugs. According to all these evidences, this work can be considered as a promotional research to favor the use of bioactive plant molecules associated with nanodiamonds for therapeutic purposes.

1. Introduction

Nanodiamonds, with size ranging from 1 to 100 nm, represent the last innovative group of chemical materials in the family of nanocarbons. These nanoparticles, that can be artificially synthesized by detonation, chemical vapor deposition or high-temperature–high-pressure procedures [1], are characterized by chemical stability, octahedral symmetry, rigid structure, large surface area and low costs of production [2,3].

Among the different kinds of nanodiamonds, the most interesting ones are produced by detonation. In fact, detonation nanodiamond (ND) can be easily modified and linked to different molecules and bio-compounds by electrostatic and/or hydrophobic interactions or covalent bonds [4]. The stability of these complexes was also proved for months after their synthesis [5]. Moreover, a series of novel technologies are based on the use of self-assembled ND in controlled structures [6], decorated with nanoparticles [7] or embedded in polymers [8].

As reported in literature, nanodiamonds, functionalized or not, can be largely used for biotechnological and medical applications. In particular, they were employed as carriers of biomolecules (DNA, RNA, proteins) and drugs inside living cells and organisms [9–12]. This phenomenon was possible because of the high compatibility between nanodiamond material and cell physiology and structure [13]. In fact, nanodiamonds were identified as the less toxic among nanocarbons (i.e. carbon blacks, fullerenes, carbon nanotubes); they generally do not induce significant oxidative stress or apoptosis during cell treatments [14].

Clathrin-mediated endocytosis was identified as the cell mechanism for nanodiamond internalization [15]. Scientific data proved that cell endocytic vesicles transport and arrange nanodiamonds everywhere in the cytoplasm, also in perinuclear area,

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but not inside the nucleus [1]. On the other hand, Martìn et al. [16] only proposed a controversial theory demonstrating that fenton-treated nanodiamonds were able to enter directly in cell nucleus. In literature, it was reported that nanodiamonds, yet absorbed in cells, could also be thrown out the cytoplasm by exocytosis. However, all these processes are not standard but depend on different factors, such as nanodiamond size, surface, concentration and treatment time but also temperature and employed cell line [17,18].

Due to their auto-fluorescent structural defects, known as nitrogen-vacancy centers, nanodiamonds can be detectable by fluorescent microscopy. Moreover, since they also have a refractive index higher than cytoplasm, nanodiamonds show a strong light scattering signal that makes them clearly distinguishable in cell compartment, even by optical microscopy with good contrast [15].

Only recently, nanodiamonds were functionalized with chemotherapeutic molecules in order to investigate if carbon materials could improve antineoplastic properties of these compounds, both *in vitro* than *in vivo* experiments. In this context, doxorubicin, one of the most common anticancer drugs, was successfully linked to nanodiamonds and introduced toward murine macrophages, as well as human colorectal carcinoma cells, preserving its efficacy [19,20]. Nanodiamond-mediated doxorubicin delivery system was also able to inhibit the generation of lung metastasis in mice, starting from 4T1 breast cancer cells [21]. Moreover, similar antitumor studies were performed by functionalizing nanodiamonds with other compounds, such as dexamethasone, 4-hydroxytamoxifen and purvalanol A. All these data suggested that nanodiamonds positively regulated the water solubility of absorbed molecules, even if highly hydrophobic, and that they did not influence drug release and activity in cell compartment [22]. In fact, Li et al. [23] showed how 10-hydroxycamptothecin, a popular chemotherapeutic agent obtained by chemical modification of natural compounds, presented much lower antineoplastic effects if compared with its nanodiamond-conjugated form.

The present study wants to highlight the prospective biotechnological application of specific nanodiamonds synthesized by our research team according to particular procedures. In particular, pristine detonation nanodiamonds were treated with three different protocols (oxidation, ND; wet chemical reduction, ND R1; plasma reduction, ND R2) that radically changed the organic groups on nanodiamond surfaces and consequently their chemical properties.

Aim of the project was the upgrade and the extension of scientific insights about nanodiamond potentialities as intracellular transporters of bioactive molecules, in order to facilitate their application for therapeutic purposes.

Firstly, this work investigated the penetration kinetics and the distribution of nanodiamonds in mammalian tumor cells. In particular, as fundamental target, we wanted to definitively understand if nanodiamonds could really enter into cell nuclear region, clarifying contrasting theories reported in literature.

On the other hand, innovative task of this study was the use of nanodiamonds as intracellular carriers of plant metabolites, an experimental approach never previously described in literature. In particular, the three types of nanodiamonds were functionalized with two natural molecules, ciproten (5,7-dimethoxycoumarin) and quercetin (3,3′,4′,5,6-pentahydroxyflavone), chosen according to their well-known pro-apoptotic and redox properties [24–29]. Therefore, main object of this research was to evaluate if these compounds improved their antiproliferative effects when adsorbed on nanodiamonds, both on human (HeLa) and murine (B16F10) cancer cells. In this context, we also wanted to assess whether and how the different surface chemical structures of nanodiamonds could determine changes in the reactivity of conjugated molecules during cell treatments.

Last but not least, since the bioactivity of plant metabolites could be modified by the interaction with nanodiamonds, the principal cell action mechanisms of pure molecules were analyzed and compared with those caused by their nanodiamond-conjugated forms: therefore, reactive oxygen species intracellular amount, cytotoxicity levels and induction of apoptosis were examined and discussed.

2. Materials and methods

2.1. Nanodiamond synthesis and drug-loading

Nanodiamonds used for the present research were synthesized by detonation and purchased from Federal Research and Production Centre 'ALTAI' (Russia). Powders were produced by disintegration and purification of extremely tight diamond aggregates, with initial size ranging from 100 to 200 nm. Final purified nanodiamond particles, with a 4–5 nm crystallite primary size, were achieved by treatments that combine bead milling with strong oxidation.

Pristine nanodiamonds were purified, via an oxidation procedure, to remove metals and sp^2 carbons from their outer shell (ND). Afterwards the oxidation protocol, NDs were opportunely modified by two different types of reducing processes: chemical reduction (ND R1) and plasma reduction (ND R2). The first process was performed resuspending a certain amount of ND in 10 mL of deionized water and adding it to 200 mg of sodium boron hydride. Then, reaction mixture was left under stirring condition for about 3 h. Finally, supernatant was removed and the product was washed with distilled water till neutral pH. On the other hand, plasma reduction was performed according to Orlanducci et al. [30] method. After that, each type of nanodiamond (ND, ND R1 and ND R2) was functionalized with ciproten (C) or quercetin (Q) by physisorption [21] and washed with the appropriate solvent (DMF for coumarin and Ethanol for Quercetin). Several cycles of centrifugation enabled to easily remove the unreacted molecules (i.e. the molecules that did not adsorb on nanodiamond surfaces) from the samples.

Powders were resuspended in phosphate buffered saline (PBS) solution for cell experiments. The loading of ciproten and quercetin on nanodiamonds was quantified by UV-Vis absorption spectroscopy after complete solvent extraction from the nanodiamond complexes. Ciproten extraction was performed in dimethylformamide while quercetin extraction was performed in ethanol. A specific amount of each conjugated complex was dispersed in the respective solvent for 24 h. Then, the supernatant was collected and analyzed. The amount of ciproten and quercetin, released from the nanodiamond complexes during this time, was measured by detecting the absorbance of the relative supernatants at 328 and 380 nm, respectively for C and Q, and by extrapolating their concentrations according to calibration curves properly obtained using adequate amount of pure C and Q as standards (Supplemental Material 0). Extraction procedures were repeated for each sample till the active molecules were totally extracted. High Resolution Transmission Electron Microscopy (HR-TEM) analysis was performed with a FEI-TITAN TEM operating from 80 to 300 kV. FT-IR analysis was performed with an HP Spectrum-ONE spectrometer. Samples were dissolved in nujol and analyzed without further manipulations. Raman studies were performed by using a XploRA ONETM Raman Microscope (Horiba Jobin Yvon) using a 532 nm excitation laser light and a 2400 gr mm^{-1} grating spectrometer coupled with an air-cooled scientific CCD. SERS analysis was carried out following the procedure illustrated by Reina et al. [31].

2.2. Cell cultures and treatments

Human cervical cancer (HeLa) and murine melanoma (B16F10) cells were grown and propagated under standard culture conditions [32,33]. Nanodiamond solutions (previously described) were sonicated before their use, in order to break up possible aggregates. Treatments were performed with pure and functionalized nanodiamond solutions at different concentrations (5, 10, 20, 50, 80, 100, 150, 200 μg/mL) for 30 min (min), 2, 4, 6, 8, 24, 48 and 72 h (h). Ciproten and quercetin were dissolved in dimethyl sulfoxide (DMSO), since the solvent concentration did not exceed 0.1% during treatments. In particular, ciproten and quercetin were used at final concentration of 100 μM for 6, 24, 48 and 72 h. Control cells were treated with DMSO (control for ciproten and quercetin) or PBS (control for nanodiamond solutions) at the same volume of the respective treatment. Staurosporine (STS), a well-known apoptotic drug, was used 2 μM for 4 h.

2.3. Confocal and optical microscopy

Cells were seeded on glass slides, previously sterilized with boiling SDS 0.1% for 2 min. After adhesion and treatment, cells were washed four times with PBS and fixed with paraformaldehyde 4% for 15 min. Then, slides were washed twice with PBS and subjected to treatment with Triton X-100 0.1% for 15 min in order to permeate cells. Other two washings with PBS were performed on samples before nucleus labeling, for 5 min, by DAPI solution (4′,6′-diamidino-2-phenylindole) 60 μg/mL. The slides were washed again with PBS and finally observed by Confocal