CELLULAR UPTAKE OF GRAPHENE ACID BY HEL AND HELA CELLS STUDIED BY UV RAMAN SPECTROSCOPY

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Abstract

The last few years have witnessed rapid development of biological and medical applications of graphene-based materials in drug delivery, biosensing, and bioimaging. Graphene acid is a novel graphene-based material with many interesting properties, including colloidal stability and biocompatibility [1]. However, mechanisms of its cellular uptake are not yet fully understood, and their study is crucial for further applications of the material. Study of a cell - nanomaterial interactions of the graphene-based nano materials is commonly performed using fluorescence imaging in a combination with respective fluorescent tags. However, labelling could potentially influence the material key properties, and new ways for label-free imaging are thus demanded. Here we developed a methodology for a label-free imaging of a cellular uptake of graphene acid by cancer (HeLa) and non-cancerous (HEL) cell lines based on Raman microscopy equipped with UV excitation laser.

Keywords: Graphene acid, Raman, imaging, cells

1. INTRODUCTION

Graphene is a two-dimensional allotrope of carbon [2]. Due to its excellent properties, such as strength, elasticity, high thermal conductivity, high electron mobility, and tuneable band gap, graphene become one of the most studied materials in last few years [2,3]. Its range of applications can be significantly extended by a surface functionalization using numerous functional groups which further enable to modulate its electronic, magnetic, and optical properties [1]. Graphene acid represents one of the strong candidates. Carboxylic groups on the surface of graphene acid significantly improve mechanical and electronic properties, sorption capacity, the dispersibility of the material in an aqueous environment and improve the stability of resulting colloidal solutions [4]. One of strong applications of graphene acid, derived from its high biocompatibility and colloidal stability, lies in the drug delivery and gene transport. However, its interactions with cells have not been fully described and understood.

Interactions of nanomaterials with cells are usually studied using fluorescent microscopy, flow cytometry and established toxicological tests [5-7]. However, there is a requirement for a utilisation of fluorescent labels in cases, where non-fluorescent materials are being studied. This can lead, because of unwanted interactions of the anchored fluorescent labels, to biased results [8,9]. The issue presents a scientific challenge worth to pursuit. Molecular spectroscopy, particularly Raman microscopy, presents an interesting alternative. Its potential has been earlier demonstrated in the imaging of graphene oxide in cell cytoplasm. However, the study was performed without an accent on a description of possible uptake mechanisms or effects on various cell lines [10,11].

Here we present an application of Raman spectroscopy for the examination of cellular uptake of novel material - graphene acid, into the different cell lines. The study was performed on two cell lines - human healthy lung fibroblasts (HEL) and human cervical cancer cells (Hela), with a variable time of uptake: 4 hours and 24 hours. We demonstrated that the UV Raman microscopy can be successfully applied in the study of the cellular

uptake behaviour of graphene acid, and we have described differences of the nanomaterial uptake between cancerous and healthy cell lines.

2. EXPERIMENTAL SECTION

2.1. Cell Culture

For analysis we used adherent HEL 12469 (human healthy lung fibroblasts) and HeLa (human cervical cancer) cell lines. HEL cells were cultivated in EMEM - Minimum Essential Medium (Sigma Aldrich) enriched by L-Glutamine and Non-essential amino acids (NEAA) and HeLa cells were cultivated in low glucose DMEM medium (Life Technologies); both at 37 °C under a 5 % CO_2 atmosphere.

2.2. Cell viability assessment

Cell viability assay was performed using BD FACSVerse flow cytometer (BD Biosciences, USA). 10,000 cells were seeded per well in a 96-well plate. Cells were incubated with various concentrations of GA (10, 25, 50, 75 and 100 µg/ml) for 24 h. After 24 h, supernatant was collected, and cells were gently washed with PBS solution (0.1 M, 7.4 pH). Then cells were detached with trypsin (0.25 % in EDTA, Sigma Aldrich), resuspended in 100 µl of culture media and added to supernatant. Viability of cells was determined by calcein-AM fluorescent probe. Cells were incubated with 2 µl of calcein-AM, diluted in DMSO (50 µM). Viable cells had active intracellular esterases that catalyzed non-fluorescent calcein-AM to highly fluorescent green calcein. The fluorescence signal of calcein was measured by flow cytometry (exc. 488/em. 527) and viability of cells was established.

2.3. Preparation of cells for Raman measurements

The cells were fixed by a simple procedure for Raman analysis. Briefly, CaF_2 substrate was put to 24 well plate, 200 µl of a cell suspension (5000 cells/slide) were added, only on the substrate area. After 24 hours cells become adhered, the medium was replaced with one containing graphene acid, prepared according to protocol published earlier by our group [1]. The concentration of the nanomaterial was set to 50 µg/ml and incubation time was 4 and 24 hours, respectively. After incubation, the cell medium on the surface of the substrate was gently washed twice with PBS buffer (Phosphate-buffered saline). Then, the substrate was carefully removed and placed on the mount. After that, 200 µl of 4 % paraformaldehyde was added and incubated for 10 minutes. Finally, cells were washed twice with PBS and once with ultrapure water and kept drying in the fridge.

2.4. Apparatus

Raman spectra of the cells were acquired using a Thermo Scientific DXR Raman Microscope (Thermo Electron, USA) equipped with laser operating at the 455 nm wavelength. The laser power on sample was set up to 3 mW and the measured range was 350-3400 cm^{-1}. The exposition time was set to 2 s with 8 expositions per spectrum. Sixteen spectra were averaged to obtain one experimental data point. Cell viability assay was performed using BD FACSVerse flow cytometer (BD Biosciences, USA).

3. RESULTS AND DISCUSSION

First, basal cytotoxicity of the graphene acid had been studied to tune its concentration before a consecutive Raman imaging. The toxicity was evaluated using a flow cytometry to avoid possible false positive or negative outcomes, which can generally occur, when a standard MTT test is applied [12,13]. The concentrations were evaluated in the range from 10 to 100 mg/L. **Figure 1** shows that only an unimportant effect of applied nanomaterial is observed. The viability decreased from 100 % for a control group to 88 % for HEL cells lines and 90 % for Hela, respectively. These results are comparable with a cytotoxicity of graphene oxide up to a

concentration of 50 mg/L. Published results indicate that graphene oxide starts to be substantially toxic at higher concentration with viability ranging from 90 % for 50 mg/L to 50 % for 100 mg/L [14,15]. Concenration of 50 mg/L of the graphene acid was selected for further experiments.

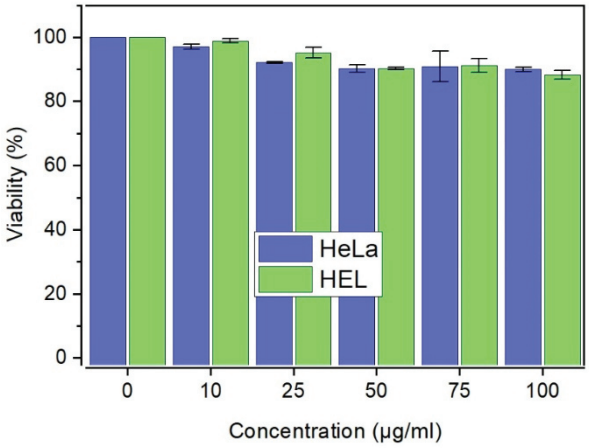

Figure 1 Cell viability of HeLa and HEL cells after 24 h of incubation with different concentrations of GA. Viability of cells was normalized to control untreated cells (100 %).

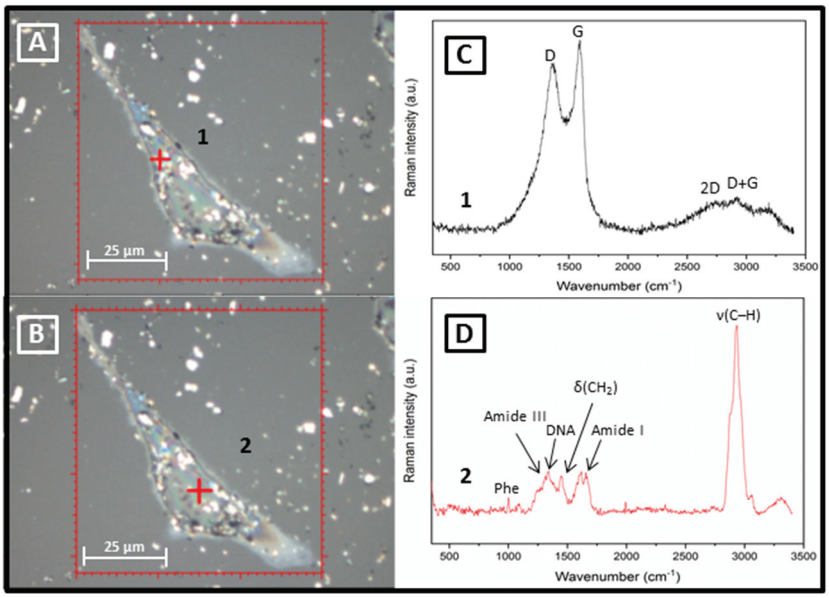

Figure 2 A), B) microscopic images of a HeLa cell.
C), D) Raman spectra obtained at different spots marked in A), B) in the HeLa cell.

Second, Raman spectra were acquired at several points on fixed cells to evaluate spectral characteristics of cells, as well as spectra characteristics of the internalized nanomaterial. **Figures 2A** and **2B** show microscopic images of the fixes HeLa cell. Figures contain two marks, labelled 1 and 2, respectively. Mark 1 corresponds to the nanomaterial (visible as light spots) and mark 2 corresponds to the cell, particularly nucleus. Raman spectrum shown in **Figure 2C** represents the mark 1, and contains two spectral bands located at 1360 and 1595 cm^{-1}, assigned to D and G spectral bands of graphene acid [11,16]. The D/G ratio of 0.8 confirms sp3 hybridisation in the graphene lattice, indicating a presence of functional carboxyl groups on its surface. **Figure 2D** represents a mark 2 and shows Raman spectrum of the nucleus with several characteristic spectral bands. The most intense band is located at 2930 cm^{-1}. This can be interpreted as C-H vibrations (v(C-H)) of -

CH_2 and -CH_3 groups presented in proteins, lipids or nucleic acids [17]. Additional Raman bands were assigned to phenylalanine at 1005 cm^{-1}, DNA backbone (O-P-O stretching) at 1092 cm^{-1}, amide III band at 1313 cm^{-1}, DNA at 1340 cm^{-1}, CH_2 bending mode found in proteins at 1450 cm^{-1}, and lipid C=C bond and amide I band of proteins at 1660 cm^{-1} [18,19].

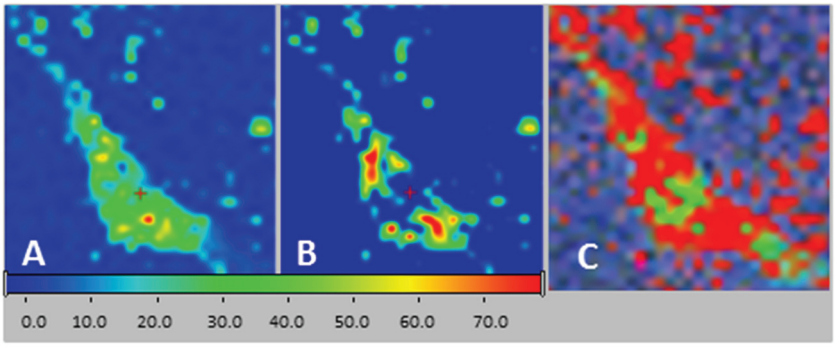

Figure 3 Raman maps of HeLa cell after the incubation with GA for 24 hours demonstrating area of: (A) cell, (B) GA, (C) image from multivariate curve resolution analysis of GA internalized in cell

Raman images obtained by the scanning of Hela cells after a 24-hour incubation with graphene acid is shown in **Figure 3**. **Figure 3A** was obtained by the univariate analysis performed at spectral band located at 2930 cm^{-1} (ν(C-H)) and reflects the spatial distribution of above mentioned compounds inside the cell. Importantly, weak Raman signal was observed also outside of the scanned cell. This phenomenon is caused by the used cell medium, which contains proteins with similar spectral characteristics. The **Figure 3B** is a result of univariate analysis performed on the spectral band localized at 1595 cm^{-1}, which is characteristic for the present nanomaterial. Most of the graphene acid is localized inside the cell, particularly in the cytoplasm. However, some particles remained outside the cell. Finally, the multivariate curve resolution was performed to visualize both signals, the cell and the nanomaterial. The result is shown in **Figure 3C**, where red colour represents the graphene acid, and the green represents the cellular structure. The resulting image confirms the uptake of the graphene acid inside the HeLa cell. Interestingly, the nanomaterial is not present in the cell nucleus, which can be caused by a combination of its negative charge and the size [20].

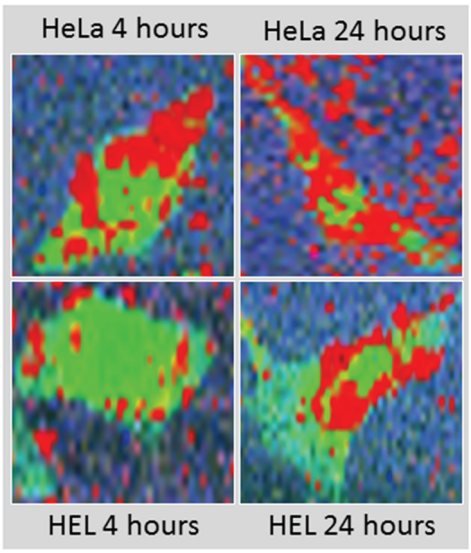

Figure 4 Raman mapping of GA internalization in the HeLa cells and HEL cells after incubation for 4 hours and 24 hours

Next, effect of the incubation time and effects of the nanomaterial on various cell lines were studied. Two cell lines were selected for the study, namely human healthy lung fibroblasts (HEL) and cancer cells (HeLa). Both lines were incubated with a total concentration of 50 mg/L of graphene acid for 4 and 24 hours. Resulting data are shown in **Figure 4** with considerable differences between healthy and cancer cell lines. The Hela cells incubated for 4 hours contained noticeably higher amount of the internalized nanomaterial, compared to normal cell lines. This can be possibly caused by the altered metabolism, characteristic for HeLa cells [21]. Nonetheless, the nanomaterial was found in the cytoplasm of both cell lines, indicating a possible exploitation of the nanomaterial in a drug delivery.

4. CONCLUSION

In summary, the new methodology for a study of the cellular uptake of graphene acid, based on the Raman microscopy, equipped with UV laser, was developed. Cellular uptake was studied on two different types of cells - healthy human lung fibroblasts (HEL) and human cervical cancer cells (HeLa). It was found that the nanoflakes of the graphene acid are easily internalized into cancer and non-cancerous cells, with a preference towards the studied cancer cell line.

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