RAMAN LABEL-FREE VISUALISATION OF TITANIUM DIOXIDE NANOPARTICLES UPTAKE IN BJ CELL LINES

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Abstract

Titanium dioxide nanoparticles represent one of the most frequently applied nanomaterials. Due to its advantageous physicochemical properties affecting the final products, use of this nanomaterial in daily used products is increasing. Beside the addition into glaze or enamels, titanium dioxide nanoparticles are found in UV protective cosmetic products applied on skin. According to the studies confirming the potential carcinogenic effect of titanium dioxide nanoparticles application of such nanomaterial may cause health risk. Cellular uptake of nanoparticles and their distribution in cell environment may play an important role in nanoparticles toxicological effect. Thus, evaluation of cellular uptake of nanoparticles is the additional step for evaluation of nanoparticles toxicology. The main objective of this study was to confirm the assumption of the cellular uptake of tested TiO_2 nanoparticles using human fibroblasts BJ cell lines and confocal Raman microscopy as a new, promising, label-free imaging technique for studying the distribution of exogenous substances in cells. The results of this study confirm that tested TiO_2 nanoparticles are uptaken by cells and distributed in intracellular environment, where form aggregates, possibly during their transport via endocytosis.

Keywords

titanium dioxide nanoparticles, cellular uptake, label-free, confocal Raman microscopy

Introduction

Nanomaterials are becoming frequently discussed ingredients of many commercially available products on the market. Due to their physicochemical properties which affects the final product suitability, the interest of manufacturers in nanomaterials is increasing.

Titanium dioxide (TiO_2) represents one of the frequently applied nanomaterials, which is used mainly for its advantageous properties – brightness, high refractive index and resistance to discolouration. Besides pigments in paints, glazes, plastics, fibers and foods or coating material in pharmaceuticals, the high percentage of TiO_2 nanoparticles is used in cosmetics as an inorganic UV filter [1]. Application of such nanomaterial into cosmetic products means the human exposure to inorganic substance effect. This fact opens the discussion about the potential health risk in humans. The toxicological threat related to skin application is not the only problem to be discussed. There is also potential of nanoparticles release which might cause an environmental risk as nanomaterials can get into surface waters and interact with living organisms or, finally, led to unwanted human exposure [1, 2].

Regardless of the way of exposure to TiO_2 nanoparticles, the cell remains the place of the toxicological action of a nanomaterial. The cytotoxicity of TiO_2 nanoparticles has been already extensively investigated [3, 4]. However, some toxicological studies performed *in vivo* have shown that metal nanoparticles do not penetrate human skin and therefore cosmetic products containing metal nanoparticles were considered safe [5]. As small particles often penetrate the cell membrane, nanoparticles might be uptaken via molecular mechanisms like endocytosis and phagocytosis. Nanoparticles in intracellular environment may

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cause the initiation of reactive oxygen species (ROS) production resulting in cytotoxicity and DNA damage of exposed tissue [6]. Thus, demonstrating the cellular uptake of nanomaterial may play the important role in evaluation of its toxicological effect.

Confocal Raman microscopy

Microscopic imaging is one of the basic procedures in any experiment observed on the cellular level. Visualisation of cell compartments or any exogenous substances in cell environment using conventional microscopic techniques is based on specific labelling, which allows us to have a look at studied structures or substances of interest because of specific reaction caused by label, e.g. fluorescent dye. The label-free imaging technique for nanoparticles uptake in cells which overlaps the labelling steps in sample preparation is confocal Raman microscopy. Herein, this microscopic technique was used to evaluate cellular uptake of tested TiO_2 nanoparticles in BJ cell lines. Other studies were performed to study the cellular uptake and distribution of nanomaterials using Raman microscopy as an imaging technique. Using the nanoparticles spectral information it was possible to conclude the polymeric nanoparticle systems cellular uptake and study the intracellular fate of this drug carrier systems in cells [7]. Polystyrene or gold nanoparticles cellular uptake was also demonstrated by Raman microscopy and nanoparticles were shown being distributed in perinuclear region in 24 h of exposure [8, 9]. Using specific spectral information from nanoparticles it is possible to visualise their cellular uptake, distribution or behaviour in intracellular environment. Considering the cellular uptake as a part of cytotoxic effect of a nanomaterial, Raman label-free visualisation of its uptake in cells provides a contribution to evaluation of nanoparticles toxicology with a minimum sample preparation.

Materials and methods

Cell culture

10^6 BJ (human fibroblasts from foreskin) cell lines were cultivated in DMEM (Dulbecco's Modified Eagle Medium) in a thermobox at 37°C and 5% CO_2 and were allowed to adhere on quartz slides for Raman spectroscopy in 24-well plates for 24 h. Cells were then incubated with a solution of titanium dioxide nanoparticles (EUSOLEX T - AVO), which was added into the medium for another 24 h. The concentration of nanoparticles in medium corresponded to hundredfold diluted IC_{50} (50% inhibitory concentration) determined using MTT assay and was estimated as 27,746; SD = 288 $mg.l^{-1}$. Cells were then washed three times with 1 ml of PBS and fixed with 1 ml of 4% formaldehyde for 10 min at room temperature. Subsequently, the cells were again washed with 1 ml of PBS and measured by Raman microscopy.

Confocal Raman measurement

In this study, confocal Raman microscope, model 300 alpha R, WITec, GmbH (Ulm, Germany) was used. During the measurement the sample was located on a piezoelectrically driven microscopic scanning table. The sample was scanned through the laser focus continuously along the lines of a selected area. Raman spectra were collected as the result of the laser excitation. Excitation was performed by a frequency doubled Nd:YAG laser (Spectra Physics Excelsior 532 nm with ~50 mW maximum output). The Zeiss EC Epiplan-Neofluar (50x/0.8 NA, WD = 0.58 mm) dry objective was used. Raman spectra were collected at a 0.5 µm grid with an integration time of 0.5 s for each Raman spectrum.

For cell visualisation, univariate Raman imaging method was used. This procedure allows to simple visualise the Raman intensity of the certain vibrational band in all Raman spectra accross the sample. Every pixel corresponds to one Raman spectrum, which reflects the molecular vibrations in corresponding area.

Raman visualisation of TiO_2 nanoparticles cellular uptake

Raman spectrum of cell is a superposition of molecular vibrations observable in the biomolecular fingerprint spectral range (~600–1800 cm^{-1}). However, the most dominating vibrational band in the Raman spectrum of cell belongs to C-H stretching vibration and lies between 2800–3100 cm^{-1} (Fig. 1A). BJ cells were visualised integrating the intensity of C-H stretching vibration in its maximum at 2935 cm^{-1} (Fig. 2A).

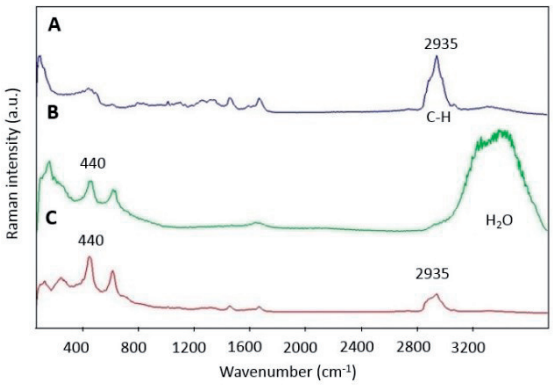

Fig. 1 Raman spectrum of biomolecules in BJ cell (A), Raman spectrum of TiO_2 nanoparticles sample in PBS (B), Raman spectrum obtained from the region with TiO_2 nanoparticles occurence (C).

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Raman spectrum of tested titanium dioxide nanoparticles sample in PBS showed some specific vibrations, which are not found in biomolecular Raman spectra of cells (Fig. 1B). Nanoparticles intracellular distribution visualised by Raman microscopy is based on their specific spectral information [7, 9]. Intensive vibrational band in TiO_2 nanoparticles Raman spectrum lies at 440 cm^{-1} and it was significantly visible in Raman spectra taken from the regions of cell cytoplasm (Fig. 1C). According to this finding, it was possible to evaluate the nanoparticles occurence in cell enviroment. To visualise TiO_2 nanoparticles, univariate images were reconstructed integrating the intensity of the most dominant vibrational band of nanoparticle sample at 440 cm^{-1} (Fig. 2B). The final images showing the distribution of nanoparticles in cell environment was created by overlaying the univariate images of the same cell reconstructed from C-H stretching at 2935 cm^{-1} and nanoparticles vibrational band at 440 cm^{-1} (Fig. 2C).

Studied nanoparticles themselves are about 100 nm in size. With a spatial resolution of approximately 300 nm, we are not able to display individual nanoparticles. The intracellular regions belonging to nanoparticles were in µm-order, and this fact confirms the nanoparticles aggregations. Aggregates could be possibly formed on cell membrane during their cellular transport via endocytosis. Nanoparticle aggregates were uptaken by BJ cells within 24 h of exposure. The distribution of nanoparticle aggregates in cells appears being significant in perinuclear regions and is probably related to different stages of endocytotic cellular pathway.

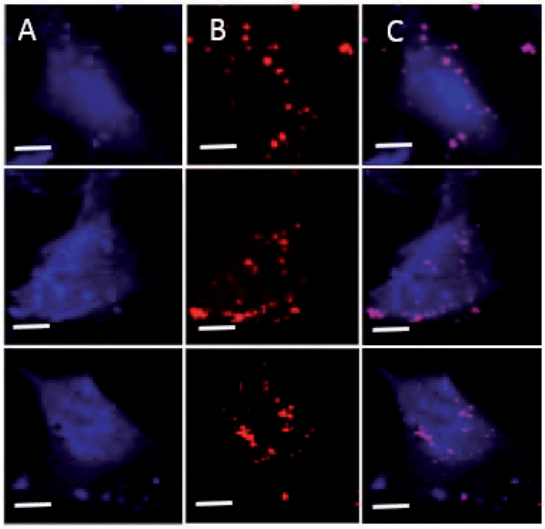

Fig. 2: Raman images of BJ cells (A), nanoparticles themselves (B) and their distribution in BJ cells (C) The scale bar shows 6 µm.

Conclusion

Cellular uptake and intracellular distribution may play an important role in nanoparticles toxicological effect. Although the cytotoxicity of titanium dioxide nanoparticles has been investigated extensively, precise mechanism through which this nanomaterial induce cell death is unclear. However, cell remains being considered as the place of nanoparticles toxicological effect. Visualisation of cellular uptake of nanoparticles may be the additional step for evaluation of nanoparticles toxicology.

Using confocal Raman microscopy as an imaging technique we were able to demonstrate that tested TiO_2 nanoparticles penetrate cellular membrane and are uptaken by BJ cells during the exposition to the nanomaterial in cell culture. This confirms the assumption of cellular uptake of TiO_2 nanoparticles, which could possibly affect the cellular metabolism.

Titanium dioxide nanoparticles have been recently classified as a possible human carcinogen by The International Agency for research on Cancer [10]. Herein we demonstrated the cellular uptake of this nanomaterial and the potential risk for fibroblasts which might come in contact via products of everyday usage.

Dedication

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