MICROSCOPIC ANALYSIS OF THE NANOSTRUCTURES IMPACT ON ENDOTHELIAL CELLS

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Abstract

Nowadays nanostructures are more and more often designed as carriers for drug delivery, especially to improve the drug pharmacokinetics and pharmacodynamics. Numerous kinds of nanostructures are considered a good prospect for medical applications thanks to their small size, acceptable biocompatibility and toxicity. Due to the fact that nanotechnology is a new field of science, every nano-scale product must be thoroughly examined regarding its toxicity to the human body. This study provides new insights into effects of exposing endothelial cells to the selected nanostructures. Dendrimers of the fourth generation (PAMAMs), multi-walled carbon nanotubes (MWCNTs) and silver nanoparticles (SNPs) were used to evaluate nanostructures influence on endothelial cells in vitro. The nanostructures were evaluated via transmission electron microscopy and dynamic light scattering technique. The cells previously exposed to the nanostructures were observed and analyzed via the atomic force microscopy and scanning electron microscopy to obtain a quantitative evaluation of the cells morphology. The presence of multi-walled carbon nanotubes and silver nanoparticles on the cells surface was confirmed by the scanning electron microscopy. Our results confirm that the surface association and/or uptake of nanostructures by the cells resulting from physicochemical and biological processes, affect the cells morphology. Morphological changes can be induced by the membrane proteins interaction with nanomaterials, which trigger a sequence of intracellular biological processes.

Keywords: *nanostructures, atomic force microscopy, transmission electron microscopy, scanning electron microscopy, cell morphology*

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Introduction

It is known that several agents (e.g. drugs, nanoparticles, cytokines) may induce changes in the cell phenotype regarding its biological and physical parameters, such as morphology, cytoskeleton reorganization, intercellular communication [1-4]. Currently, nanoparticles (NPs) are intensively studied on cells or in animal models considered as potential drug delivery or angiogenesis inhibition agents [5] thanks to their ability to improve the pharmacokinetics and pharmacodynamics of drugs. However, due to the complexity of the *in vivo* systems there are still several issues inhibiting the efficiency of NPs that need solutions to fully exploit their theoretical potential [6]. Given their small size, good biocompatibility and low toxicity, many nanoparticles are considered a promising prospect for biological and medical applications e.g. the gold [5] and the silver ones [7]. Dendrimers, due to their internal structure, can be potentially used for drug delivery, yet their metabolism by cells, tissues and organs has not been investigated enough so far [8]. Some of nanostructures, such as multi-walled carbon nanotubes (MWCNTs), are examined in nanoscience and nanotechnology for application in electronic circuits as field emitters or for hydrogen storage [9]. Still, they can be also investigated in terms of biomedical applications [10,11].

Endothelial cells (ECs) play a key role in nanomaterials delivery and their functionality. Lining the interior of the entire vascular system, ECs represent a major barrier for therapeutic agents being transferred from the bloodstream to the target tissues. One of the crucial parameters which provide information about the cells functional status, i.e. proliferation, migration or even capillary formation, are morphological changes [5].

Atomic Force Microscope (AFM) is a powerful tool to develop and assess nanoparticles [12] and characterize the properties of nanostructures [13]. Additionally, it provides nanomedicine with a versatile platform by combining the nanometer-scale resolution and the unique ability to visualize single biomolecules in their native environment [1-4] as well as nanoparticles exposed to environmental conditions. Over the last decade, AFM techniques and atomic force spectroscopy (AFS) were employed to verify the physiological state of cells [1-2], including dysfunctions after hormones, cytokines or drugs application [14-16]. An AFM probe was also postulated as a diagnostic nanosensor for cancer investigation [17-19]. Scanning electron microscopy (SEM) is yet another method to evaluate cells morphology after the nanomaterials application and to confirm the nanostructures presence on the cells membrane is [20].

The presented work studies the influence of three kinds of nanomaterials: polyamidoamine dendrimers (PAMAMs) of 4.0 generation, silver nanoparticles (SNPs) and multi-walled carbon nanotubes (MWCNTs) on the EA.hy926 endothelial cell line. The selected nanomaterials represent three main groups of nanomaterials: polymers, metals and carbon nanomaterials. The obtained results contribute to the already published works [21] on the transcriptome and proteome analysis of endothelial cells under stress conditions induced by the nanomaterials. The comprehensive analysis of the nanoparticles influence on cells morphology is vital to understand the endothelium response to drugs and other agents delivery upon the nanoparticles application.

Materials and Methods

Cell culture

The EA.hy926 endothelial cell line formed by the fusion of the HUVEC and A549 human lung carcinoma cell lines was obtained from American Type Culture Collection (ATCC® CRL-2922™) and cultured at 37°C in the humidified atmosphere of 9% CO_2 in Dulbecco's Modified Eagle Medium (DMEM, ATCC) supplemented with 10% of Fetal Bovine Serum (Gibco) and Penicillin/Streptomycin solution (ATCC). The cells were harvested for experiments before reaching 90% of confluence. Trypsin-EDTA (ATCC) was used as a dissociation solution, followed by the neutralization with the 10-fold volume of complete DMEM medium. The cells were seeded on the proper dishes for selected measurements (sterile cover glasses for AFM and SEM, cell culture dishes for TEM) and cultured for 24 h. Next, the solution of PAMAM dendrimers, SNPs or MWCNTs, in the serum-free cell culture medium was added in final concentrations, respectively (TABLE 1). The application of nanostructures at the selected concentrations caused the final toxicity of the cells - 10 and 25% [21] marked as EC 10 and EC 25.

TABLE 1. Selected concentration for AFM and SEM investigation [21].

Designation	MWCNTs [µg/ml]	SNPs [µg/ml]	PAMAM [µg/ml]
EC 10	51.7	2.8	0.07
EC 25	74.1	74.8	0.17

Dynamic light scattering

The measurements of hydrodynamic radius (R) of silver nanoparticles and dendrimers were performed using dynamic light scattering device Dyna Pro Nano Star (Wyatt). After the sonication and filtration processes (filter pores of 0.1µm), the SNPs and dendrimers suspensions in mQ water were placed in disposable cuvettes and the size distribution values (5 times with 10 acquisitions) were collected. The regularization fitting analysis to the autocorrelation functions was employed for the size calculation.

Atomic force microscopy

The atomic force microscopy was used to characterize changes in the endothelial cells morphology. The cells cultured on the cover glasses and exposed to nanostructures were fixed with a mixture solution of glutaraldehyde (2.5%) and formaldehyde (3.7%) in the phosphate buffer at pH 7.4 for 10 min. Having been fixed, the samples were rinsed with the phosphate buffer and immediately subdued to the atomic force microscopy measurements performed in a liquid chamber filled with the PBS solution. The morphology was evaluated using NTEGRA scanning probe microscope (NT-MDT, Russia) with soft cantilevers (CSG01, with the nominal spring constant 0.003 N/m and the tip radius curvature less than 10 nm, NT-MDT Probes, Russia). The scan size was 45 µm x 45 µm and the image resolution was 512 points per 512 lines.

The values of volume and surface area of the cells central part (i.e. above half of the cell height) were obtained with MountainsMap Premium 5.0 (Digital Surf) software. Additionally, the roundness parameter of the cell central part was calculated according to the formula:

$$R = Area/4\pi D^2_{max}$$

where: Area is the surface area of the apical cell part, D_{max} is the maximal diameter of the central part.

The mean value and the standard mean deviation of the morphological parameters were calculated for each experimental point (the cells exposed to the nanostructures at selected concentrations).

Additionally, the roughness parameter of 2 µm x 2 µm AFM topography scans, including the density of summits (Sds) was examined (Nova PX software). For each experimental point at least 16 images of the cells were used for calculation. The acquired data was compared with the results obtained for the control and statistical analysis of the data was performed using the variance ANOVA test to determine differences between the means for each group and the control group at the levels (*) $P<0.05$ and (**) $P<0.01$.

Scanning electron microscopy

For the scanning electron microscopy analysis, the samples were fixed for 24 h and then dehydrated with the alcohol series. Next, the samples were dried in the nitrogen stream at room temperature and a thin layer of gold (about 4 nm) was sputtered on their surface with the Leica AM ACE600 sputter.

The cell morphology was analyzed with the scanning electron microscope Quanta FEI 250 equipped with Everharta-Thornley'a (ETD) detector of secondary electrons. The microscope was working at room temperature with an accelerating voltage of 10 - 20 kV and magnification of 4000x.

Transmission electron microscopy

The silver nanoparticles and multi-walled carbon nanotubes were characterized with Transmission Electron Microscope Talos F200X (FEI) at the acceleration voltage of 200 kV working in the Scanning Transmission Electron Microscopy (STEM) and Energy Dispersive Spectroscopy (EDS) modes. The SNPs and MWCNTs were suspended in 100% ethyl alcohol and milli-Q water, respectively. After sonication, the drop of each sample was placed onto the carbon-coated copper grids (300 mesh) and dried at room temperature.

Results and Discussion

The studied nanostructures were characterized by DLS technique, TEM and EDS methods. FIG. 1 presents the results of the size distribution of PAMAM dendrimers and silver nanoparticles obtained by the DLS technique. The average value of hydrodynamic radius of PAMAM is about 1.9 nm, while for SNPs two populations of 4.3 nm and 54.9 nm are observed.

FIG. 2 presents the TEM images of SNPs where both spherical and longitudinal shapes of silver nanoparticles can be observed. The size distribution of SNPs is polydisperse, which confirms the polydispersity of SNPs observed with the DLS technique. The chemical composition of SNPs was examined with STEM (FIG. 2C) and EDS (FIG. 2D) methods. The EDS spectrum confirms a high silver content and the copper presence in the spectrum results from the use of Cu TEM grids. The other light elements are typical trace contamination.

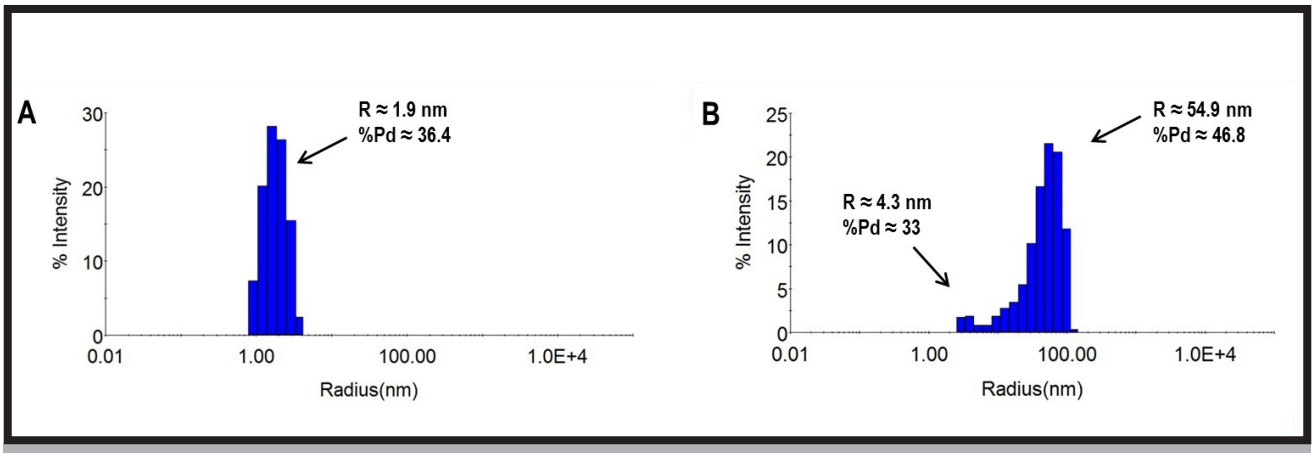

FIG. 1. Hydrodynamic radius distribution determined with DLS method in milli-Q water for PAMAMs (A) and SNPs (B). Mean value of radius (R) and % of polydispersity (%Pd) are provided.

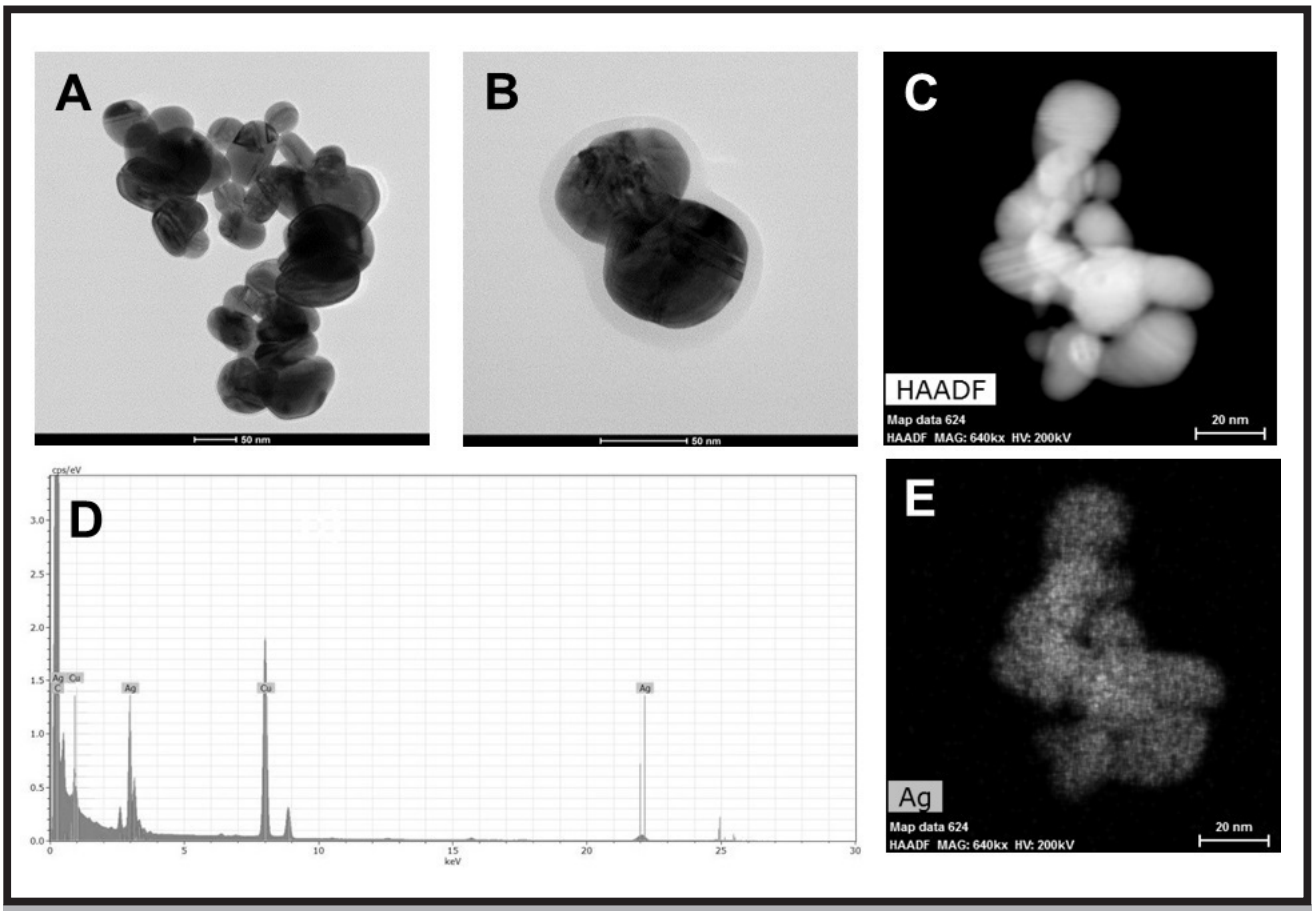

FIG. 2. TEM images of SNPs (A,B), STEM image (C), EDS spectrum (D) and EDS map of silver distribution (E).

In FIG. 3 the TEM images of MWCNTs are presented. The nanotubes are multiwalled (FIG. 3C) and the external diameter is below 20 nm. The main signal peak in the EDS spectrum (FIG. 3D) is assigned to carbon, confirming a high material purity. Copper is present in the spectrum due to the Cu TEM grids.

The cell morphology of randomly selected cells determined by the AFM method is presented in FIG. 4. The white arrows indicate agglomerates of nanostructures accumulated on the cell membrane. However, these nanostructures could also have been moved by the AFM probes while scanning the topography.

FIG. 5A presents the mean values of the surface area and the volume of the cells central parts. The statistically significant increase in the apical surface area was observed for the cells exposed to SNP or MWCNT at EC 10 and EC 25 when compared to the untreated cells (white bars, FIG. 2A). Interestingly, these nanostructures did not induce relevant changes in the cells volume (dark bars, FIG. 2A). Furthermore, the decrease in the cell roundness was observed for the cells exposed to SNP or MWCNT at EC 10 (FIG. 2B). PAMAMs dendrimers did not induce significant morphological changes at the applied concentrations.

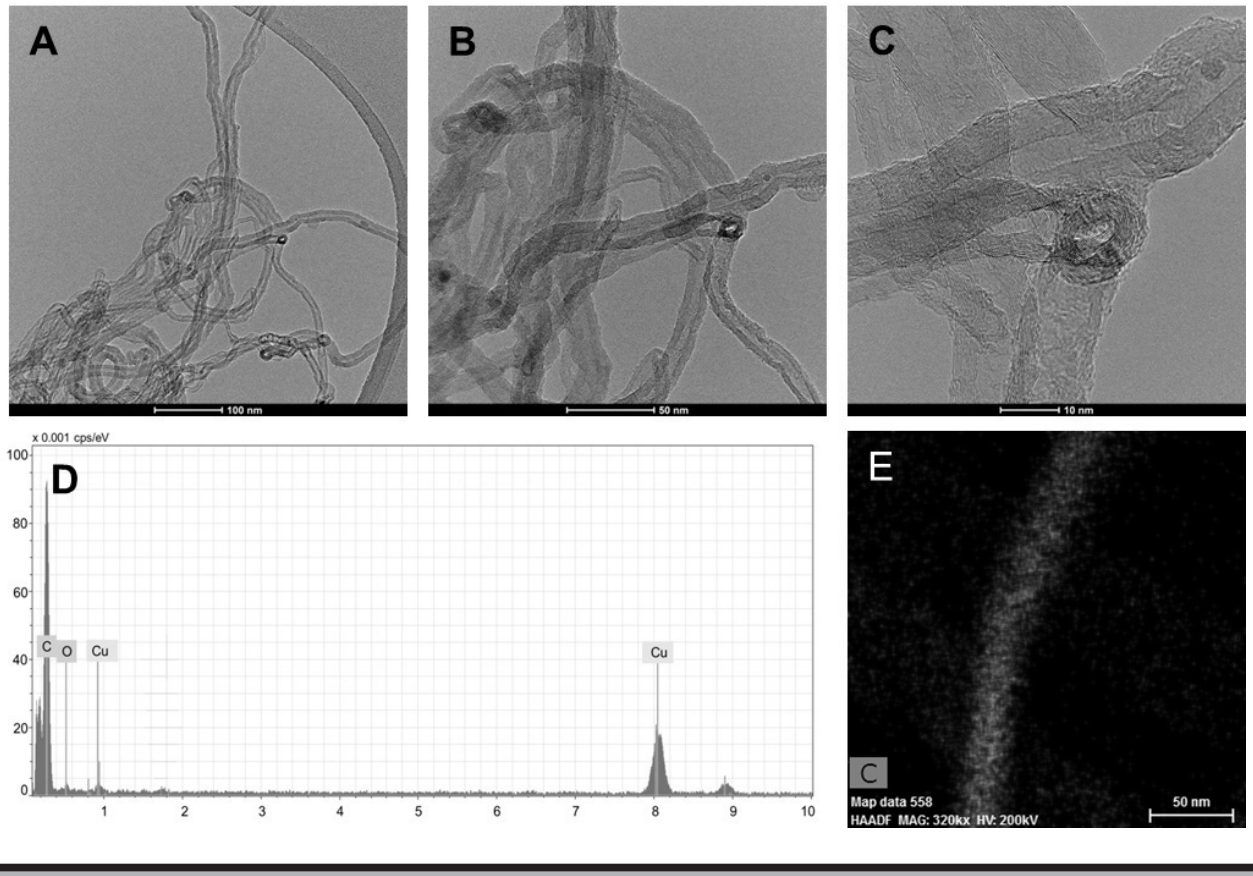

FIG. 3. TEM images of MWCNTs obtained at different magnifications: 74 000x (A), 190 000x (B), 630 000x (C), EDS spectrum (D) and EDS map of carbon distribution (E).

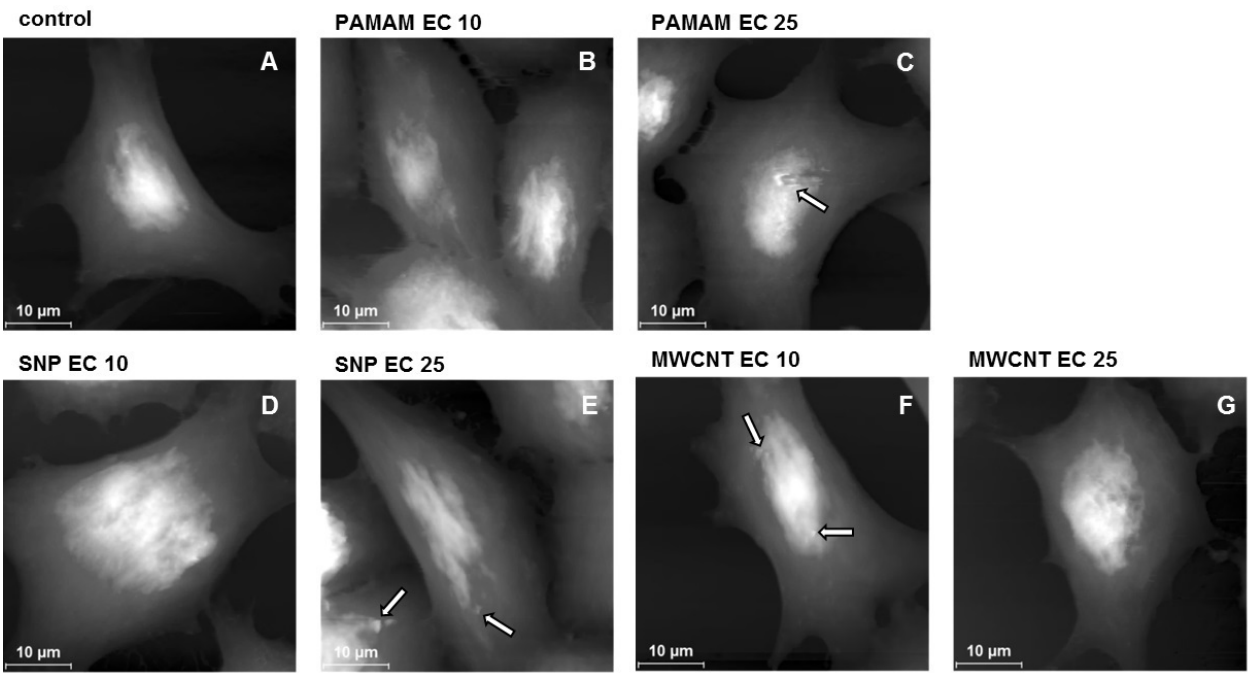

FIG. 4. AFM images (45 µm x 45 µm) of endothelial cells incubated with nanostructures. Arrows indicate agglomerates of nanostructures.

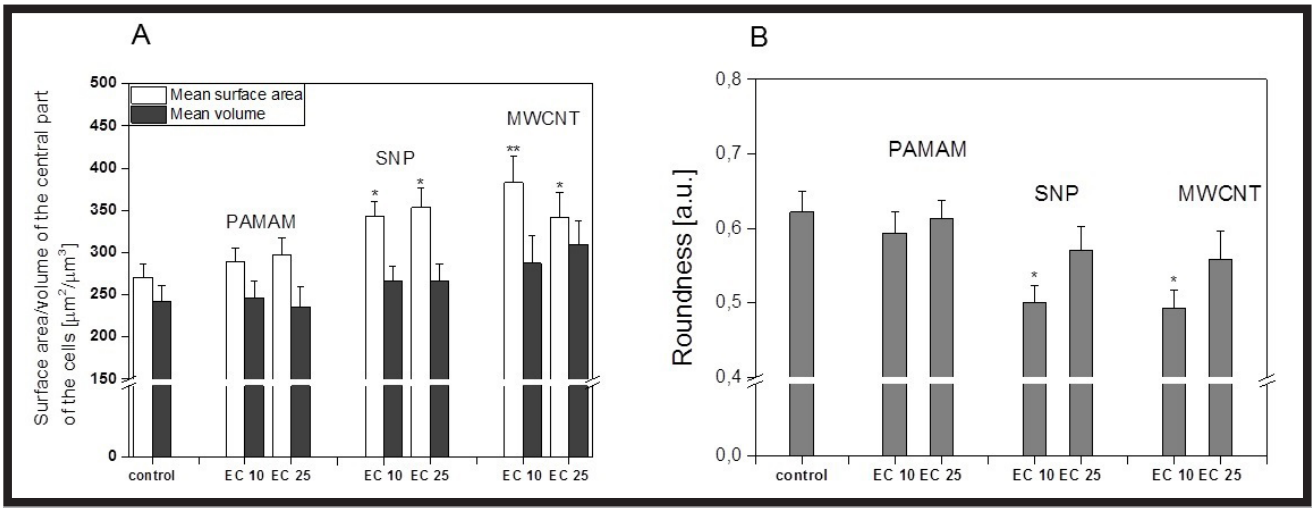

FIG. 5. Mean values of the apical volume of the cells (dark bars) and the apical surface area (white bars) (A) and mean values of the roundness of the cells (grey bars) (B). Statistically significant differences untreated cells are marked as: (*)P < 0.05 and (**)P < 0.01.

TABLE 2. Values of Sds parameter (mean ± standard mean deviation) for cells exposed to nanostructures at EC 25.

Sample	Sds [1/μm²]
Reference	155 ± 56
PAMAM EC 25	239 ± 60
SNP EC 25	294 ± 99
MWCNT EC 25	401 ± 69 (**)

The roughness analysis (TABLE 2) indicates a significant increase in Sds value for the cells exposed to MWCNTs. This phenomenon may suggest the distinct changes in the cellular granularity related to the actin and myosin fibres reorganization and/or the nanotubes accumulation under the cell membrane.

The SEM images of the control and the cells exposed to nanoparticles at EC 10 and EC 25 are presented in FIG. 6. The huge agglomerates of MWCNTs or SNPs in the endothelial cells exposed to these nanostructures were observed (the zoom area of the attached nanostructure on the cell membrane are presented in FIGs 6 F, H).

The alterations in the sample height are visible via the AFM microscopy. However, the AFM imaging does not provide clear information about the nanomaterials localization within the cell - whether nanomaterials are on the cell surface, just below the cell membrane or inside the cell. Moreover, in some cases certain cell structures or nanomaterials and their agglomerates move along the scanning direction, that may suggest their presence on the cell surface (FIG. 4C). The AFM topography measurements were performed on the cells lacking the huge agglomerates of nanoparticles, which allowed to avoid the AFM tip damage or increase in the estimated curvature radius. For the SEM imaging, the cells with larger NMs agglomerates were selected to present the sample diversity that is necessary for cells measurements. These microscopic techniques were selected to show the effect of nanomaterials on endothelial cells in their most desirable environment. Apart from the bovine serum, cellular media contain other factors (antibiotics, L-glutamine, glucose) that can affect the agglomeration of nanostructures but maintain the cell culture in its proper conditions. It is also worth noticing that the alterations in ionic strength change the radius of dendrimers [22].

Morphological changes might be induced by the membrane proteins interaction with nanomaterials which may trigger a sequence of intracellular biological processes. Morphological changes can be linked to: the gene expression regulating cells growth and proliferation, several biological processes related to the proper protein folding, and/or cytoskeletal formation and reorganization. NPs can evoke a cytotoxic or genotoxic effect in the cell [23,24], change certain signalling pathways or alter the gene or protein expression [25].

Regarding the MWCNTs influence on mammalian cells, several papers reported that these nanomaterials lacking the surface modification were cytotoxic [26-28]. The obtained morphological results showed an increase in the apical surface area and the density of summits parameter that may be associated to the MWCNTs agglomeration, as well as the single molecules binding to the cell membrane. Similarly, when the endothelial cells were exposed to SNP at EC 10 and EC 25 concentrations, the increase in the cell surface area and the decrease in the cell roundness (at lowest concentrations) were observed. The SNPs, similarly to MWCNTs, are accumulated on the cell membrane (FIG. 6 D, E, F) and they penetrate inside, thus causing the observed changes in the cells morphology. The flow cytometry tests from our previous study confirmed the uptake of SNPs by endothelial cells which was revealed by an increase in the side (SSC) scattered light parameter [20]. In the case of the PAMAMs at EC 10 and EC 25 concentrations, there were no morphological changes as compared to the control cells.

Conclusions

The microscopic techniques can be an additional source of information characterizing the cells response to external factors present in their environment (e.g. drugs, nanomaterials). Due to the fact that nanotechnology is gaining more and more recognition in the field of medicine, every nano product should be thoroughly examined regarding its toxicity to the human body. Often, the standard methodology which includes cyto- and/or genotoxicity tests, is not sufficient and does not allow a definitive conclusion whether a given material is safe. Despite the satisfactory response of the cells in these tests, nanoparticles can exert a significant effect on the cell at the molecular level, inducing changes in the signalling pathways, expression of genes or proteins responsible for performing key functions for the cell life, which has been confirmed in numerous reports [29-32].

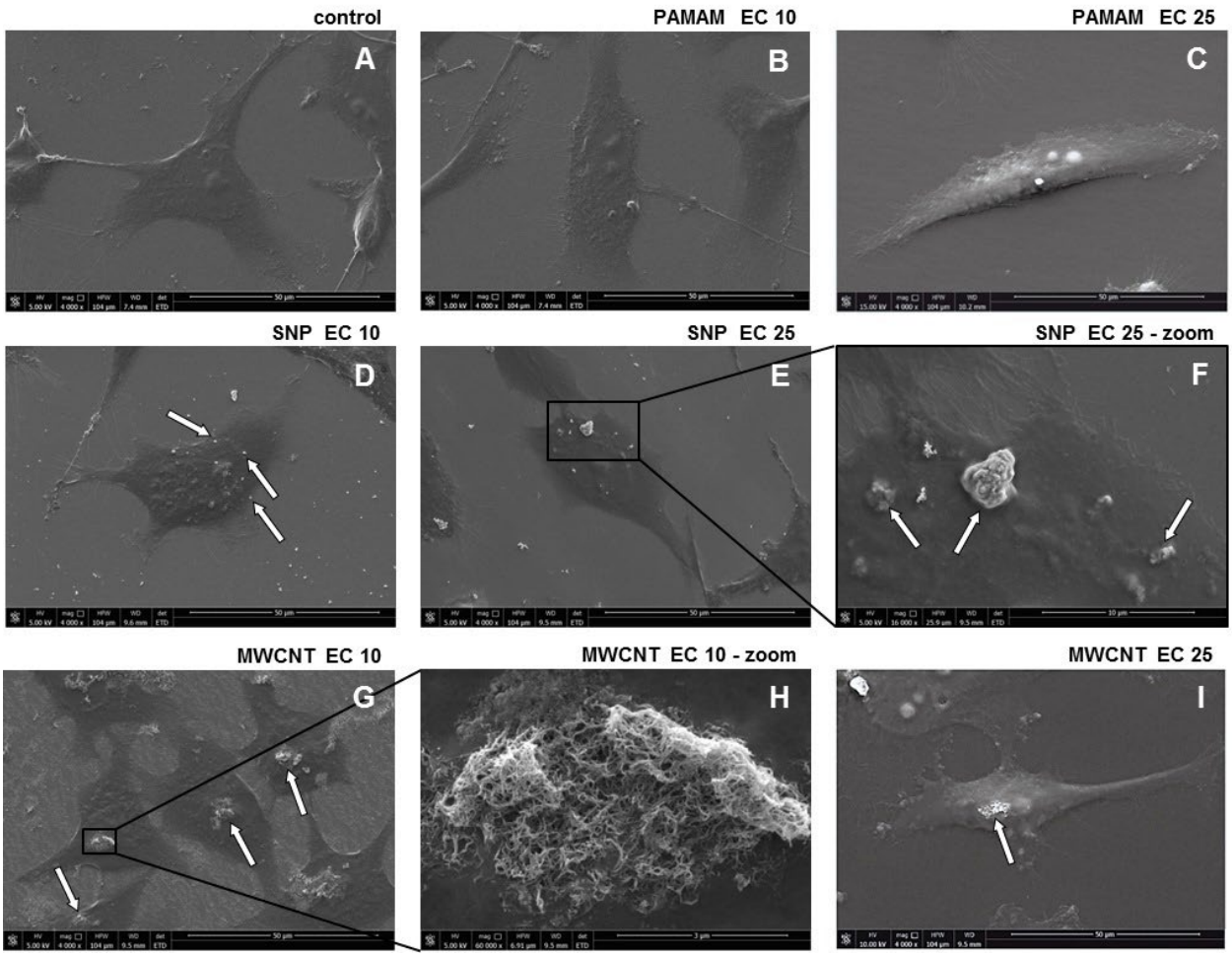

FIG. 6. SEM images of control cells (A) and cells incubated with nanostructures at EC 10 (B, D, G, H) and EC 25 (C, E, F, I). Arrows indicate examples of agglomerated nanomaterials on the cell membrane.

In our recent paper [21], it was proved that the EA.hy926 cell line after interaction with SNPs, MWCNTs and PAMAM dendrimers shows changes in the expression of numerous mRNA transcripts. Similar observations were performed while assessing the proteomic profile of these cells - the expression of proteins was significantly changed in terms of the cell growth and proliferation, apoptosis, reorganization of the cytoskeleton, synthesis of other proteins and their degradation, DNA repair, regulation of transcription, as well as the stress response.

The presented study provides new insights into evaluation of effects of exposing endothelial cells to the selected nanostructures. Both silver nanoparticles and polyhedral carbon nanotubes, used at concentrations corresponding to EC 10 and EC 25, have a significant impact on the endothelial cell morphology. Despite the widely reported toxicity of PAMAM dendrimers, our observations do not confirm such an influence of these nanostructures on endothelial cells, if used at relatively low concentrations (EC 10 and EC 25),

The microscopic techniques presented in this work may contribute to the characteristics of the cells exposed to nanomaterials, and help to establish whether the cell undergoes harmless adaptive changes, or whether the defence response to stress caused by the presence of nanomaterials in their environment begins.

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