Nanoscale

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Cite this: DOI: 10.1039/c0xx00000x

www.rsc.org/xxxxxx

ARTICLE TYPE

Metallic oxide nanoparticle translocation across human bronchial epithelial barrier – Supplementary information.

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Supplementary Information

1. NP Synthesis.

Materials and reagents

NP synthesis is performed with Tetraethoxysilane (TEOS) (99+%) and 3-aminopropyltriethoxysilane (APS) (99%), N-(trimethoxysilylpropyl)ethylenediamine (EDPS) (97 %), Fluorescein isothiocyanate (FITC) (98%), tetrabutyl titanate (TBT) (97%) and anhydrous 1-butanol (99.8%) (Sigma-Aldrich, Saint Quentin Fallavier, France).

Size-controlled fluorescent silica nanoparticles (NPs) production

16 nm-SiO_2-FITC NPs production was performed in one step by adding a solution of 5 mL of fluorescent organosilane solution previously prepared mixed with 240 mL of ethanol and 5 mL of TEOS into a 2 L round bottom flask containing 650 mL of EtOH, 2.4 mL of water and 24.1 mL of ammonia. The solution was let to react overnight at room temperature (RT) in the dark. 16 nm-SiO_2-FITC NPs were purified against ultrapure water (18 MΩ) by tangential ultrafiltration with an Amicon system (Millipore, Billerica, USA) equipped with a membrane of 100 kDa cut-off until disappearance of fluorescence in the filtrate. Batches of bigger sizes were purified by centrifugation. Before, ammonia and ethanol were removed from the medium by rotative evaporation at 40°C at the end of the synthesis. The fluorescent particles were extensively washed by centrifugation against ultrapure water at 13,000 g for 15 min until disappearance of fluorescence in the supernatant. Estimation of silica concentrations in the dispersion was carried out by Inductively Coupled Plasma (ICP) using an ICP Optical Emission Spectrometer (ICP-OES) and a gravimetric method.

For the production of 50- and 100 nm-SiO_2-FITC NPs, please refer to Vranic et al., 2013a[1].

Chemical surface modification of SiO_2-FITC-NPs by EDPS

Starting from the NP developed surface area and given that the area on the NP surface covered by the organosilane coupling agent is assumed to be nominally 55 Å² per molecule, it is possible to calculate the amount of organosilane needed for a given NP size. After it was left to react overnight, 100 mL of glycerol was added and the ammonia and ethanol were evaporated at low boil under a moderate vacuum. In order to promote the condensation of the polysiloxane film onto the silica surface, the reaction was followed by a thermal treatment in vacuum at 100-110°C. An equal volume of water was then added which induces the colloidal destabilisation. Flocculates were then washed three times by centrifugation, once with acetone and twice with water, to remove excess of reactants. Aminated silica NPs were peptidized by addition of chlorhydric acid in the solution to achieve a pH of 6 in order to promote the dispersion of the colloids. Estimation of silica concentration was carried out again by ICP-OES and gravimetric method for each colloidal dispersion.

Production of TiO_2-coated SiO_2-FITC-NPs

SiO_2-FITC-NPs were coated by an ultrathin layer of TiO_2 by hydrolysis/condensation of terbutyl titanate (TBT) in hydroalcoholic medium. First, the silica dispersions were diluted in ethanol in order to limit the volume fraction of water under 2%. This can be performed by centrifugation or tangential ultrafiltration. The amount of TBT was calculated in order to reach a thickness of 2 nm around the SiO_2-FITC-NPs. Then, the corresponding volume of 10% TBT solution in 1-butanol was added at once into the silica dispersion under vigorous stirring. After 4 h stirring at RT, TiO_2-coated SiO_2-FITC-NPs were washed with water by centrifugation.

All data have been summarized in the Supporting Figure S1.

2. NP Characterization.

Material and methods:

DRIFT was achieved by using a Bruker IFS Equinox 55 spectrometer and measured in a Selector Graseby Specac reflection cell. The sample was prepared by drying 2 mL of a nanoparticle solution at 80°C under vacuum. 9 mg of the dried sample was added to 281 mg of equally dried KBr (spectroscopy grade). The mixture was pestled in an agate mortar and the powder was deposited on the sample holder. Thirty minutes after the installation of the sample in the cell an infrared spectrum was recorded by the acquisition of 128 measurements in the range of 400-4000 cm^{-1}.

Inductively Coupled Plasma (ICP) analysis was carried out by using an ICP Optical Emission Spectrometer (OES) Varian 720-ES equipped with a Varian SPS-3 Sample Preparation System Autosampler. Samples were prepared by dissolving of silica or titanium oxide/silica dispersions by hydrogen

fluoride (HF ; 40%) followed by neutralization with ammonia and by dilution 20 to 100 times in borate buffer in order to enter the values in calibration range performed with certified standards. Each measurement was repeated 3 times.

Based on laser Doppler interferometry, the Pz measurement was used to assess the electrophoretic mobility of NPs. Measurements were performed for 20 s using a standard capillary electrophoresis cell. The dielectric constant of solvent (water) was set to 80.4 and the Smoluchowsky constant f(ka) was 1.5. NP sample dispersions were prepared at various pH with a concentration of 0.1 mg/mL in order to determinate their isoelectric point (IEP) before and after surface modification.

Results and discussion:

Analysis by DRIFT were first performed for SiO_2-FITC-NPs before and after silanization or coating with titanium oxide (Supporting Information S2A). SiO_2-FITC-NPs spectrum displayed all the vibration bands of naked colloidal silica[2]: those from 400-1200 cm^{-1} are characteristics of Si-O-Si vibrations, band at 1872 cm^{-1} could be identified as a typical silica overtone band and the intense broad band in the range from 3400 to 3700 cm^{-1} is due to the OH stretching vibrations of hydrogen bonds of Si-OH groups covering the surface of the colloidal silica and H bonded H_2O. It can be noticed the presence of weak bands at 2933 and 2862 cm^{-1} attesting the hybrid nature of the core composed of fluorescent organosilanes. SiO_2-FITC-NP^+ spectrum revealed additional bands in the range from 1500 to 1390 cm^{-1}. The bands at 1358 and 1402 cm^{-1} are attributed to C-N deformation vibrations and a band at 1450 cm^{-1} attributed to C-H deformation vibrations for a carbon chain attached to a primary or a secondary amine group. TiO_2-coated SiO_2-FITC-NP spectrum revealed the absence of the band for vibrations of the Si–O–Ti bonds usually observed at 949 cm^{-1} for thermally treated dehydrated materials[3], suggesting that the TiO_2-coating was just physically adsorbed onto silica surface certainly separated by a thin chemisorbed water layer. Characteristic bands of Ti-O vibrations in TiO_2 lattice[4] usually observed between 800-1400 cm^{-1} are not visible here due to the superposition of Si-O vibrations predominantly present in this region.

Fluorescent SiO_2-FITC-NPs displayed the same surface features as pristine silica particles with an isoelectric point (IEP, Supporting Information S2B) around 2.6 in agreement with values of the literature[5]. After silanization of the silica surface, the IEP value shifted to 9 close to pKa values of primary and secondary aminoalkyl groups. This result shows that the silica surface is entirely covered by the aminated polysiloxane film with a high degree of condensation. The value of the TiO_2-coated SiO_2-FITC-NP isoelectric point was measured at 4.3 which is in good agreement with those already observed for amorphous titanium oxide prepared by similar sol-gel route i.e. 4.5[6]. A thin layer of TiO_2 is therefore sufficient to carry the physicochemical surface characteristics of amorphous TiO_2 prepared by sol-gel.

Supporting Information S2C gives the ratio between Si and Ti elements measured by ICP-OES for 140 nm TiO_2-coated SiO_2-FITC-NPs with the calculated values of the thickness. The size magnitudes of TiO_2 thickness for greater particles are quite close to those expected.

3. Morphological characterization of Calu-3 epithelium.

Characterization of Calu-3 epithelium by optical and confocal microscopy (Supporting Figure S3). Visualisation of MUC5AC, ZO-1, CLD-4 and actin proteins.

4. NP cytotoxicity and pro-inflammatory response.

To evaluate NP cytotoxicity Calu-3 cells were seeded in 96-well plates at 50,000 cells/well. Cells were treated with 16- 50- 100 nm-SiO_2 or SiO_2^+-FITC-NPs or 140 nm-TiO_2 coated SiO_2-FITC-NPs in a concentration range from 0 to 50 µg/cm² for 24 h. Metabolic activity was assessed using the WST-1 cell proliferation reagent (Roche, Meylan, France) according to the manufacturer's recommendations (Supporting Figure S4A). For this purpose, cells were rinsed after NP treatment and incubated for 2 h with WST-1 reagent. Using a microplate photometer Elx800 (Biotek, Colmar, France) we measured by spectrophotometry the absorbance at 450 nm of supernatants only to avoid interference of internalized NPs.

To estimate NP pro-inflammatory response after NP treatment, apical and basolateral media were removed, and centrifuged at 10,000g for 10 min at 4°C to eliminate cellular debris and NPs and then stored at -20°C. IL-8 amounts were evaluated using an ELISA kit (R&D Systems, Supporting Figure S4B). The optical density was measured at 450 nm with a microplate photometer Elx800 (Biotek).

5. Modulation of cellular permeability after NP treatment and NP retention inside Calu-3 monolayer after 1 week of exposure.

After NP treatment, the efficiency of the monolayer tightness was determined by TransEpithelial Electric Resistance (TEER) measurement (Supporting Figure S5A).

Complete cell culture medium was changed before each TEER measurement. After 1 h of incubation at 37°C, TEER was determined using the STX2 electrode (World Precision Instruments, Hertfordshire, United Kingdom) and the electronic circuit of the Epithelial Voltohmmeter (EVOM). Mean TEER values of twelve wells were calculated. The mean resistance of a cell-free TF (100 Ω/cm²) was subtracted from the resistance measured across each cellular monolayer to yield TEER value of the cell monolayer.

To evaluate NP retention after 24 hours of treatment Calu-3 cells treated with 50 nm-SiO_2-FITC-NPs at 5 µg/cm² were rinsed two times by PBS and maintained in culture during 7 days more (Supporting Figure S5B). After 21 days of culture cells were fixed for confocal microscopy experiments.

6. NP translocation through TF in absence of cells

in function of NP concentration, composition or corona.

TF were exposed to NPs for 24 h at 5 and 10 µg/cm² ± FCS or ± DPL to determine NP ability to cross TF in absence of cells (Supporting Figure S6). After 24 h of exposure 100 µL of each apical and basolateral media as well as the serial NP dilutions were deposited inside a white 96-well plate with clear bottom (Greiner, Courtaboeuf, France). FITC fluorescence (488 nm / 521 nm) coupled to NPs was quantified using FluoStar Galaxy. Fluorescence background value (medium without NPs) was subtracted from sample fluorescence. Fluorescence values were converted into final NP concentrations in each compartment by using a standard curve, and then in percentage of the initial NP concentration. For the "filter" compartment values were determined by subtraction of the apical and basolateral media from the initial NP concentration applied.

To ensure that the TF on which cells grew was not a limitation to NP translocation from the apical to the basolateral compartment, TF were treated in absence of cells with 16- 50- 100 nm-SiO_2 and SiO_2^+-FITC-NPs and 140 nm-TiO_2-coated SiO_2-FITC-NPs at 5 and 10 µg/cm² for 24 h. All NPs were detected in the basolateral side but SiO_2^+-FITC-NPs and 140 nm-TiO_2-FITC-NPs used at 5 µg/cm² crossed more than NPs used at 10 µg/cm². This was not the case for SiO_2-FITC-NPs where the NP concentration did not influence NP translocation. NP treatment at 10 µg/cm² led to a higher final NP basolateral concentration than NP treatment at 5 µg/cm² (for example for 16 nm-SiO_2-FITC-NPs$^+$ 2.7 µg/cm² compared to 3.3 µg/cm² respectively), but final NP basolateral proportion was higher for the lower concentration compared to the higher concentration (54.1% compared to 32.7% respectively). There was not a huge difference among NPs of different sizes within the same type. However there was a clear difference among the different types of NPs: SiO_2-FITC-NPs translocated less than SiO_2-FITC-NPs$^+$ and TiO_2-coated SiO_2-FITC-NPs suggesting the role of NP charge in their trapping inside the TF.

This role of NP surface charge has already been determined for polystyrene carboxyl- or amine-modified NPs[7] where charges modulated the NP translocation across a TF in absence of a cellular monolayer.

7. Confocal microscopy observations of NCI-H292 cells exposed to fresh and conditioned media.

The human bronchial epithelial cell line NCI-H292 were purchased from the ATCC and grown in RPMI (Roswell Park Memorial Institute) medium 1640 culture medium with phenol red (Life Technologies), containing 1% glutaMAX, 10% FCS, 1% PS, and 0.5% amphotericin B. All experiments were performed with these cells from passages 13 to 20. Cells were grown in T75-flasks for cellular expansion and seeded at 50,000 cells/well in 8-well Labtek (Millipore, Billerica, USA) for conditioned-media experiments (Supporting Figure S7).

Cell cultures were maintained in a humidified atmosphere of 5% CO_2 and 95% air at 37°C.

1/ Calu-3 cells were treated with 16 nm-, 50 nm- and 100 nm-SiO_2-NPs at 10 µg/cm². Basolateral media, subsequently referred as conditioned-media, were recovered from Calu-3 translocation experiments after 24 h of treatment. At 70% of confluence, NCI-H292 cells were exposed to these conditioned-media for 6 h. After exposure, conditioned media were removed and cells were rinsed two times with PBS for fixation for Confocal Microscopy experiments.

2/ At 70% of confluence, NCI-H292 cells were exposed to free FITC (1.2 ng/mL final in culture medium, Sigma Aldrich), or treated with 16 nm-, 50 nm- and 100 nm-SiO_2-FITC-NPs for 6 h either at the same concentration initially applied on Calu-3 cells (10 µg/cm²) or at the estimated Calu-3 basolateral NP concentration after 24 h of treatment (0.86, 1.45 and 0.79 µg/cm²). After exposure media were removed, cells were rinsed and fixed for confocal microscopy observations.

8. Quantification of NP internalization by Calu-3 cells by flow cytometry.

Calu-3 cells were seeded in 12-well plates at 40,000 cells/cm². Cells were treated with 16- 50- 100 nm-SiO_2 or SiO_2^+-FITC-NPs or 140 nm-TiO_2-coated SiO_2-FITC-NPs at 5 µg/cm² for 24 h. After treatment cells were washed by PBS and were harvested by trypsination whose action was stopped with 10% FCS. Before flow cytometry analysis cells were incubated with complete cell culture medium containing 0.11% Trypan Blue (stock solution at 0.4%, Sigma Aldrich) in order to quench the FITC-fluorescent signal coming from NPs absorbed to the cell surface according to the method described by Vranic et al., 2013[1]. Cell-associated fluorescence was detected using a CyAn ADP LX (Dako Cytomation, Beckman Coulter, Villepinte, France) flow cytometer. Laser excitation wavelength was 488 nm and fluorescence emission was detected using a band-pass filter of 530 ± 20 nm. A minimum of 10,000 cells was analyzed after exclusion of cellular debris from the analysis by gating on the 575 nm Log versus Forward Scatter area graph. Results are reported as mean fluorescence intensity (MFI) obtained by analyzing 10,000 cells in the gate (Supporting Figure S8).

Notes and References

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