Proteomic study of human bronchial epithelial cells exposed to SiC nanoparticles

Caroline Tokarski¹, Seishiro Hirano², Christian Rolando¹

¹Miniaturisation pour l'Analyse, la Synthèse & la Protéomique (MSAP), USR CNRS 3290, and Protéomique, Modifications Post-traductionnelles et Glycobiothèque, IFR 147, Université de Lille 1 Sciences et Technologies, 59655 Villeneuve d'Ascq Cedex, France

²National Institute for Environmental Studies, 16-2 Onogawa, Tsukuba, Ibaraki 305-8506, Japan

E-mail: Christian.Rolando@univ-lille1.fr

Abstract. The presented work proposes an optimized methodology for the study of cell exposure to nanomaterials at protein level. The study was investigated on proteins extracted from human bronchial epithelial cells exposed and non-exposed to silicon carbide nanoparticles (SiC). The analytical strategy was based on high resolution measurement using Fourier transform mass spectrometer 9.4 T. The methodology proposed succeeds in identifying over 300 proteins; most of the identified proteins are present in both exposed and non exposed cells to SiC nanoparticles. More interestingly, cytokines as Macrophage migration inhibitory factor protein could be identified only in the cells exposed to SiC nanoparticles indicating cell inflammatory response.

1. Introduction

Nanotechnology allows the ability to design many new materials [1-4] and devices with multiple applications, such as in medicine e.g. nanoparticles can represent drug delivery vectors [5]. However, nanotechnology also raises several concerns about the environmental impact [6] and the toxicity [7] of nanomaterials. In particular, the literature showed that toxicity of nanoparticles is highly related to the particle size and its chemical composition. For example, cytotoxicity studies related to the in vitro response of eukaryotic cells to TiO₂ nanoparticles exposure, showed that intracellular reactive oxygen species are generated [8,9]. Other study exposing human aortic endothelial cells to different metal oxide nanoparticles as iron oxide, yttrium oxide or zinc oxide showed induced cell inflammation depending on particle composition [10]. In this way, transcriptomic studies using cobalt nanoparticles pointed out increased transcription and activities of some matrix metalloproteinases [11]. Additional study based on electron microscopy shown that multi-walled carbon nanotubes associate with the plasma membrane of macrophages and disrupt the integrity of the membrane [12].

The presented work aims to highlight consequences of cell exposure to nanomaterials at the protein level using adapted proteomics methodology. The study was investigated on proteins extracted from human bronchial epithelial cells (BEAS-2B) exposed and non-exposed to silicon carbide nanoparticles
(SiC). As low abundant proteins like pro-inflammatory cytokine are mainly researched, very sensitive methodology was developed based on high resolution Fourier transform mass spectrometer 9.4 T.

2. Experimental section

2.1. Silicon carbide nanoparticles
The SiC nanoparticles were obtained from the Commissariat à l'Energie Atomique, CEA Saclay, Gif-sur-Yvette, France. The nanoparticle composition was 69.2% Si, 30.1% C and 0.88% O. The particle size and aggregation measurements were done by dynamic light scattering. In distilled water, depending on the ultrasonic duration from 2' to 6', mono disperse populations were measured with an average size from 222 nm to 182 nm. The elementary size of the particle is 36.8 nm. The density was measured at 3.09 g/cm³. The BET surface was 52.75 m²/g.

2.2. Cells
A human bronchial epithelial cell line BEAS-2B was cultured to early confluence in Dulbecco's modified minimum essential medium (DMEM) according to the detailed procedure published by Dr Seishiro Hirano et al. [13]. BEAS-2B cells were exposed to SiC at a concentration of 10 µg/ml for 24 hours according to the detailed procedure [13].

2.3. Protein extraction
Cells were washed 3 times using PBS buffer. Proteins were extracted using lysis solution containing 0.05 M tris(hydroxymethyl)aminomethane pH 8, sodium dodecyl sulfate 2%, 0.2 M dithiothreitol, 50 mM glycerol-2-phosphate, 5 mM sodium fluoride, 1 mM sodium orthovanadate, 1 tablet of protease inhibitor for 50 mL of buffer. A solution containing 0.05 M MgCl₂, DNase 2000U/mL, RNase 750 U/mL, 0.05 M tris(hydroxymethyl)aminomethane pH 8 was added in 1/10 proportion (v/v). The supernatant was separated from the cell membranes using centrifugation at 15 000 rpm during 30 min. Proteins were quantified using the commercial PlusOne 2-D Quant kit (GE Healthcare, Uppsala, Sweden); bovine serum albumin was used as calibrating agent.

2.4. Gel electrophoresis
The extracted proteins from the BEAS-2B cells exposed and non exposed to SiC nanoparticles were dissolved in Laemmli buffer [14]. The stacking gel was 4% and the separating gel was 12%. A quantity of 40 µg of extracted proteins per condition was run on 1D gel. Electrophoresis was carried out with running buffer containing 25 mM tris(hydroxymethyl)aminomethane, 0.1% sodium dodecyl sulfate (w/v), and 192 mM glycine, at 10 mA per gel until the bromophenol blue front had reached the bottom of the gel. Proteins were stained with SYPRO Ruby. Stained 1D gels were digitized using a Typhoon 9000 scanner (GE Healthcare). Image acquisition was performed at 550 V with a blue laser (λexc= 488 nm/λem= 610 nm).

2.5. Protein digestion
The protein digestion was investigated with preliminary strong protein reduction and alkylation [15]. Briefly, the gels were sliced all over in 40 bands. Each band was washed with 100 µl of 50 mM ammonium bicarbonate, pH 8.8, by agitation for 15 min with a vortex mixer. The proteins were reduced with 10 mM dithiothreitol for 2 h and alkylated with 55 mM iodoacetamide in the dark for 1 h. The supernatant was removed and the gel pieces were dehydrated with 100 µl of acetonitrile for 15 min and washed with 100 µl of 50 mM ammonium bicarbonate, pH 8.8, for 15 min. This operation was repeated three times. Gel pieces were completely dried with a Speed Vacuum (Concentrator 5301; Eppendorf, Hamburg, Germany) for 20 min. The dried gel pieces were rehydrated with 50 µl of 50 mM ammonium bicarbonate, pH 8.8, 5% acetonitrile containing 20 µg/µl of trypsin. The supernatant was removed and replaced by 50 mM ammonium bicarbonate, pH 8.8, 5% acetonitrile.
The hydrolysis was done overnight. Samples were completely dried with Speed Vacuum and 5 µl of deionized water, 0.1% formic acid, and 5% acetonitrile were added.

2.6. Peptide analysis

The peptide sequence spectra were obtained using nanochromatography (Ultimate LC system, Dionex, LC-Packings, Amsterdam, Netherlands) on-line with an Apex Qe 9.4 T Fourier Transform Ion Cyclotron Resonance Mass Spectrometer (Bruker Daltonics, Bremen, Germany). Starting from a volume of 1 µl of the protein digest solution, peptides were desalted and concentrated on a C18 preconcentration column (0.5 cm x 300 µm) and separated on a Pepmap C18 column (15 cm x 75 µm) at 200 nl/min solvent flow. The elution was performed using gradients of solvent A (95% H2O, 5% ACN, 0.1% formic acid) and solvent B (20% H2O, 80% ACN, 0.1% formic acid): 15 min in 100% solvent A, solvent B was increased to 100% in 130 min, then kept at 100% for 15 min, and finally decreased to 0% in 5 min. The column was allowed to equilibrate for 15 min between runs. The FT-ICR mass spectrometer is equipped with a nano-electrospray source. Detection was carried out in positive mode. A potential of 1.6 kV was applied on the needle. The detection parameters are broadband detection, 512 K acquisition size, and start mass at m/z 300. Fragmentation was triggered for any multiply charged species with a relative collision energy calculated in function of the selected species charge state by the Bruker AutoMS method in ApexControl software. Once selected and fragmented, eluted species were excluded from the MS/MS selection for 10 min.

2.7. Protein identification

The peptide monoisotopic mass was extracted from each MS and MS/MS spectrum using DataAnalysis software (Bruker Daltonics, Bremen, Germany). The generated mass data in Mascot format were post calibrated using an in-house program before extensive database search. Calibrated files were then submitted to local MASCOT software containing SwissProt database (release 56.8 of 10 February 2009: 410,518 sequences) for protein identification. The interrogations were performed with a restriction to the human species. Mass tolerance was set at 5 ppm on precursor ions and 5 mmu on fragment ions. A tolerance of three missed cleavage sites was set. Methionine oxidation and carbamidomethylation were entered as variable modifications.

The peptides related to the Macrophage migration inhibitory factor protein (Human species, MIF_HUMAN, P14174) from in silico trypsin digestion were search in the MS and MS/MS spectra from experiments related to cells non exposed to SiC using Extracted Ion Chromatograms (DataAnalysis software).

3. Results and discussion

The proposed study is based on multi-steps proteomic analysis including a first separation of extracted protein according to their molecular weights using 1D gel electrophoresis. To identify low abundant proteins, each step of the methodology had to be optimized. In details, proteins were extracted from human bronchial epithelial cells (BEAS-2B) exposed and non exposed to SiC nanoparticles using adapted protocol detailed in the experimental section part 2.3; it can be noted that sodium dodecyl sulfate, a strong detergent, was used due to its very high solubilizing power. The extracted proteins from exposed and non exposed cells to SiC were separated independently on 1D SDS-PAGE gel. The Figure 1 presents the separated proteins extracted from BEAS-2B cells exposed to SiC at a concentration of 10 µg/ml for 24 h. The gel was totally sliced in 40 band spots for each condition (exposed and non exposed cells) and each band was enzymatically hydrolyzed using trypsin; the digestion protocol was optimized, in particular, strong protein reduction and alkylation before digestion were as detailed in the part 2.5. The generated peptides were analyzed using on-line nanoLC nanoESI-Qh-FT-ICR MS/MS. The nanoLC column used in this study was a C18 column of 75 µm inner diameter; C18 precolumn was also used to desalt and concentrate the sample. The chromatographic run was particularly optimized to obtain the highest number on identified peptides for each condition. The best results were obtained using 3 h experiment for each gel band as detailed.
in the experimental section part 2.6. The mass spectrometer settings were adapted to the sample composition, i.e., time cycle of an experiment (MS or MS/MS) including accumulation, transfer, excitation, detection and quench, run on ~3 s. In details, ions were accumulated 1 s in the hexapole, 0.01 and 2 s in the quadrupole collision cell respectively for MS and MS/MS; 0.0016 s was set for optics transfer and 0.01 s for electronic dwell time. The detection parameters were broadband detection, 512 K acquisition size, start mass at m/z 200 leading to 0.5243-s transient duration, allowing theoretical resolution of 190 000 at m/z 400. The obtained spectra were calibrated and treated using the bioinformatic tools (see part 2.7) for protein identification.

The optimized proteomic methodology detailed here succeeds in identifying more than three hundred proteins. Most of the identified proteins were present in exposed and non exposed cells to SiC nanoparticles. However, some proteins were identified only in the cells exposed to SiC nanoparticles as for example pro-inflammatory cytokines as the Macrophage migration inhibitory factor protein (MIF). The Figure 2 presents the MS/MS spectrum allowing the identification of the sequence PMFIVNTNVPR relating to the position 2-12 of the MIF sequence using y_4$^+$ to y_10$^+$ ions. It can be noted that previous antibody-based study on BEAS-2B cells exposed to multi-walled carbon nanotubes (MWCNT) showed increased MIF concentrations in a MWCNT-dose dependent manner [13]. Based on high accuracy measurement MS and MS/MS data (Δm < 1 ppm on precursor ions and fragment ions), the lack of MIF in the cells non exposed to SiC was confirmed using extracted ion chromatograms of MIF constitutive peptides.

Figure 1. SDS-PAGE gel presenting the BEAS-2B cells exposed to SiC at a concentration of 10 µg/ml for 24 h (on the right) and molecular weight markers (on the left).

The molecular weights of marker proteins are indicated in kDa from 10 to 260 kDa.

Figure 2. MS/MS spectrum of the peptide eluted at 14.7 min allowing the identification sequence PMFIVNTNVPR (precursor m/z 652.345, Δm< 1 ppm). The peptide represents the position 2 - 12 of the Macrophage migration inhibitory factor from Human species (MIF_HUMAN). The detected peptide is oxidized on the methionine amino acid.
4. Conclusion

The presented optimized proteomics methodology based on high resolution mass spectrometry allowed identifying cytokines in cells exposed to SiC nanoparticles indicating the pro-inflammatory reaction.

Acknowledgements

The Mass Spectrometry facilities used for this study are funded by the European community (FEDER), the Région Nord-Pas de Calais (France), the IBISA (Infrastructures en Biologie Santé et Agronomie) network, the CNRS, and the Université Lille 1 Sciences et Technologies. We acknowledge Carole Sentein and Frédéric Schuster for providing nanoparticles and The International Team in NanosafeTy members (TITNT).

References

[1] Sentein C, Guizard B, Giraud S, Yé C and Ténégal F 2009 J. Phys.: Conf. Ser. 170 012013
[2] Schuster F 2010 Clefs C.E.A. Eds Commissariat à l'énergie atomique, Paris 59 4
[3] Maximilien R, Schuster F, Sicard Y and Tardif F 2010 Clefs C.E.A. Eds Commissariat à l'énergie atomique, Paris 59 84
[4] Sentein C, Schuster F and Tardif F 2009 J. Phys.: Conf. Ser. 170 011001
[5] Ibeth G L, Czosnek C, Smycz A, Janik J F and Kozik A 2009 J. Phys.: Conf. Ser. 146 012022.
[6] Nowack B and Bucheli T D 2007 Environ. Pollution 150 5
[7] Hirano S 2009 Environ. Health Prev. Med. 14 223
[8] Barillet S, Jugan M L, Simon-Deckers A, Leconte Y, Herlin-Boime N, Mayne-l’Hermite M, Reynaud C and Carrière M 2009 J. Phys.: Conf. Ser. 170 012016
[9] Barillet S, Simon-Deckers A, Herlin-Boime N, Mayne-L’Hermite M, Reynaud C, Cassio D, Gouget B and Carrière M 2010 J. Nanopart. Res. 12 61
[10] Gojova A, Guo B, Kota R S, Rutledge J C, Kennedy I M and Barakat A I 2007 Environ. Health Perspect. 115 403
[11] Wan R, Mo Y, Zhang X, Chien S, Tollerud D J and Zhang Q 2008 Toxicol. Appl. Pharmacol. 233 276
[12] Hirano S, Kanno S and Furuyama A 2008 Toxicol. Appl. Pharmacol. 232 244
[13] Hirano S, Fujitani Y, Furuyama A and Kanno S 2010 Toxicol. Appl. Pharmacol. 249 8
[14] Tokarski C, Cren-Olivé C, Fillet M and Rolando C 2006 Electrophoresis 27 1407
[15] Tokarski C, Fillet M and Rolando C 2011 Anal. Biochem. 410 98