Mathematical nanotoxicoproteomics: Effects of multi-walled carbon nanotubes and TiO₂ nanobelts

Mathematical nanotoxicoproteomics: Quantitative characterization of effects of multi-walled carbon nanotubes (MWCNT) and TiO₂ nanobelts (TiO₂-NB) on protein expression patterns in human intestinal cells

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Abstract

Various applications of nanosubstances in industrial and consumer goods sectors are growing rapidly because of their useful chemical and physical properties. Assessment of hazard posed by exposure to nanosubstances is essential for the protection of human and ecological health. We analyzed the proteomics patterns of Caco-2/HT29-MTX cells in co-culture exposed for three and twenty four hours to two kinds of nanoparticles: multi-walled carbon nanotubes (MWCNT) and TiO₂ nanobelts (TiO₂-NB). For each nanosubstance cells were exposed to two concentrations of the material before carrying out proteomics analyses: 10 µg and 100 µg. In each case over 3000 proteins were identified. A mathematically based similarity index, which measures the changes in abundances of cellular proteins that are highly affected by exposure to the nanosubstances, was used to characterize toxic effects of the nanomaterials. We identified 8 and 25 proteins, which are most highly affected by MWCNT and TiO₂-NB, respectively. These proteins may be responsible for specific response of cells to the nanoparticles. Further 14 reported proteins are affected by either of the two nanoparticles and they are probably related to non-specific toxic response of the cells. The similarity methods proposed in this paper may be useful in the management and visualization of the large amount of data generated by proteomics technologies.
1. Introduction:

In the post-genomic era, the omics sciences, viz., genomics, proteomics, metabolomics, lipidomics, etc. [1-4] have made great strides in generating high dimensional data or so called “big data” that facilitate deep understanding of chemical-biological interactions at the molecular level. The technology of proteomics, which generates information on thousands of cellular proteins, has emerged as a promising research area in biochemical pharmacology and toxicology. This technique provides a snapshot of cellular protein expression status and how this picture may be altered when cells are exposed to agents like drugs, toxicants or nanosubstances [5-7]. As compared to the microarray technology which provides information exclusively on the state of cellular transcription, proteomics technology gives information on the quantitative picture of the cellular status of proteins that maintain the metabolic and functional integrity of the cell.

Applications of nanosubstances in industrial and consumer goods sectors are growing rapidly because of their useful chemical and physical properties. Multi-walled carbon nanotubes (MWCNT) are used in superconductor materials, optical devices and biomedical applications [8, 9]. TiO2 nanobelts (TiO2-NB) are being considered as photocatalysts [10]. Such widespread applications of nanosubstances will increase the likelihood of occupational and consumer exposure to these substances. So, there are concerns about the potential risks posed by such exposures [11, 12].

In order to identify patterns of expression that may indicate high versus low NP toxicity, Tilton et al. [13] carried out global transcriptome and proteome analyses using human intestinal cells exposed to two high aspect ratio NP types. They generated data on the unique patterns of gene and protein expressions for different exposures to multi-walled carbon nanotubes (MWCNT) and TiO2 nanobelts (TiO2-NB).

An important objective of toxicoproteomics research is to study the perturbation of protein expression of cells exposed to toxicants. However, characterizing patterns consisting of thousands of proteins is a daunting task. It requires rigorous mathematical/statistical methods for a thorough and objective analysis of such patterns. Using 2-D gel data generated in Frank Witzmann’s lab, our group has been involved in the characterization of toxicoproteomics patterns applying four different techniques: a) invariants of graphs associated with proteomics maps [14], b) spectrum-like representations of proteomics maps based on projections of the 3-D space (mass, charge, and abundance) onto three (xy, yz, and xz) planes [15], c) selection and use of toxicologically-relevant spots in predictive toxicology based on robust statistical methods [16], and d) information-theoretic characterization of the pattern of protein spots of the 2-DE gel [17]. In this paper, we have mathematically characterized differential protein expression patterns generated by the exposure of Caco-2/HT29-MTX intestinal cells in co-culture to MWCNT and TiO2-NB and quantified by label-free quantitative mass spectrometry (LFQMS).

2. Methods and Materials
2.1 Experimental

The Caco-2/HT29-MTX cells co-culture has been described in a previous study [13]. Briefly, Caco-2, human colorectal adenocarcinoma cells (ATCC HTB-37, Lot 57863838) were maintained in EMEM media supplemented with 10% FBS (ATCC cat# 30-2003 & 30-2020) and incubated at 37°C – 5% CO2. HT29-MTX,
human colon adenocarcinoma cells treated with methotrexate were obtained from Dr. Thécla Lesuffleur (INSERM, Paris, France) and were maintained in DMEM with Glutamax and 10% Hi-FBS (Invitrogen) at 37°C - 5% CO₂. Cells were counted using a hemocytometer and then mixed together at a 75:25 Caco-2:HT29-MTX ratio and placed in the HT29-MTX medium with 1% penicillin-streptomycin. Combined cells were mechanically mixed and 1 mL was added to 6-transwell plates to obtain 0.4 x 10⁶ cells per well (8.9 x 10⁴ cells/cm²). Prior to NP exposure, cells were incubated at 37°C - 5% CO₂ for 14 days, replacing the medium every other day, to achieve post-confluent differentiation, polarization, and tight-junction formation. For NP exposures, 1.5 ml NP suspensions at 10 or 100 µg/ml were placed in the top compartment (apical) of the 6 Transwell™ plate containing the 14 days co-cultured cells. The bottom compartment (basolateral) contained 2.5 ml of fresh medium. Control groups received only fresh medium in both compartments. The treated cells were incubated at 37°C – 5% CO₂ for 3 and 24 h for proteomics analysis.

MWCNT and TiO₂-NB preparation/dispersion, characterization, and toxicity assessment have been published previously [13].

**Proteomics analysis.** Global proteomics were conducted for each cell type 3 and 24 h post exposure to 10 or 100 µg/ml MWCNT or TiO₂-NB (n=5 biological replicates per condition). Briefly, cell lysates were tryptic digested for global LC-MS/MS analysis. Proteins were identified against the International Protein Index (IPI) database (ipi.HUMAN.v3.69) using SEQUEST (v. 28 rev. 12) and validated by PeptideProphet [19] and ProteinProphet [20] in the Trans-Proteomic Pipeline (TPP, v. 3.3.0). Only proteins and peptides with (a) protein probability ≥ 0.9000, (b) peptide probability ≥ 0.8000, and (c) peptide weight ≥ 0.5000 were used in the quantitation algorithm. Protein abundance was determined using a label-free platform, IdentiQuantXL™ [18].

2.2. Mathematical analysis of proteomics data

Our mathematical analysis is based on the comparison of protein abundances between control and treated samples. We denote two measurements by \(a\) and \(b\). To compare two measurements we apply the similarity index \(s^{a,b}\) defined as [15]:

\[
s^{a,b} = \frac{1}{N} \sum_{i=1}^{N} \frac{z_i^a z_i^b}{\max(z_i^a z_i^b)^2}
\]

Eq.(1)

Here, the \(N\) is the number of considered proteins, \(z_i^a\) and \(z_i^b\) are intensities (abundances) of \(i\)-th protein and \(\max(z_i^a z_i^b)\) the maximal value of both intensities.

From Eq. (1) some properties of similarity index \(s\) are straightforward.

1. If protein abundances do not change the similarity index is equal 1.
2. \(s^{a,b} = s^{b,a}\).
3. If all reported proteins are different the similarity index is zero.
4. It measures the changes in abundance and each individual spot and it is not sensitive on sign of difference. In the first step we calculate the similarity indices between control and treated samples for individual proteins. (In this calculation N is set to 1.) For each protein reported in measurements: NT 3 h, NT 24 h, TiO$_2$ 3 h, TiO$_2$ 24 h we calculated two comparative indices:

1. Similarity index between Control sample and treated sample with 10 µg
2. Similarity index between Control sample and treated sample with 100 µg

In the second step we selected proteins, which show above-average response. The criterion for the selection was that one of the similarity indices has to be below the limit:

$$\text{Limit} = \text{average} - 3 \times \text{standard deviation}$$

The average (standard deviation) in the expression above is the average (standard deviation) of similarity indices calculated for each of eight measurements (NT 3 h 10 µg, NT 3 h 100 µg, NT 24 h 10 µg, NT 24 h 100 µg, TiO$_2$ 3 h 10 µg, TiO$_2$ 3 h 100 µg, TiO$_2$ 24 h 10 µg, TiO$_2$ 24 h 100 µg).

In the next we focus on three classes of proteins, first, on proteins affected equally from either nanoparticle types, and second, on proteins, which are affected with one kind of them.

3. Results and discussion

The major objective of this study was to compare the effects of two types of nanosubstances, viz., MWCNT and TiO$_2$-NB, at two doses (10 and 100 mg) and two time courses (3 and 24 hours) of exposure on the cellular proteome. We used a mathematical measure of similarity to quantify the proteomics-based differences between the protein patterns of the Caco-2/HT29-MTX cells.

Data in Table 3.1 show that the average similarity indices both for MWCNT and TiO$_2$-NB exposure for a three hour time period are pretty high, ranging from 0.8875 to 0.9347. This probably indicates the short term 3 hour exposure does not perturb the overall bulk protein patterns of the exposed Caco cells significantly. But for both of the nanoparticles tested and for both 10 mg and 100 dose levels, after 24 hour exposure the similarity index goes down substantially (Table 3.1). This probably indicates that the response to of the cell to the nanosubstances increase over time through perturbation of many more proteins. The proteomic changes reflect "acute" responses (i.e. oxidative stress responses) in the proteome that are different from "long-term responses" [13].

It is altogether a different picture when one considers the selected set of highly perturbed proteins. Here similarity indices have much smaller magnitude as compared to the average similarity indices. This
Mathematical nanotoxicoproteomics: Effects of multi-walled carbon nanotubes and TiO₂ nanobelts happens because the similarity indices in the selected protein row are not diluted by proteins which are not affected by the exposure of the cells to the nanosubstances.

A comparison of the number of highly perturbed proteins shows that for both the nanosubstances the number goes down from the 3 hour exposure to the 24 hour exposure. For example, for TiO₂ 100 mg dose and three hour duration, the number is 57 and that number goes down to 29 after a 24 hour exposure.

In all eight cases the similarity indices are significantly lower for selected proteins. In a further step we found the proteins which are most affected by treatment in at least four cases. We found 14 proteins which are equally affected with MWCNT and TiO₂ particles (Table 3.2). Contrary to this we found eight proteins are strongly affected by exposure to MWCNT, and 25 proteins affected by exposure to TiO₂ particles (shown in Tables 3.3 and 3.4). Detailed analysis of selected proteins, which may indicate a general or specific response of cells to exposure to NP are ongoing in our labs and such results will be communicated subsequently.

References:
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Table 3. 1. The similarity matrix shows similarity indices. (Average: average values of similarity index over all proteins; Selected protein similarity: similarity index for selected proteins)
Table 3.2: Selected proteins, which similarity indices are below the limit in at least four cases. (Indicator = 1 if the similarity index is below limit.)
Table 3.3: Selected proteins, which are specific for MWCNT particles.
### Table 3.4: Selected proteins, which are specific for TiO₂ particles.

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