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Mathematical nanotoxicoproteomics: Quantitative characterization of effects of multi-walled carbon nanotubes (MWCNT) and TiO_2 nanobelts (TiO_2-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 tgat 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]. TiO_2 nanobelts (TiO_2-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 TiO_2-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% CO_2. 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% CO_2. 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/cm^2). Prior to NP exposure, cells were incubated at 37°C - 5% CO_2 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% CO_2 for 3 and 24 h for proteomics analysis.

MWCNT and TiO_2-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]:

$$s^{a,b} = \frac{1}{N}\sum_{i=1}^{N}\frac{z_i^a z_i^b}{\max(z_i^a z_i^b)^2} \qquad \text{Eq.(1)}$$

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:

Limit = average - 3*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

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 TiO_2 100 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 TiO_2 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 TiO_2 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.

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	MWCNT (3h)		MWCNT (24h)		TiO_2 (3h)		TiO_2 (24h)	
	10μg	100μg	10μg	100μg	10μg	100μg	10μg	100μg
# of selected proteins	61	56	39	32	57	57	42	29
Average	0.9347	0.8875	0.8265	0.8374	0.9228	0.9154	0.861	0.8773
Selected protein similarity	0.693	0.63	0.5029	0.5267	0.6797	0.704	0.5713	0.6044

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)

			3h-MWCNT 10ug/ml	3h-MWCNT 100ug/ml	24h-MWCNT 10ug/ml	24h-MWCNT 100ug/ml	3h-TiO2 10ug/ml	3h-TiO2 100ug/ml	24h-TiO2 10ug/ml	24h-TiO2 100ug/ml
	Protein ID	**Gene name**	**Indicator**	**Indicator**	**Indicator**	**Indicator**	**Indicator**	**Indicator**	**Indicator**	**Indicator**
1	IPI00640611	XXXXXX	1	0	1	0	0	1	1	1
2	IPI00012048	NME1;NME2,etc.	1	1	0	0	1	1	0	0
3	IPI00013976	LAMB1	1	0	0	1	1	0	1	0
4	IPI00072534	UNC45A	0	1	1	1	0	0	1	1
5	IPI00922096	XXXXXX	0	1	0	0	1	0	1	1
6	IPI00445108	KARS	0	0	1	1	0	0	1	1
7	IPI00107531	RAD50	0	1	1	1	0	0	1	1
8	IPI00304214	FAM118A	0	0	1	1	0	0	1	1
9	IPI00936387	RPN2	0	0	1	0	1	1	1	0
10	IPI00514831	RBM39	0	0	0	1	1	0	1	1
11	IPI00005084	ASNSD1	0	0	1	1	0	0	1	1
12	IPI00023987	PPCS	0	0	1	1	0	0	1	1
13	IPI00030320	DDX6	0	0	1	1	0	0	1	1
14	IPI00219616	PRPS1	0	1	1	1	0	0	1	0

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.)

			3h-MWCNT 10ug/ml	3h-MWCNT 100ug/ml	24h-MWCNT 10ug/ml	24h-MWCNT 100ug/ml	3h-TiO2 10ug/ml	3h-TiO2 100ug/ml	24h-TiO2 10ug/ml	24h-TiO2 100ug/ml
	Protein ID	**Gene name**	**Indicator**	**Indicator**	**Indicator**	**Indicator**	**Indicator**	**Indicator**	**Indicator**	**Indicator**
1	IPI00219038	H3F3A	1	1	0	0	0	0	0	0
2	IPI00908503	PLS3	1	1	0	0	0	0	0	0
3	IPI00719366	ATP6V1E1	1	1	1	0	0	0	0	0
4	IPI00033025	SEPT7	1	1	0	0	0	0	0	0
5	IPI00012912	CPT2	1	1	0	0	0	0	0	0
6	IPI00005711	HDAC6	1	1	0	0	0	0	0	0
7	IPI00215918	ARF4	0	0	1	1	0	0	0	0
8	IPI00218829	GSPT1	1	1	0	0	0	0	0	0

Table 3.3: Selected proteins, which are specific for MWCNT particles.

			3h-MWCNT 10ug/ml	3h-MWCNT 100ug/ml	24h-MWCNT 10ug/ml	24h-MWCNT 100ug/ml	3h-TiO2 10ug/ml	3h-TiO2 100ug/ml	24h-TiO2 10ug/ml	24h-TiO2 100ug/ml
	Protein ID	**Gene name**	**Indicator**	**Indicator**	**Indicator**	**Indicator**	**Indicator**	**Indicator**	**Indicator**	**Indicator**
1	IPI00291946	USP10	0	0	0	0	0	0	1	1
2	IPI00438170	SNX12	0	0	0	0	1	1	0	0
3	IPI00453473	HIST2H4B;etc.	0	0	0	0	0	0	1	1
4	IPI00966206	PDCD6	0	0	0	0	0	0	1	1
5	IPI00925702	XXXXXX	0	0	0	0	0	0	1	1
6	IPI00791054	XXXXXX	0	0	0	0	0	0	1	1
7	IPI00019888	ALDH5A1	0	0	0	0	1	1	0	0
8	IPI00029737	ACSL4	0	0	0	0	0	0	1	1
9	IPI00877773	MAP4	0	0	0	0	1	1	0	0
10	IPI00025869	GLA	0	0	0	0	1	1	0	1
11	IPI00793498	PA2G4P4	0	0	0	0	0	0	1	1
12	IPI00910126	REEP6	0	0	0	0	0	0	1	1
13	IPI00025273	GART	0	0	0	0	0	0	1	1
14	IPI00023234	UBA2	0	0	0	0	0	0	1	1
15	IPI00384863	XXXXXX	0	0	0	0	0	0	1	1
16	IPI00305092	WIBG	0	0	0	0	0	0	1	1
17	IPI00909984	ANK3	0	0	0	0	0	0	1	1
18	IPI00185146	IPO9	0	0	0	0	0	0	1	1
19	IPI00072044	C11orf54	0	0	0	0	0	0	1	1
20	IPI00895865	ETFA	0	0	0	0	1	0	1	0
21	IPI00844539	XXXXXX	0	0	0	0	1	0	1	0
22	IPI00465315	CYCS	0	0	0	0	0	0	1	1
23	IPI00793920	XXXXXX	0	0	0	0	0	0	1	1
24	IPI00947319	PDHB	0	0	0	0	0	0	1	1
25	IPI00005040	ACADM	0	0	0	0	0	0	1	1

Table 3. 4: Selected proteins, which are specific for TiO_2 particles.