Framing the nano-biointeractions by proteomics

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ABSTRACT

Knowledge of the molecular mechanisms underlying the interactions between nanomaterials and living systems is fundamental for providing more effective products for nanomedicine and drug delivery. Controlling the response of cells/bacteria (such as activation of inflammatory processes or apoptosis/necrosis in tumor cells or pathogenic bacteria) by tuning specific properties of the nanomaterials is ultimately the challenging goal. Notably, this may also provide crucial information in the assessment of any toxic risks induced by nanoparticles on humans. However, in studying the nano-biointeractions, it is imperative to take into account the dynamic evolutions of nanoparticles in the biological environments (in terms of protein corona formation, size and charge changes) in synergy with the dynamic events occurring in cells, including signal transduction, metabolic processes, homeostasis and membrane trafficking. In this context, we discuss the impact of analytical technologies, especially in the field of proteomics, which can provide major insights into the processes affecting the NPs surface as well as the cells and bacteria functionalities. In particular, we show that a precise control of the chemical-physical characteristics of the interacting nanoparticles or nanostructures may impact the cells by inducing changes in the proteomic profiles with direct consequences on their viability.

Keywords: proteomics, nanoparticles, protein corona, 2D-DIGE, bacteria proteome, biomarkers

INTRODUCTION

Since its definition[1], proteomics has been recognized as the new post genomic science with great impact in life science, biology and medicine. Although the lack of a stoichiometric correspondence between the encoding gene and its protein product (due to the numerous and, in some cases, still unknown protein post-translational modifications), ideally, proteomics allows to the identification of the entire set of proteins expressed by genes at a precise temporal cell state, revealing precious information on specific biological processes (e.g., disease processes or drug induced effects), molecular mechanisms for genes regulation, proteins activities, protein-protein interactions, and large scale mapping of subcellular and tissue protein distributions[2]. As such, proteomics has been demonstrated to be an effective tool for toxicology, providing insights in the toxic molecular mechanisms induced by drugs or toxicants in cells or specific tissues (toxicoproteomics).[3] Moreover, by applying one and two dimensional gel electrophoresis techniques (1- and 2-DE) coupled to mass spectrometry (MS), significant differences between control and treated samples (e.g., differential display proteomics) can be quantified, giving information on the different protein expression profiles of cancer cells with respect to normal cells, and on the molecular mechanisms inducing cancerogenicity at protein levels.[4] Finally, whole protein mapping and biodistribution in diseased or normal tissues as well as protein biomarkers discovery may be also explored by using highly advanced proteomic tools such as LC-MS/MS and quantification methods such as iTRAQ[TM], SILAC and others[2]. From a technological point of view, a plethora of technologies and analytical instrumentations, in the field of electrophoresis, mass spectrometry and sensors (*e.g.,* 2-PAGE, 2-DIGE, MALDI-TOF, LC-Q-TOF, SELDI, antibody microarray, etc.) have been developed and improved to address proteomics. Figure 1 reports just some examples of possible proteomic pipelines. Generally, to be successful, such techniques must fit, at least, two principal conditions: separation of complex protein mixtures (for example, whole cellular or tissue lysates, specific cellular components, etc.) and quantification and identification, with the help of bioinformatics tools, of the protein of interest, along with the related biological functionality (if known) associated to its gene.

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However, all the techniques mentioned in Figure 1 present advantages and limits. In our study, we mainly focused on the use of 1- and 2-DE techniques coupled to MS analyses. Whereas in the case of 1-DE, proteins are separated on the basis of their molecular masses, in the second approach, proteins are discriminated according to their isoelectric point and molecular size. Traditionally, the identification of statistically significant differences between two or more proteomes by 2D-PAGE (Polyacrylamide gel electrophoresis) requires the running and analyses of many gels. In any case, the reproducibility of this method is often hindered by technical variations in sample preparation and gels running conditions that render difficult an appropriate proteins matching between the patterns of treated and control samples (with a consequent difficult quantification of the identified "up" and "down" regulated proteins). 2D-Differential-In-Gel-Electrophoresis (2D-DIGE) is an effective approach to reduce gel-to-gel variation, by allowing multiplex analyses (by staining proteomes with different dyes) (Figure 1) of two distinct proteomes onto the same gel. Protein matching and the relative comparison are made against a pooled internal standard sample that is used, in all the gels, as an anchor to achieve a better normalization among the gels.[5]

Toxicoproteomics applied to nanotechnology is now emerging as an attractive tool to address the still unanswered questions dealing with nano-biointeractions and NPs-induced toxicity in living systems. For example, it has been reported that NPs suspended in biological fluids adsorb a set of different proteins (referred to as protein corona),[6-7] whose composition and affinity kinetics strongly depend on particles surface, size, and concentration. Hence, protein coating, surrounding and hiding NPs surface, plays a pivotal role in determining the effective size, surface charge, aggregation state of the newly formed hybrid bio-nanomaterials, influencing biodistribution and, finally, triggering the beneficial or hazardous biological effect.[8-9] The exposed protein corona may, in turn, activate specific epitopes on the cells surface, thus allowing the recognition and the activation of specific receptors and cellular functions.[10] Furthermore, it has been shown that poly (acrylic acid)-conjugated gold nanoparticles, selectively binding fibrinogen contained in the plasma, induce its unfolding and the subsequent specific activation of Mac-1 integrin receptor. This activation primes the NF-kB signaling pathway, leading to the release of inflammatory factors.[11] Finally, by applying "omic" techniques and in particular 2D-DIGE, it has been recently demonstrated that AuNPs induce toxicity by a direct damage of the cellular endoplasmic reticulum (ER).[12]

In this scenario, we describe herein the application of different proteomic approaches to characterize the protein corona involved in the interaction of AuNPs with different cellular media and to frame the proteomic response of *E. Coli* bacteria upon interaction with nanostructured gold substrate. In particular, we show the application of different proteomic workflows. In the first case, we applied the 1-DE/MS analyses to characterize the temporal evolution of protein coronas of differently sized gold NPs and their modulating effects in inducing cellular toxicity. Importantly, in the second approach, by 2D-DIGE and LC-MS/MS, we demonstrate how nanoscale changes in the nanoroughness of a gold substrates may impact on the bacteria functionality by triggering changes at post-translational levels, in the bacteria proteome. Finally, many proteins involved in the molecular mechanisms leading to the loss of bacteria fimbrial structures are highlighted.
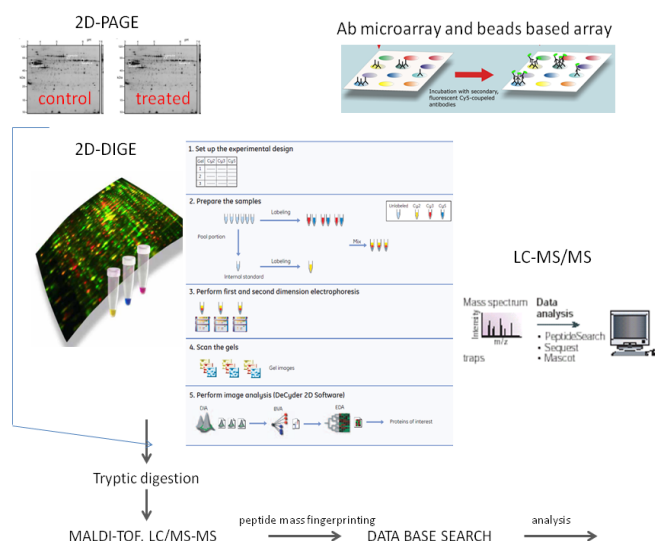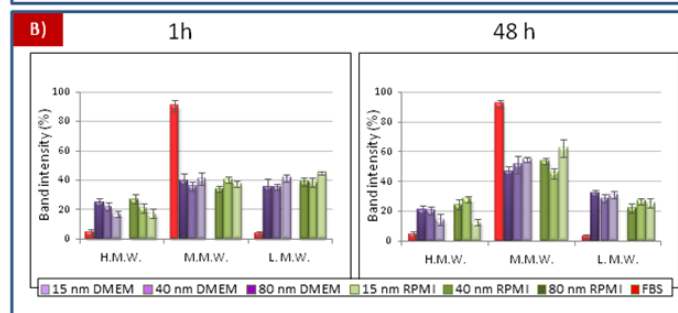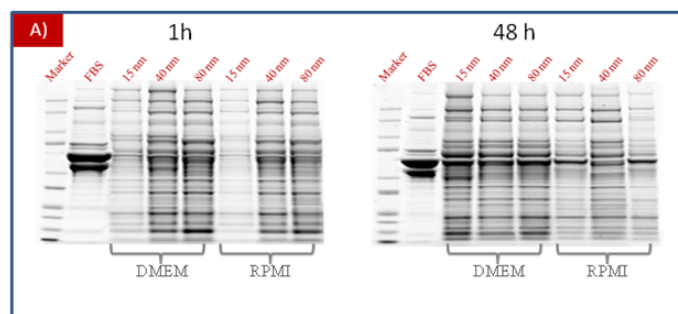

Figure 1 Examples of analytical platforms for proteomics studies

RESULT AND DISCUSSIONS

1.1 Characterization of the time-dependent evolution of hard protein corona of AuNPs by proteomics

Many methods have been applied to investigate protein corona *ex situ*, all showing advantages and limits. Among them, centrifugation method has been recently recognized to provide useful information on what is usually called "hard protein corona".[7] In general, once the NPs are incubated with serum proteins, the resulting protein pellet is isolated by centrifugation and accurate washing. Afterwards, the stable protein/NP complexes are analyzed by following the proteomic workflow reported in Figure 2 that explores the use of SDS-PAGE for proteins separation and MS analyses for protein identification. In particular, in our study, we aimed at the characterization of hard protein corona obtained from differently sized AuNPs (15, 40 and 80 nm) in the presence of two cell culture media (DMEM, RPMI) enriched with FBS, as proteins source. Furthermore, a semi-quantitative description of the temporal evolution of protein coronas out of cellular medium solution (*ex situ)* was performed by applying a semi-quantitative approach based on densitometry analysis (Figure 2). Hence, after centrifugation, we collected pellets at two specific temporal points (namely, 1 h and 48 h) that, on the basis of previously reported spectroscopic measurements of protein coronas in solution,[13] are known to correspond to the starting and plateau phases of protein corona formation onto the NPs surfaces. Such procedure likely reproduces an off-line snapshot of the kinetic of NPs corona in solution. Importantly, in order to compare the different protein electrophoretic lines, we incubated batches of AuNPs at concentrations giving constant values of the total surface area of the NPs for all the sizes. Representative gels (Figure 2A) showed rich protein profiles for all the AuNPs sizes in both the cell culture media. Densitometric analyses indicated that, whereas at 1 h of incubation the total amount of adsorbed proteins roughly increases as a function of the nanoparticle sizes, with no particular differences between the two media, after 48 h (plateau phase), DMEM incubated AuNPs produced a higher total amount of hard protein coronas (as also previously evidenced by TEM images).[13] These data are in good agreement with our previous results obtained by means of *in situ* characterization (*i.e.*, in medium solutions),[13] supporting the recent idea that inspection of protein corona by separation methods may be useful to study the complex dynamics underlying proteins/NP interactions in solution.[14] We analyzed the relative distribution of protein classes as a function of NPs size and cellular media. Interestingly, gel images and densitometric analyses revealed that protein patterns strongly differ with respect to the relative distribution of serum proteins (data not reported). In particular, bands belonging to 1-9 classes (280-100 KDa) were grouped in the high molecular weight proteins (H.M.W.), 10-21 classes (100-35 kDa) in the medium molecular weight proteins (M.M.W.), bands 22-31 (35-10 kDa) in the low molecular weight (L.M.W.) range. On the basis of their electrophoretic mobility and mass, we can argue that most represented proteins of FBS, such as BSA and Fibrinogen, are in 12-15 classes range. We can observe that such species are not predominantly represented in the gel lines of protein coronas. This suggest that, in the serum mixture, complex mechanisms of competition among highly affine protein molecules lead NPs to act as concentrators of less abundant serum proteins, while BSA and Fibrinogen only partially interact with the NPs surface (Figure 2).[7,15-19] Such an effect was found to be similar in both the cellular media, with a slight dependence on the NPs size. Moreover, histograms relative to the band intensity of the three grouped classes (*i.e.*, H.M.W, M.M.W and L.M.W) revealed further differences in the kinetics of proteins distribution in the hard protein corona (Figure 2B). In particular, at 1 h, H.M.W. and L.M.W. proteins are strongly up-represented with respect to fetal bovin serum (control), while M.M.W. proteins are down represented (Figure 2B). After 48 hours, the intensity of M.M.W. protein classes increases at expense of the L.M.W. proteins (Figure 2B), while layers composed by high molecular weight protein do not show particular changes over time.[14,18-21] This suggests that this latter class is less susceptible than the other groups to the dynamic protein exchange occurring during the formation of protein corona. Since increasing evidences suggest that the specific molecular composition of protein corona may play a fundamental role in triggering biological responses,[7,9,15,16] the above characterizations are of particular interest.

The gel bands of interest were excised, digested and analyzed by LC-MS/MS. The main identified protein species adsorbed onto the metallic NPs surfaces are reported on the Table in Figure 2. We found out important proteins involved in key biological processes, such as transport and trafficking (Apolipoprotein A1, Transferrin, Vitamin D-binding protein, etc.), blood coagulation (Protein C inhibitor, antithrombin III, Coagulation factor V, etc.) and tissue development (Fibulin 1, periostin, Thrombospondin-1, Galectin 3 binding protein, etc.).[7,18,19,22] On the basis of their molecular weight, such proteins are present especially in the M.M.W. and L.M.W. groups. Hence, they may be susceptible to the exchange kinetics evidenced in Figure 2B. Such changes, occurring within the experimental temporal windows typical of cell viability tests, might differently affect the outcomes of nanotoxicity tests, due to the specific biological influence of protein coronas.

Figure 2: Proteomic workflow for the "ex situ" characterization of the protein corona onto AuNPs in the presence of the cell culture media, enriched with FBS as proteins source. A) Representative SDS-PAGE of protein corona obtained from 15, 40 and 80 nm AuNPs, after incubation with DMEM and RPMI at 37 °C for 1 hour and 48 hours. (B) Semiquantitative densitometry analyses of gel bands at two different time points of protein corona formation (1 h and 48 h). Approximately 30 gel bands were experimentally detected as single peaks, whose intensity percentages were plotted *vs* band electrophoretic position (data not reported). Classes 1-31 are ranged starting from high to low molecular weights. Plot of band intensity vs pooled gel bands, which were grouped as reported: 1-9 as H.M.W., 10-20 as M.M.W. and 21-31 as L.M.W., according to the experimental position. Data are reported as mean values of three independent experiments. Identified proteins by LC-MS/MS and their relative biological functions were reported in the Table.

M.W. class	UniProt a.n.	Protein Name	Biological process	M.W. (Da)
H.M.W.	gi/119900517*	Predicted: Talin 1	Cell-cell junction assembly, cellular movement, cytoskeletal anchorage	269780*
	Q28107	Coagulation factor V	Blood coagulation	248983
	Q2UVX4	Complement C3	Complement pathway, immune response, inflammatory response	187253
	A8E647	Alpha-2-macroglobulin	Endopeptidase inhibitor activity, protein binding	167576
	Q28085	Complement factor H	Complement alternate pathway, immune response, innate immunity	140374
	Q28178	Thrombospondin-1	Cell adhesion	129534
	A5D7R6	Inter-alpha globulin inhibitor H2 polypeptide	Hyaluronan metabolic process	106187
	P01030	Complement C4	Complement pathway, immune response, inflammatory response	101885
M.M.W.	Q2KJC7	Periostin, osteoblast specific factor	Cell adhesion, extracellular matrix organization, tissue development	86859
	gi/268607679*	Coagulation factor XIII chain A precursor	Blood coagulation	82675*
	Q3SX14	Gelsolin	Actin filament capping	80731
	Q29443	Serotransferrin	Ion transport, iron transport, transport	77753
	A5D7S8	Fibulin 1	Extracellular matrix organization	77530
	P00735	Coagulation factor II or protothrombin	Blood coagulation, acute phase	70506
	B0JYQ0	ALB protein	Transport	69293
	P02769	Serum albumin	Transport, homeostasis, maintenance of mitochondrion location	69293
	A6QQA8	QSOX1 protein	Cell redox homeostasis	62975
	A7E3W2	Galectin 3 binding protein	Cell adhesion	62127
	Q3ZBS7	Vitronectin	Cell-matrix adhesion, extracellular matrix organization	53575
	Q3MHN5	Vitamin D binding protein	Transport	53342
	P41361	Antithrombin III	Blood coagulation	52347
	P50448	Factor XIIa inhibitor	Blood coagulation	51723
	gi/2232299*	IgM chain constant region secreted form	Immune sistem	47915*
	P34955	Alpha-1 antiproteinase	Inhibitor of serine proteases	46104
	Q9N2I2	Protein C inhibitor	Blood coagulation	45297
	gi/76673754*	Predicted: similar to Pleckstrin	Actin cytoskeleton reorganization, platelet aggregation,	40057*
L.M.W.	P12763	Alpha-2-HS-glycoprotein	Mineral balance	38419
	P15497	Apolipoprotein A-I	Cholesterol – lipid – steroid metabolism, lipid transport	30250
	P02081	Hemoglobin fetal subunit beta	Oxygen transport, transport	15859
	P01966	Hemoglobin alpha chain	Oxygen transport, transport	15184

1.2 Bacteria proteome is influenced by bacteria/nanorough films interaction

To characterize the nano-biointeractions we explored another proteomic approach using the combination of 2D-DIGE technique and LC-MS/MS. This proteomic workflow (Figure 3) led us to investigate the entire proteomic profile of *E. coli*, upon interaction with substrates at controlled levels of nanoroughness. 2D-DIGE is a well established and powerful method that allows understanding and clarifying specific metabolic pathways of cells by highlighting up- or down-expression of proteins.[23-25] Figure 3(top) shows a representative 2D gel (in single channels and overlaid image) in which ~1500 protein spots of *E. coli* grown on both glass and nanorough gold substrates were identified. We used strips with a 4-7 pH gradient for the first dimension to better resolve the bacterial proteomic profile. The quality of our 2D-DIGE experiments was demonstrated by a variance below 15% for 1500 spots within the biological replicates;[26] at the same time, also the Principal Component Analysis (PCA) (Figure 3bottom) clearly displayed two well resolved populations. Yellow spots (which represent the population of control proteins pool) are in fact grouped together in the left side of the Spot Maps and are well separated from the red spots (representing the treated population)[27] (Figure 3 bottom). Among the ~1500 protein spots in the 2D gel, we identified 15 of them which were differentially expressed (up- or down-regulated) with significant statistical validity [t-test < 0.02 between glass (control) and nanorough (treated)] (no significant differences were detected between glass and flat gold surface). These 15 significant spots, representative of 15 proteins, showed up and down trends of regulation, as also quantified by the Extended Data Analysis (EDA) (data not reported). Protein identification was performed by picking up the 15 significant spots, from the corresponding preparative gel, and carrying out LC-MS/MS mass spectrometry analyses. We observed that the presence of nanotopography alone, without chemical or biological external stimuli/surface modifications, induces a different expression of proteins involved in transcription regulation, transport of oligopeptides and aminoacids, energy metabolism, stress response, and synthesis and modification of macromolecules (Table II). In particular, we found that some proteins involved in the general stress response were regulated in the nanorough samples. For instance, the DNA protection during starvation protein (Dps), that represents one example of defense against oxidative DNA damage in actively growing cells,[28] was up-regulated. At the same time, also the OsmC stress-inducible membrane protein (whose transcription is typically induced at elevated osmotic pressure) codified by the osmC gene[29] was up-regulated. Such latter finding is also confirmed by the down-regulation of the H-NS transcriptional regulator, which is a histone-like protein able to repress the expression of numerous genes, including osmC.[30] We also found that several proteins involved in general cell processes were regulated; in this frame, the over-expression of the YaeT complex is of particular interest, as it is required for the Outer Membrane β-barrel Proteins (OMPs) assembly, that is important for a proper maintenance of the outer membrane, required for cell viability.[31] In addition, the NusA protein, involved in transcription termination in the tryptophan operon of *E. coli*,[32] and the Periplasmatic Histidine Binding Protein (HBP), that is an initial receptor in the process of active transport across cell membranes and/or chemotaxis,[31] are both up-regulated in the bacteria interacting with the nanorough surfaces. On the other side, the Oligopeptide transport OppA,[33] the Outer Membrane Protein A (OMPA), that plays a structural role in the integrity of the bacterial cell surface,[34] and Protein YgiW are down-expressed. These observations indicate that nanostructured surfaces strongly affect *E. coli* cells, leading to general stress processes in bacteria, which activate defense mechanisms against DNA or membrane damage. Moreover, we detected proteins important for the synthesis and modification of macromolecules. The β-subunit of the Glycyl-tRNA synthetase, an important enzyme in protein synthesis,[35] and the Dihydrodipicolinate synthase, a crucial target enzyme of many antibiotics,[36] resulted over-expressed. On the other hand, the Uracil phosphoribosyltransferase, which is the precursor for all pyrimidine nucleotides (pyrimidine salvage enzyme),[37] is down-regulated. Finally, we found that the energy metabolism proteins Transketolase 2[38] and Pyruvate kinase[39] were both up-regulated in treated samples. Overall, such protein pattern suggests that *E. coli* undergoes important changes in the metabolic pathways upon interaction with nanostructured surfaces, confirming that nanoroughness alone causes a general stress condition in the bacteria. A possible explanation is that the nanotopography causes a significantly reduced contact area of the bacteria with the underlying substrate, likely perturbing their anchoring mechanisms. This might also lead to damages of the outer membrane of adherent *E. coli*, which consequently rearrange its protein expression profile up-regulating enzymes involved in the protection of DNA, aminoacid synthesis, energy production, regulation and rearrangement of the external membrane; at the same time, *E. coli* down-regulates some transport proteins and enzymes related to DNA synthesis, possibly trying to avoid mistakes and/or damages during base synthesis.
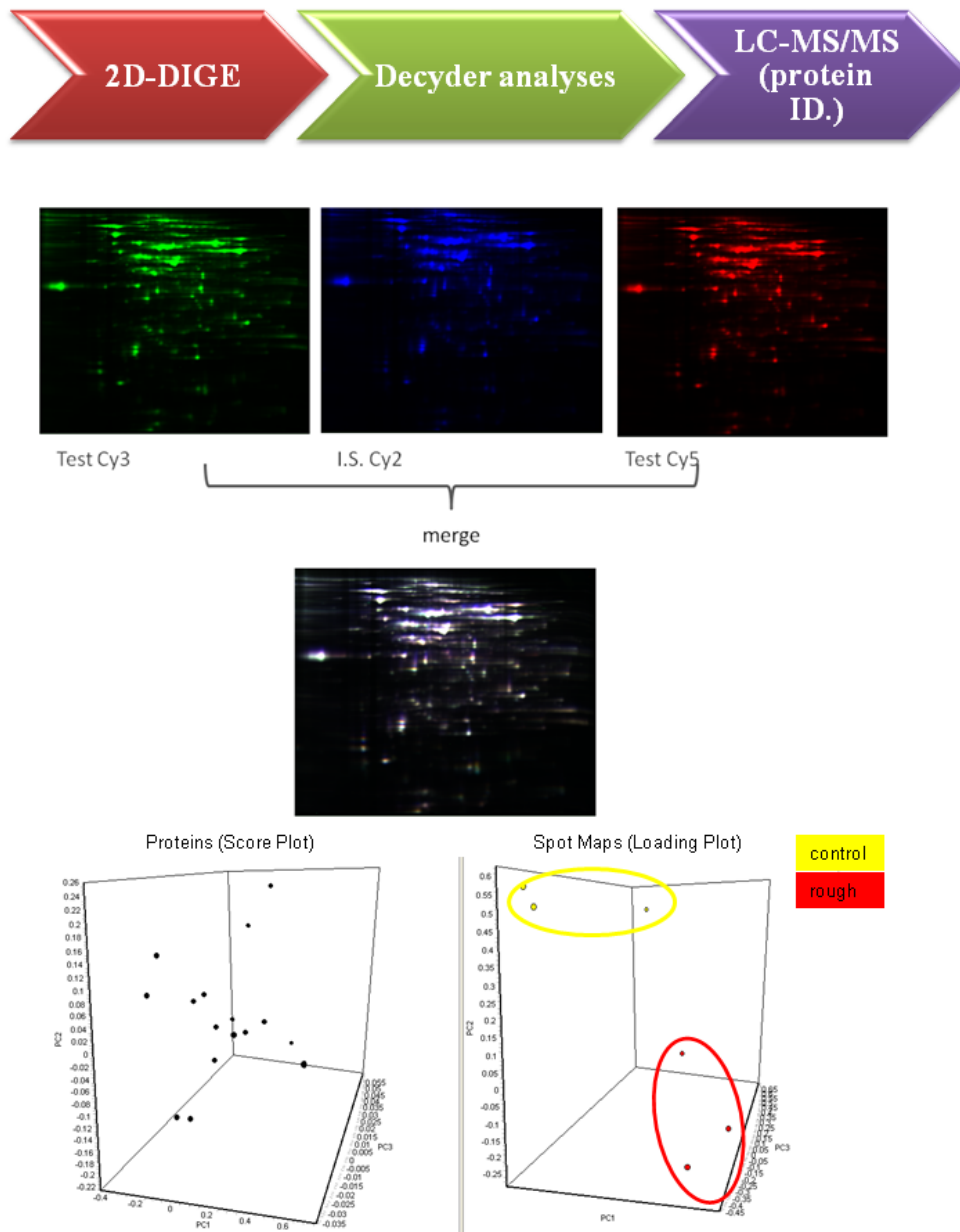
Figure 3 2-D DIGE and Principal Component Analysis (PCA) of bacteria extracts upon treatment with control and nanorough gold substrates. Top): overlay of whole proteome of bacteria treated onto flat (Cy3) and rough gold substrate (Cy5), and pooled internal standard (Cy2). The three samples show overlap based on distinct color of protein spots; bottom) Clustering analysis of proteins by PCA. A filter including all proteins that were present in >75% spot maps was applied. Previously, a subset considering those proteins whose expression varied within the 95th confidence level (Student's t test; $p < 0.02$) was created.

Master spot N.	Protein ID	Protein name	Av. ratio[a]	t test[a]	Score[b]	Coverage[c]	Theoretical mass (KDa)[c]; pI[c]

Cell Processes

Master spot N.	Protein ID	Protein name	Av. ratio[a]	t test[a]	Score[b]	Coverage[c]	Theoretical mass (KDa)[c]; pI[c]
11	gi/15799859	Outer membrane protein assembly factor yaeT	+1.19	0.0071	1668	45	90.6; 4.93
43	gi/15803711	Transcription elongation factor NusA	+1.19	0.0083	1159	56	55; 4.53
157	gi/170080870	Oligopeptide transport periplasmatic subunit	-1.34	0.0094	863	52	61; 6.05
155	gi/15801469	Oligopeptide transport	-1.32	0.0009	638	51	61; 5.95
658	gi/194436225	Periplasmatic histidine binding protein (HBP)	+1.23	0.0067	859	62	28.5; 5.30
1080	gi/15800816	Outer membrane protein A	-1.38	0.0148	309	26	37.2; 5.99
1235	gi/15803566	Hypothetical protein Z4376	-1.45	0.0045	466	49	14; 5.08

Synthesis and modification of macromolecules

Master spot N.	Protein ID	Protein name	Av. ratio[a]	t test[a]	Score[b]	Coverage[c]	Theoretical mass (KDa)[c]; pI[c]
47	gi/16131430	Glycyl-tRNA synthetase	+1.3	0.0079	1111	47	76.9; 5.29
482	gi/15803001	Dihydrodipicolinate synthase	+1.07	0.0058	508	40	31.6; 5.98
913	gi/38704095	Uracil phosphoribosyltransferase	-1.17	0.0024	650	78	22.5; 5.32

Energy metabolism

Master spot N.	Protein ID	Protein name	Av. ratio[a]	t test[a]	Score[b]	Coverage[c]	Theoretical mass (KDa)[c]; pI[c]
56	gi/16130390	Transketolase 2	+1.24	0.014	763	30	73.2; 5.86
220	gi/15802267	Pyruvate kinase	+1.3	0.0074	99	13	51.5; 6.44

General stress response

Master spot N.	Protein ID	Protein name	Av. ratio[a]	t test[a]	Score[b]	Coverage[c]	Theoretical mass (KDa)[c]; pI[c]
1060	gi/15800564	DNA protection during starvation protein	+1.82	0.0146	585	73	18.6; 5.72
1127	gi/15801465	DNA-binding protein H-NS	-1.05	0.0049	448	54	15.5; 5.43
1220	gi/15801655	Osmotically inducible, stress-inducible membrane protein	+1.52	0.0057	96	20	15.2; 5.57

Table II. Summary of protein identification based on peptide mass fingerprinting/peptide sequencing and database searching approaches using LC-MS/MS analyses. The protein are classified according to their biological function, with the average ratio and statistical data (obtained by DeCyder software), and the data from Mascot identifications.

[a] Average volume ratio (treated/control) and t test p value, quantified by DeCyder BVA module. The average ratio value indicates the standardized volume ratio between the two groups (indicated as log of the true ratio measurements). The values are displayed in the range from $-\infty$ to -1 for decreased expression and from +1 to $+\infty$ for increased expression.

[b] Score: Mascot search algorithm reports ion scores as $-10*\log(P)$, where P is the probability that the observed match is a random event. Scores > 38 indicate identity or extensive homology (p < 0.05).

[c] Calculated automatically by Mascot.

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