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Structure-activity relationship models for hazard assessment and risk management of engineered nanomaterials

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Abstract

The widespread use of engineered nanomaterials (ENMs) for commercial purposes made human exposure to these materials almost inevitable. Moreover, the number of *in vivo* and *in vitro* studies reporting the potential adverse effects of exposure to ENMs is growing rapidly. Consequently, there is an urgent need to understand the interactions between ENMs and biological/environmental systems. Although the need to improve our understanding of the adverse health effects of ENMs has been recognised for some time, it has not been fully met to date. There are many reasons that have caused the hazard assessment of ENMs to fall behind the innovations in nanotechnology such as knowledge gaps exist in the field of nanotoxicology, difficulties in categorization of ENMs for toxicological considerations and uncertainties regarding the evaluation and regulation of potential risks of nanoparticles. The presence of a large number of ENMs with unknown risks has led to increased interest in the use of fast, cost-effective and efficient computational methods for predicting the toxic potential of ENMs. To that end, the potential use of *in silico* techniques, such as quantitative structure-activity relationship (QSAR), to model the relationship between biological activities and physicochemical characteristics of ENMs is investigated in this paper. The focus of this paper is on defining the current level of knowledge in (Q)SAR modeling of potential hazards of ENMs and demonstrating the use of (Q)SAR to predict the potential risks specific to ENMs with a case study. Moreover, it presents an overview of the (1) existing barriers currently limiting the development of robust nano-(Q)SAR models, (2) the current obstacles to regulatory acceptance of these models and (3) the integration of (Q)SARs into the risk assessment process. The result of this study demonstrated that the use of (Q)SAR modeling approach to model the toxicity of ENMs based on specific structural and compositional features greatly facilitates (1) filling knowledge gaps regarding the effect of specific parameters on the biological activities of ENMs, (2) predicting the potential risks associated with the exposure to ENMs, (3) classifying the ENMs according to their physicochemical properties and potential hazard degree and (4) reducing the risk by modifying ENMs based on the observed correlations between structural features and biological responses.

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1. Introduction

The physical and chemical properties of materials exhibit extraordinary behaviors when the size of particles is reduced to the nano-scale (e.g. the colloidal gold nanoparticles range in color from red to purple depending on their particle size). These exceptional features of engineered nanomaterials (ENMs) make them ideal for several applications in almost every industrial field. As a consequence, there are now hundreds of nanotechnology-based products on the market, most of which do not carry nano-labels since the labelling of nano-scale ingredients in the products has been very recently turned into a legal obligation. The size-dependency of nano characteristics also suggests that the biological activity (i.e. toxicity) of materials may vary depending on the size of particles in the nano-range. Therefore, the safety of materials containing nano-sized particles should be carefully checked before their commercial use in order to prevent the negative consequences that may arise due to inadequate evaluation of health risk posed by potentially toxic nanoparticles (NPs). As the number of ENMs and their commercial use increase, it becomes more and more difficult to individually evaluate the toxicity of all newly developed nano-products. This motivates the development and use of alternative, cost and time efficient methods to assess the potential risks associated with exposure to ENMs. It is believed that the integration of computational methods, such as quantitative structure activity relationship analysis (QSAR), with nanotoxicology will facilitate the risk assessment of the large number of ENMs and their variants.

1.1. The use of (Q)SAR in hazard modelling of engineered nanomaterials

There is some confusion regarding the definition of structure-activity relationship (SAR) and QSAR models. When the activity being modelled is a class like category (e.g. active / inactive or toxic/nontoxic), the method is often called SAR, but more correct term might be qualitative SAR (qSAR). On the other hand, QSAR aims to establish a statistical model of the biological activity in a quantitative manner. It is also plausible to consider QSAR as a special case of SAR, in which the modelled relationship is attempted to be quantified. Overall, SAR analysis is based on the categorical data while QSAR is concerned about deriving a mathematical model that relates structural features to biological activity from continuous data. The both approaches, SAR and QSAR, are collectively referred as (Q)SAR in this paper.

(Q)SAR analysis is based on the assumption that the toxicity of compounds can be predicted based on its particular characteristics. Different biological responses of ENMs, such as cytotoxic and inflammatory effects, can be modelled based on their physico-chemical properties, such as size, size distribution, surface area, surface charge and crystal structure. The structural and compositional toxicity-predictors used in (Q)SAR analysis can be obtained through experimentation or theoretical calculation. As the properties of nanoscale materials are remarkably different from conventional ones, their toxicity may also be associated with different features. Therefore, development of novel and interpretable descriptors which can capture the specificity of NPs is one of the major research needs in the field of nano-(Q)SAR. Although nano-(Q)SAR is a relatively new field of research, (Q)SAR modelling of ENM toxicity has been already attempted by several researchers[1-7] and reviewed by a number of authors [8-13].

1.2. Integration of (Q)SAR models with risk assessment and mitigation strategies

The hazardous effects of the NPs-containing products on both human health and the environment must be carefully assessed to ensure the safe use of these newly developed products and to gain the confidence of existing users/future costumers. As the social acceptance is the ultimate warranty of the sustainability of a new emerging technology, it is desired to develop a modified risk assessment approach for ENMs which is responsive to the

specificity of NPs. Risk and hazard are often used interchangeably but they have slightly different meanings. Hazard is the possibility of something causing harm while the risk is the probability of a consequence occurring. To put it differently, risk exists only if the exposure to a hazardous component occurs. There are two possible ways of minimizing the risk: hazard or exposure control. Although there are some inspiring studies on the assessment and control of ENM exposure [14-18], development of exposure control strategies for ENMs is currently a difficult target to reach due to the absence of nano-specific exposure metrics and the difficulties in the identification, characterization and quantification of exposure to ENMs. Another approach that can be taken to develop risk reduction strategies for ENMs is to make use of generated hazard and NP characterization data with the aim of achieving hazard control through active engineering of nanomaterials. The basic idea behind this approach is that if the physico-chemical properties leading to toxicological responses are identified, then the toxicity of ENMs can be minimized or eliminated by modifying these toxicity-related properties, without affecting the desired characteristics of materials. This is where (Q)SAR comes into play, to meet the need of discovering knowledge based-rules and relationships between physico-chemical characteristics of ENMs and their biological activity. The knowledge regarding the potential causative factors of nanotoxicity can be obtained through (Q)SAR analysis and can be further employed with the aim of hazard minimization. If successfully applied, (Q)SAR may facilitate the risk assessment and regulatory decision-making process by providing hazard information (i.e. hazard characterization and identification).

Before considering the capabilities of nano-(Q)SAR analysis, we should focus on what is really needed to assist risk assessment of ENMs. The first question that should be asked before anything else is "how can (Q)SAR be of help and contribute to the risk evaluation of ENMs, despite all limitations ?". The answer of this question is directly related to the definition and the scope of (Q)SAR. (Q)SAR analysis can be broadly defined and differently applied, depending on the purpose of the modeller. In general, all investigations seeking to find some explanations for the observed biological activity of a compound based on its identical characteristics can be included in the scope of (Q)SAR studies. It does not matter if a regression method is employed to provide some definite answers or not.

In nano-(Q)SAR world, there are some well-recognized barriers, such as the scarcity of the high-quality datasets and nano-specific descriptors, which make the straightforward extension of traditional (Q)SAR to nano cases difficult. However, the demand for high-quality datasets to derive robust nano-(Q)SAR models does not imply that the available nanotoxicity data is completely useless for hazard assessment. Even if the existing data is far from the ideal, we can still learn from it and use it for risk assessment and minimization purposes. In the view of current knowledge and available data, it seems that the best approach would be to develop nano-specific knowledge-based systems and property-based NM libraries in order to make the best use of all existing data in different sources. This integrated approach provides a focused environment which significantly increases the usefulness of (Q)SAR analysis for nanotoxicology studies and helps (Q)SAR to reach its realistic potential.

1.3. Regulatory acceptance of (Q)SAR models

The REACH (Registration, Evaluation, Authorization and Restriction of Chemicals) regulation aimed at ensuring the safe production, use and import of substances has entered into force in June 2007. Although there is still no specific framework for the regulation of ENMs, REACH legislation covers ENMs under the definition of "substance". The involvement of (Q)SAR specialists in nano-safety researches has become more prominent since the EU's REACH regulation has promoted the use of *in silico* techniques, such as (Q)SAR, for the purpose of risk assessment, classification and prioritization of ENMs. However, this encouragement does not warrant that the outcome of all of the nano-(Q)SAR models will be accepted by the regulatory authorities and the end-users. In fact, nano-(Q)SARs are at least a few years away from getting regulatory approval. At this stage, we should rather focus on the important issues that needs to be addressed to achieve and broaden the regulatory acceptance of nano-(Q)SAR models in the near future. The confidence in nano-(Q)SAR analysis can be only gained through the establishment of (Q)SAR models with scientific validity. The construction of reliable and statistically significant nano-(Q)SAR models requires more training together with the availability of high quality data and customized data collection systems. There are also other key issues that need to be considered in order to improve the regulatory acceptance of the nano-(Q)SAR models. First of all, the uncertainties in the constructed (Q)SAR models and the model's applicability domain should be clearly and transparently reported. In addition to the extracted knowledge and established equations, the model builder should also attempt to provide some probabilistic reasoning to justify

the results obtained. The ability to interpret the observed correlations to be used for external toxicity predictions is as important as building statistically significant mathematical models. Finally, the modeler/reporter should use intelligible language, instead of complex technical terms, considering the background of end-users (i.e experimentalists, industrial partners or regulating authorities), who should have a clear understanding of the model and its applicability in order to avoid misuse of it.

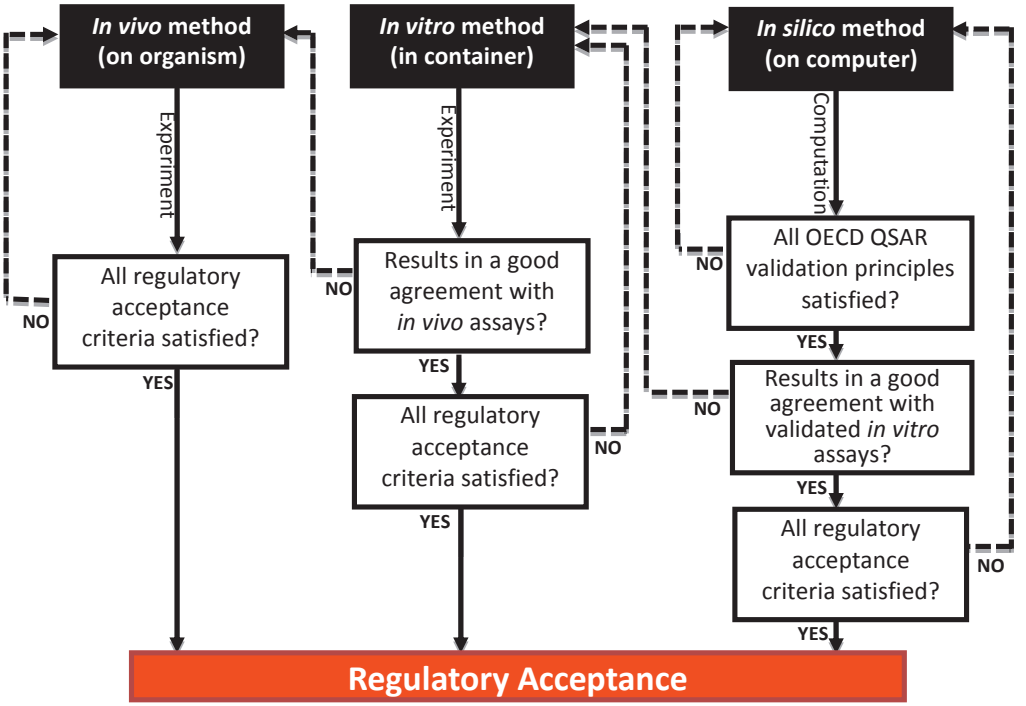

Fig.1. Regulatory acceptance of *in vivo*, *in vitro* and *in silico* methods

The (Q)SAR analysis has a great potential to provide an alternative, fast and cheap way of screening/evaluating the toxicity of ENMs in the risk assessment process and prioritizing/categorizing ENMs according to their toxicity potential. However, the implementation and future success of nano-(Q)SAR models directly rely on the level of acceptance it gets from the potential users and regulators. There is no doubt that *in silico* toxicity assessment methods are currently not as accepted as animal testing by the authorities. In order to replace animal testing with computational methods in nanotoxicology, it should be proven that the outcome of (Q)SAR models are as valid and reliable as existing *in vivo/vitro* tests. However, it does not imply that all nano-(Q)SAR models are useless if their validity and credibility is not as high as experimental findings. The certainty of the results of (Q)SAR model should be indicated by the model builder and this value should be compared with the degree of accuracy demanded by the regulating agency. If the accuracy of (Q)SAR model is lower than the required level, then it can be used for non-regulatory purposes such as initial toxicity evaluation/screening and prioritization.

It is very likely that the regulatory acceptance of nano-(Q)SAR models will be based on a case by case basis with consideration given into the validity/reliability of the model and the endpoints being modelled. The road to regulatory acceptance of different testing (*in vivo* and *in vitro*) and non-testing (*in silico*) methods is shown in Figure 1. It should also be mentioned that science would never guarantee %100 risk free materials. As the nano-specific regulatory guidelines are still in preparation, the materials of concern and the most likely exposure scenarios should be assessed with the best of current knowledge.

1.4. Existing barriers in the (Q)SAR modelling of ENMs

Although (Q)SAR is regarded as an affordable and quick way of evaluating the toxicity of ENMs, there are several barriers that need to be addressed to ensure reliable and safe use of computational techniques in the risk assessment process. Almost all of the existing papers on nano-(Q)SAR emphasize the scarcity of high-quality experimental data. However, the acquisition of high-quality and consistent data can only be possible with the availability of common metrics, standardized protocols and validated testing methods. Unfortunately, there are currently no validated in *vitro/in vivo* methods for ENMs. Moreover, the long term effects of ENMs are mostly unknown. The use of high or overload doses in *in vitro* studies is another critical issue. Ideally, the side effects of ENMs should be evaluated only at the relevant doses while investigating their dose-response relationships. At this point, nano-specific guidelines are needed to define or express "overload doses" and to decide what is realistic and what is not. The list of appropriate dose metrics should also be included in these guidelines to improve the consistency of the reported findings.

The stability is one of the most important factors that need to be considered in the scope of toxicity modelling. The small particles may be no longer small as a result of aggregation/agglomeration in the exposure media. The sharpness of particle may also be affected by aggregation. The observed correlations to size and shape may become questionable when aggregation occurs. Therefore, agglomerated samples should be carefully investigated.

Since (Q)SAR is a data-driven computational method, its performance is directly related to the quality of input data from which the model is developed and also to the technique used for model construction. Figure 2 summarizes the issues (i.e. input data and tool requirements) that need to be considered when developing a nano-(Q)SAR model.

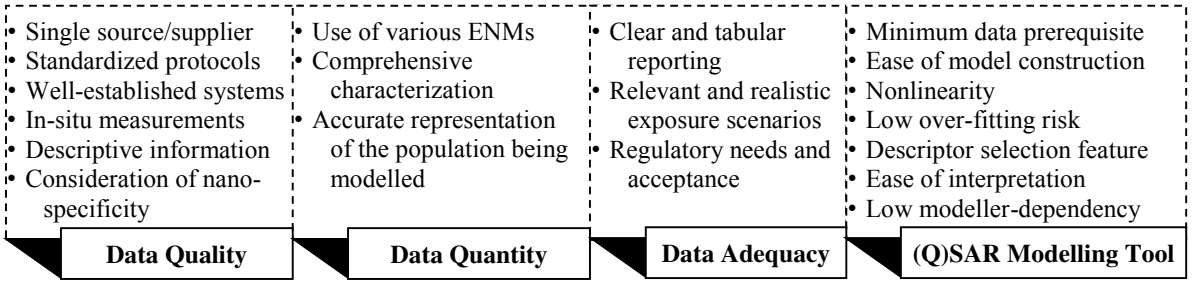

Fig. 2. Input data and modeling tool preferences for the establishment of robust nano-(Q)SAR models

Although the development of predictive (Q)SAR model with a wide applicability domain is only possible with the availability of comprehensive datasets, a single data point can also be useful, maybe not for external predictions, but for the researchers or manufacturers dealing with the same type of ENMs with similar properties. The family of ENMs is quite heterogeneous. Therefore, it is difficult to create the full list of descriptors that drive the toxicity in all cases. The choice of descriptor may depend on structural variability. In other words, the list of characteristics that may contribute to the toxicity of ENMs can vary between material types. Finally, the existing challenges are not all scientific. The self-concentrated disciplines and the lack of communications, motivations and integrations lead to repetition and confusing literature in nanotoxicology. More focused researches, integrated processes and more dialogue are needed.

2. Case studies

2.1. Dataset

The dataset used in this study includes 33 descriptors measured for 10 different metal oxide ENMs (Table 1). A range of *in vitro* cytotoxicity assays including Lactate Dehydrogenase Release assay (4 doses), apoptosis/necrosis/viability studies (4 doses for each), haemolysis assay and MTT assay have been performed on these characterised ENMs. For more detailed information about the experimental procedures and the measurement techniques (i.e. physicochemical and cytotoxic characterization), please refer to [5].

Table 1: A set of NPs (NP1-10), descriptors (x1-33) and in vitro toxicity assays (y1-33) used in this study

Code	NP Name	Code	Descriptor Name	Code	Toxicity assay
N1,2,3	Aluminium oxide (7, 50, 300nm)	**x1-7**	7 LD size statistical meas.	**y1,2,3,4**	LDH (4 doses)
N4	Cerium oxide	**x8-10**	84 LD size distribution meas. (replaced by 3PCs)	**y5,6,7,8**	Apoptosis (4 doses)
N5	Nickel oxide	**x11-12**	2 SEM/TEM meas.	**y9,10,11,12**	Viability (4 doses)
N6	Silicon oxide	**x13-14**	2 EPR meas.	**y13,14,15,16**	Necrosis (4 doses)
N7	Zinc oxide	**x15-27**	13 BET meas.	**y17**	Haemolysis
N8,9	Titanium dioxide (rutile and anatase)	**x28**	1 Reactivity meas.	**y18**	MTT
N10	Silver	**x29-33**	Metal content meas.		

2.2. Modelling method: Partial Least Squares (PLS)

PLS is a linear regression technique which can be considered as an effective tool in handling large datasets associated with nanotoxicology researches. It can be used as a visual aid to identify the key features that are potential sources of the observed toxicity and to understand the possible relationships between the physico-chemical properties (descriptors) of ENMs and their biological activity (endpoints). PLS is preferred as a method of choice in this study since it can handle inter-correlated descriptors and can model multiple endpoints simultaneously. Additionally, PLS has the advantage of easy implementation and interpretation while the main disadvantage of this method is its inability to capture nonlinear correlations. In this study, pre-processing of data (mean-centering and unit-variance scaling) and PLS have been carried out using SIMCA P10 software.

2.3. Case study 1

In the first case study, the relationship between 33 physicochemical descriptors and 18 toxicological responses is modelled simultaneously using PLS method. The resulting graphs, PLS score and weight plots, provide an overview of the relationship between descriptors and toxicity endpoints.

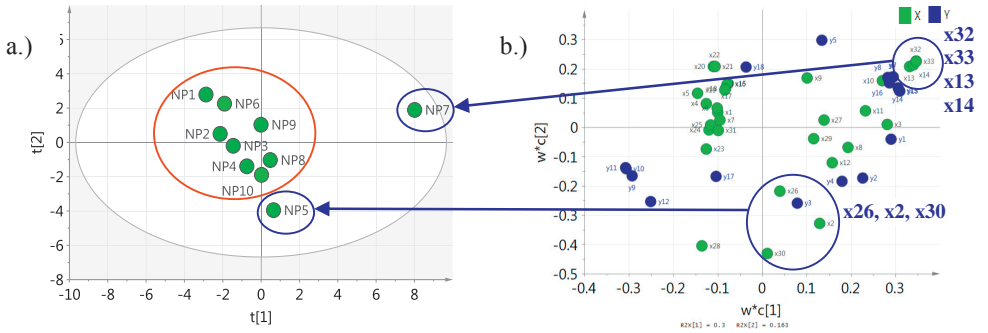

Fig. 3. (a) PLS t_1/t_2 score plot which reveals relationship between observations (i.e. nanomaterials); (b) PLS weight plot (loading plot) corresponding to Fig. 3a

Although not shown here, it is clear from the raw cytotoxicity data that zinc oxide (N7) has high toxicity value in LDH release, apoptosis and necrosis tests while nickel oxide (N5) has high toxicity value in LDH and haemolysis

assays. In this sense, the t_1/t_2 score plot given in Fig.3a looks as excepted as the low toxicity NPs are located in the main cluster while the high toxicity particles, nickel oxide (N5) and zinc oxide (N7), are separated from this cluster. If we look at the PLS weigh plot (Fig.3b) showing how the x-variables are combined to form PLS X-scores (t_1/t_2), we can identify the descriptors contributing to the positioning and separation of NPs. By comparing these two plots given in Fig. 3, it can be concluded that the particle density (x26), the laser diffraction size measurement (x2) and the nickel content (x30) are associated with the differentiation of nickel oxide NPs (N5) while the zinc content (x32), the cadmium content (x33), and the oxygen-centred free radical activities (x13 and x14) are the main reasons for the separation of zinc oxide (N7) from the main cluster formed by low toxicity NPs.

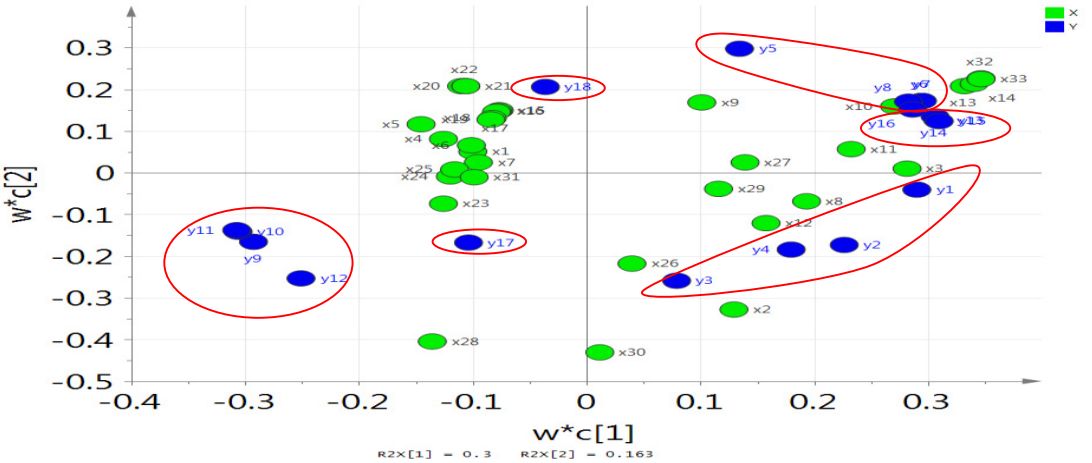

Fig.4. PLS weight plot which showing the inter-relatedness among 33 descriptors (x variables) and 18 biological responses (y variables)

PLS weight plot given in Fig. 3b can be further employed to identify the structure-activity relationships between 33 measured descriptors and 18 toxicity and also activity-activity relationships between different toxicity endpoints (Fig. 4). It can be seen form Fig. 4 that the influences of nano-characteristics on specific types of toxicity endpoints are different. Therefore, it may not be possible to identify an exact set of physicochemical descriptors that drive different types of adverse effects. This finding confirms that (Q)SAR modelling studies should concentrate on a single toxicological endpoint at a time since the parameters contributing to the particular types of side effect are (likely) different. By further examining the weight plot, we can see that the same types of cytotoxic effects measured at different doses are clustered together, as expected. Moreover, the strong correlation between necrosis (y13-y16) and apoptosis (y5-8) assays, the moderate correlation between necrosis (y13-y16) and LDH release (y1-4) tests, and the relatively lower correlation between viability (y9-12) and haemolysis (y17) values can be observed. To conclude, in addition to structure-activity correlations, PLS can also be used as a tool for exploring activity-activity relationships which allow the estimation of some biological activities based on particular toxicity endpoints.

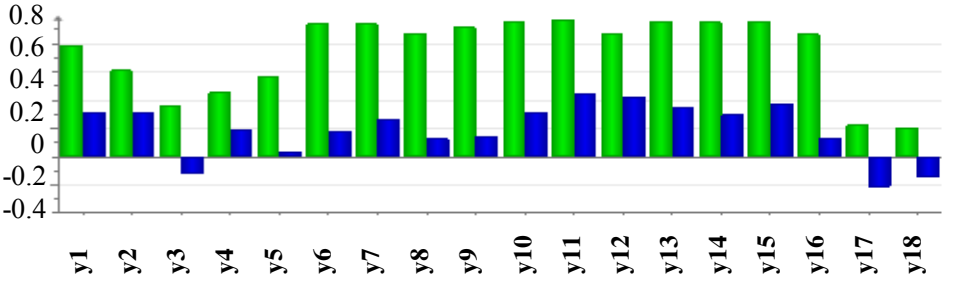

Fig. 5. R^2 (green) and Q^2 (blue) values which show the goodness of fit and the goodness of prediction, respectively.

The cumulative R^2 and Q^2 values of the each variable are given in Fig. 5. After the computation of three PLS components, R^2Y (cum) was determined as 0.606. The Q^2 values indicating the goodness of predictive ability are not really a square. The negative Q^2 values revealed by cross validation denote that the model is not predictive. Although the value of the goodness of prediction is extremely low, it is mainly caused by the simultaneous modelling of multiple toxicity endpoints and the different nature of the each toxicity endpoint being modelled. At this point, it has been decided to focus on a single toxicity assay, viability (y9-12), in order to improve the model's statistics.

2.4. Case study 2

In the second case study, PLS is performed on a dataset including a set of independent variables, x1-33 (33 descriptors), and one toxicity assay, viability (y9-12). The cell viability results (measured at four different doses) are replaced with a new single variable (y1), principal component, that accounts for 95% of the total variation.

PLS score plots given in Fig. 6a and b, t[1]/t[2] and u[1]/[u2], show the relationships among observations in the X space and Y space, respectively. It is clear from the score plots of the model that zinc oxide (N7) is separated from the main cluster, in the X and Y space. From the weight plot shown in Fig. 6d, it can be seen that the zinc content (x32), the cadmium content (x33), and the oxygen-centred free radical activities (x13 and x14) are responsible for this separation. The inner relation between these score plots (i.e. the correlation between X and Y) is displayed in Fig. 6c. From this graph, the outlier (NP7) and the slight non-linearity between the descriptors and the response variable can be seen. Although not in the scope of this study, different data pre-processing methods (i.e. log transform) could be applied to make the relationship linear before model construction.

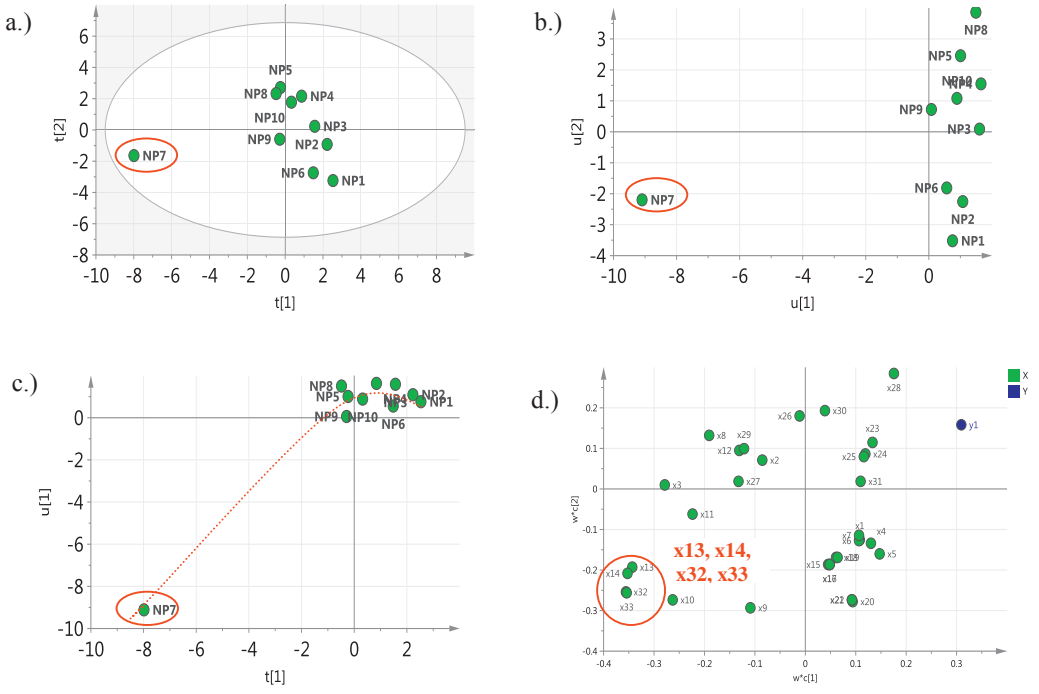

Fig. 6. (a) PLS t_1/t_2 score plot which reveals relationships between observations (i.e. nanomaterials) in the X space; (b) PLS u_1/u_2 score plot which reveals relationships between observations in the Y space; (c) PLS t_1/u_1 score plot; (d) PLS weight plot corresponding to Fig. 6a, b and c.

The weight plot given in Fig. 7 demonstrates the inter-relatedness among thirty three descriptors and one biological response (viability). In order to identify the correlation between x variables and y variable, one can

imagine a line passing through the origin and the point y1. The x variables should be projected onto this imaginary line to facilitate interpreting. The computed distance from the origin determines the impacts of the predictors on the response. The variables that are close to the origin have no or near-zero impact while the ones that are far out from the origin have large influence. Therefore, the impacts of variables on the viability can be summarized as follows:

- Variables that have zero or near zero contribution to toxicity: BET particle density (x26), BET surface area (15,16,17) and porosity measures (x18 and x19)
- Variables that have large contribution to toxicity: zinc content (x32), cadmium content (x33), oxygen-centred free radical activities (x13 and x14), specific surface area (x3), size statistical measurement (x10) and reactivity (x28)

To conclude, it is confirmed that the high level of zinc and cadmium content, oxygen-centred free radical activities, surface area and reactivity can contribute to the toxic effects. After the computation of three principal components, the goodness of fit (R^2) and the predictive ability of the model (Q^2) were determined as 0.99 and 0.80, respectively, by cross-validation.

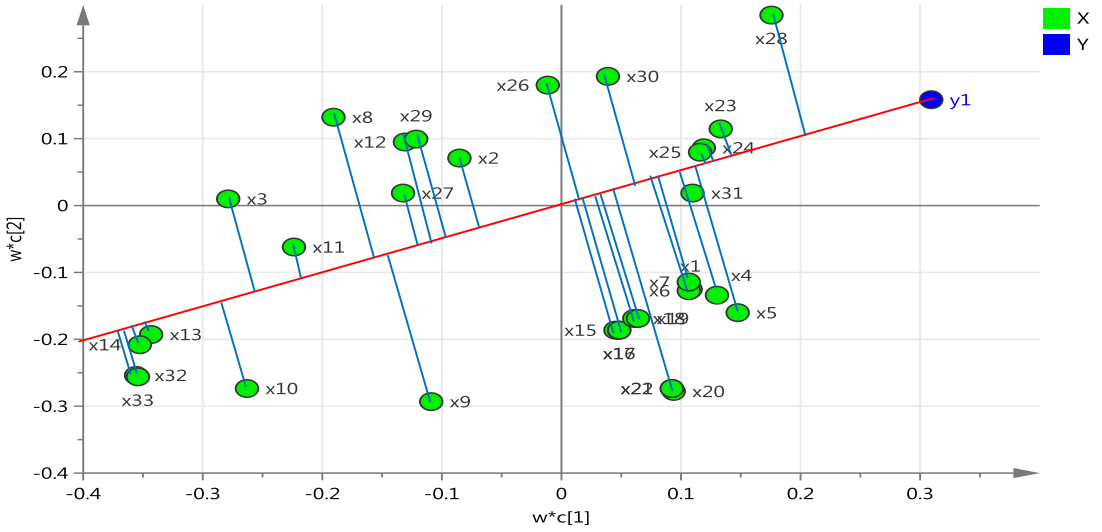

Fig.7. PLS weight plot with an illustration of how to interpret a weight plot (w*c [1]/ w*c [2])

3. Conclusion and future recommendations

As the commercialization of nanoproducts expands, the demand for alternative decision making processes for assessing the potential side effects of ENMs has reached great proportions. It is mostly agreed that particles behave differently at the nanoscale and hence existing risk assessment frameworks and guidelines need major updates to cover the specificity of ENMs. However, there are still many open questions that need to be addressed for the reliable adaptation of existing risk assessment frameworks to these newly developed ENMs.

In this paper, the use (Q)SAR modelling approach to uncover the potential relationship between the toxicity and a number of structural and compositional features was introduced. In the first case study, the correlation between three descriptors (i.e. particle density, laser diffraction size measurement and nickel content) and the toxicity of nickel oxide NPs was found. It was also demonstrated that there were four parameters (i.e. zinc and cadmium contents, and oxygen-centred free radical activities) potentially relevant to the toxicity of zinc oxide NPs. In addition to structure-activity correlations, PLS was also used to explore activity-activity relationships. Some strong and moderate correlations between different toxicity endpoints were observed. However, it was also observed that the influences of nano-characteristics on different types of toxicity endpoints were different. This finding confirms that (Q)SAR modelling studies should concentrate on a single toxicological endpoint at a time since the parameters

contributing to the particular types of side effects are (likely) different. In the second case study, similar findings with a statistically much better model (R^2Y=0.99, Q^2=0.80) were obtained. Although some case-specific correlations between the properties of ENMs and their biological activity were observed, it was not possible to generalize these findings for external ENMs. Creating a full list of descriptors that drive the toxicity in all cases is a very difficult task, if not impossible, due to the heterogeneity of ENM family.

Overall, it has been shown that (Q)SAR tools are useful for identifying the properties that influence the toxicity of ENMs. Indeed, this modelling approach has a great potential to provide an alternative, fast and cheap way of screening/evaluating the toxicity of ENMs. Once the toxicity mechanisms of ENMs and the physico-chemical properties leading to toxicological responses are fully identified by means of (Q)SAR-like analysis methods, it will be possible to control the hazard of ENMs through engineering approaches without affecting the desired characteristics of materials. If successfully applied, (Q)SAR may also facilitate the risk assessment and regulatory decision-making process by providing hazard information (i.e. hazard characterization and identification).

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