NanoSAR: In Silico Modelling of Nanomaterial Toxicity

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The candidate confirms that the work submitted is her own and that appropriate credit has been given where reference has been made to the work of others.

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Abstract

The number of engineered nanomaterials (ENMs) being exploited commercially is growing rapidly, due to the novel properties of ENMs. Clearly, it is important to understand and ameliorate any risks to health or the environment posed by the presence of ENMs. However, there still exists a critical gap in the literature on the (eco)toxicological properties of ENMs and the particular characteristics that influence their toxic effects. Given their increasing industrial and technological use, it is important to assess their potential health and environmental impacts in a time and cost effective manner. One strategy to alleviate the problem of a large number and variety of ENMs is through the development of data-driven models that decode the relationships between the biological activities of ENMs and their physicochemical characteristics. Although such structure-activity relationship (SAR) methods have proven to be effective in predicting the toxicity of substances in bulk form, their practical application to ENMs requires more research and further development. This study aimed to address this research need by investigating the application of data-driven toxicity modelling approaches (e.g. SAR) that are beneficial over animal testing from a cost, time and ethical perspective to ENMs. A large amount of data on ENM toxicity and properties was collected and analysed using quantitative methods to explore and explain the relationship between ENM properties and their toxic outcomes, as a part of this study. More specifically, multi-dimensional data visualisation techniques including heat maps combined with hierarchical clustering and parallel co-ordinate plots, were used for data exploration purposes while classification and regression based modelling tools, a genetic algorithm based decision tree construction algorithm and partial least squares, were successfully applied to explain and predict ENMs' toxicity based on physicochemical characteristics. As a next step, the implementation of risk reduction measures for risks that are outside the range of tolerable limits was investigated. Overall, the results showed that computational methods hold considerable promise in their ability to identify and model the relationship between physicochemical properties and biological effects of ENMs, to make it possible to reach a decision more quickly and hence, to provide practical solutions for the risk assessment problems caused by the diversity of ENMs.

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Abbreviations

AES	Auger electron spectroscopy
AFM	atomic force microscopy
ANNs	artificial neural networks
APO or Apop.	Apoptosis
ATP	adenosine triphosphate (ATP)
BET	Brunauer–Emmett–Teller
BSAI	biological surface adsorption index
CNTs	carbon nanotubes
DCF	dichlorofluorescein
DCS	dynamic centrifugal sedimentation
DLS	dynamic light scattering
DTs	decision trees
EC_{50}	half maximal effective concentration
EDA	exploratory data analysis
EL	ensemble learning
ELS	electrophoretic light scattering
EM	expectation maximisation
ENMs	engineered nanomaterials
FCS	fluorescence correlation spectroscopy
FlFFF	flow field flow fractionation
GA	genetic algorithm
HR-TEM	high-resolution transmission electron microscopy
HTS	high-throughput screening
IR	infrared spectroscopy
kNN	k nearest neighbours
LC_{50}	lethal concentration 50
LDA	linear discriminant analysis
LDH	lactate dehydrogenase

LGR	logistic regression
LR	linear regression
MCO	Monte Carlo optimisation
Mito	mitochondrial potential
MLR	multivariate (or multiple) linear regression
MO	metal oxide
MWCNTs	multi-walled carbon nanotubes
NB or NBC	naive Bayes classifier
Nec.	Necrosis
NHECD	nano health and environmental commented database
NMs	nanomaterials
NMR	nuclear magnetic resonance spectroscopy
NNet	neural networks
NNeig	nearest neighbours
NPs	nanoparticles
NPTA	NP tracking analysis
NTA	nanoparticle tracking analysis
OECD	organisation for economic cooperation and development
PBPK	physiologically based pharmaco-kinetic
PC	principal component
PCA	principal component analysis
PCR	principal component regression
PCS	photon correlation spectroscopy
PI	propidium iodide
PLB	polystyrene latex beads
PLS	partial least squares
(Q)SAR	(quantitative) structure–activity relationship
REACH	registration, evaluation, authorisation, and restriction of chemicals

RED or Red	reducing equivalents
RT	regression trees
ROS	reactive oxygen species
SAED	selected-area electron diffraction
SAR	structure–activity relationship
SAXS	small-angle X-ray scattering
SEM	scanning electron microscopy
SLR	simple linear regression
SOM	self-organising map
STM	scanning tunnelling microscopy
SVM	support vector machine
TEM	transmission electron microscopy
UV–Vis	ultraviolet–visible spectrophotometry
XPS	X-ray photoelectron spectroscopy
XRD	X-ray diffraction

Chapter 1
Introduction

There has been much interest recently in monitoring and assessing the potential effects of ENMs on human health and the environment. There is now a significant amount of research highlighting that although not all ENMs necessarily have toxic effects, certain types of ENMs can pose risks to human health and the environment (Sharifi *et al.* 2012; Holgate 2010; Buzea, Pacheco and Robbie 2007). Evidently, the toxicity of a nano-sized and bulk material can substantially differ, despite their identical chemical composition (Karlsson *et al.* 2009; Jeng and Swanson 2006). Although it is agreed by most researchers in this field that some ENMs display toxicological behaviour different from that of the conventional materials, their modes of toxic action and the factors that makes particular ENMs toxic are still not fully identified. Clearly, large gaps in knowledge still exist in fields that are essential for assessing and managing the risk of all ENMs (Fadel *et al.* 2015; Dhawan and Sharma 2010b).

The current toxicity testing approach primarily relies on animal-based (in-vivo) testing that is very time and cost demanding and ethically problematic. Considering the high number of ENMs requiring toxicity screening, the use of alternative approaches such as in silico tests relying on computational modelling methods are needed to predict health risks of a range of ENMs with less cost and time compared to animal testing. Although the need for the development of intelligent testing strategies based on in silico methods to assess the toxicity of ENMs has been emphasized by many scientists and regulators (Puzyn and Leszczynski 2012; Gajewicz 2012; Gallegos, Burello and Worth 2009), scientific investigation of their applications as predictive tools for toxicological evaluation of ENMs has not received much attention. To address this research gap and devote systematic attention to this subject, this study is focused on investigating whether the computer-based structure-activity relationship methods are applicable to predict the toxicological effects of ENMs. The ultimate aim here is to contribute to moving the nanotoxicology research forward from individual assessments toward a

more integrated hazard screening approach that can predict the toxicity potential of ENMs based on their structural and physical characteristics.

This chapter sets the scene for this study, describes the motivation for conducting the research, provides contextual and theoretical background to the research problem being investigated, and defines the main aims of the work. It addresses where the gap in scientific knowledge related to the toxicological effects of ENMs is and how this study advances knowledge in the field.

1.1 Motivation

Nanotechnology is a broadly applicable science with considerable potential for breakthroughs in a wide variety of fields. It has impact in almost all branches of engineering, resulting in a rapid increase in the number of ENMs being exploited commercially. However, the distinctive characteristics of ENMs not only make them a material of choice for various applications, but also affect their toxicity potential and present a challenge for the existing regulatory systems. Undoubtedly, it is important to understand and ameliorate any risks to health or the environment posed by the presence of ENMs. The immediate goal is to regulate without hampering public perception on the benefits of nano-enabled products, but scientific findings have not yet provided any clear answers on the toxicity of ENMs.

Clearly, there is a gap in scientific knowledge relating to the toxicological effects of ENMs, which makes it difficult to assess and manage risks associated with ENMs. Considering the ever increasing production and use of ENMs, it will soon be impossible to individually assess the toxicity of a vast number of ENMs. One strategy to alleviate the problem of the large number and great variety of ENMs is through the development of data-driven models that decode the relationships between the biological activities of ENMs and their physicochemical characteristics (Oksel *et al.* 2015). The main assumption behind this approach is that similarities in the structure of different materials that may create predictable patterns of particular biological activity. This approach, so called (Q)SAR analysis, allows the biological activity of untested chemicals to be estimated by assuming that the biological activity is linked to their physicochemical characteristics. The ability to predict the

biological activity of ENMs based on their structural and compositional properties, as determined by descriptors such as particle size, shape, surface charge and topology, provides an effective, affordable and rapid way of assessing ENM's toxicity and helps to close knowledge gaps in this context. The value of using such alternative toxicity assessment methods is also reinforced by the European Union's Registration, Evaluation, Authorisation, and Restriction of Chemicals (REACH) regulation that is intended to ensure the safe use of materials in Europe. Additionally, the (Q)SAR analysis is currently the only method available that can generate quantitative predictions of the biological effects of multifarious ENMs in very complex biological or ecological 'real world' environments.

1.2 Theoretical Background

Over the past decade, computational modelling has emerged as a powerful tool to underpin parameters that potentially control properties and effects of chemical substances on the basis of (Q)SAR. Such in silico models are now being routinely used by researchers, industry, and regulators to estimate physicochemical properties, human health and (eco)toxicological effects, environmental behaviour and the fate of a wide range of chemical substances (Kruhlak *et al.* 2007; Cronin and Dearden 1995; Veith, Call and Brooke 1983). Although the traditional (Q)SAR method has been widely used to estimate the biological activity of discrete molecules and materials in bulk form, nano-(Q)SAR modelling is relatively new and still developing. One of the main issues that complicate the adaptation of computational toxicity approaches to nanotoxicology is the scarcity of consistent and high-quality experimental data, which hinders the development of robust and predictive nano-(Q)SAR models (Oksel, Ma and Wang 2015). The scarcity of such data is mainly caused by the lack of standardized nanotoxicity testing procedures and characterisation conditions for physicochemical properties, reflecting that the scientific community is still learning to test ENMs. Clearly, there is a need to identify possible factors affecting the toxicity of ENMs and to have practical guidelines for the development of the nano-(Q)SAR models.

The earliest studies in nano-(Q)SAR research were less than ten years ago (Durdagi *et al.* 2008), and there has been an increasing interest in the application of these methods to ENMs in the last few years (Fourches *et al.* 2010; Sayes and Ivanov 2010; Puzyn *et al.* 2011a; Epa *et al.* 2012; Liu *et al.* 2013b; Zhang *et al.* 2012; Wang *et al.* 2014). The common problem in the majority of published nano-(Q)SAR studies is that they have limited robustness and predictivity, and the interpretation of the generated models can be problematic. Given the scarcity of the comprehensive datasets on ENM toxicity and characterisation, new computational modelling tools or new ways of using existing tools are required to model the relatively sparse and sometimes lower quality data on the biological effects of ENMs.

1.3 Objectives

The main objective of this work is to investigate the application of computational approaches such as (Q)SAR to the prediction of the potential risks of ENMs. The focus is on computational methods that can not only provide toxicity estimations (e.g. predictive models) but can also give valuable information on nanotoxicity (e.g. prioritisation of ENMs for further toxicity testing, identification of toxicity-related properties, etc.). It is also aimed at identifying the primary sources of nano-(Q)SAR data and assessing the available literature data on nanotoxicity in terms of their quality and usefulness for computational studies. Lastly, this study attempts to take the issue of risk assessment of ENMs a step further and explore risk control measures that are appropriate for reducing the risks of ENMs to an acceptable level.

1.4 MARINA (Managing Risks of Nanomaterials) Project

This work has been conducted as part of collaborative research project, MARINA, funded by the European Commission under FP7, aiming at addressing the health and safety concerns associated with ENMs by developing and validating risk management methods for ENMs (MARINA 2011). The project involved a global consortium of 47 partners from academia (e.g. Karolinska Institute, University of Parma, Aarhus University, University College Dublin), industrial (e.g. BASF, Nanocyl), governmental organisations

(e.g. Finnish Institute for Occupational Health, National Institute for Public Health and the Environment (RIVM)) and independent institutions (e.g. National Centre for Nanoscience and Technology of China, Institute of Occupational Medicine, National Physical Laboratories).

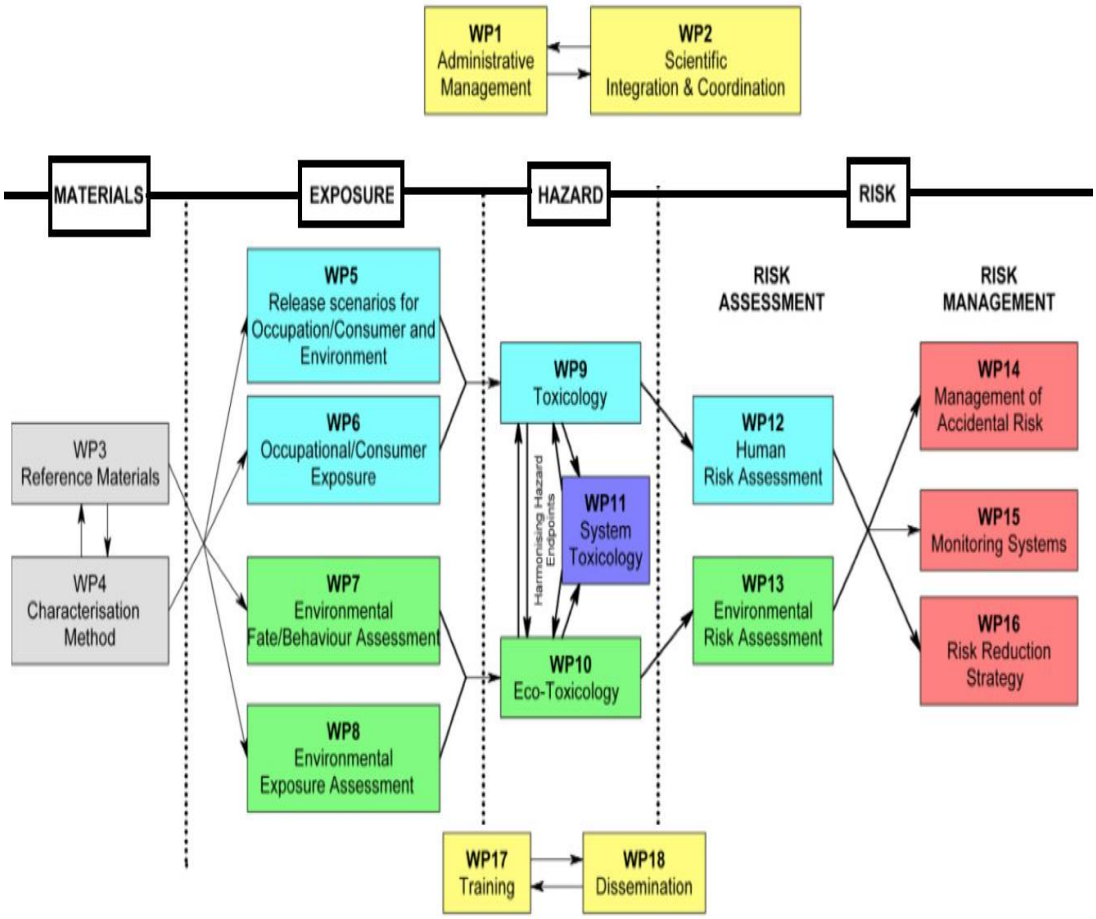

Figure 1.1 Flow chart of the Marina project (MARINA 2011)

The specific themes included in the MARINA project are: Materials, Exposure, Hazard, and Risk. Fig. 1.1 illustrates the four major themes in MARINA and the corresponding Work Package (WP). This work was a part of WP16 which contributed towards the identification of the most harmful ENMs through computational approaches and development of strategies to reduce the risk around potentially toxic ENMs. More specifically, WP16 was focused on the development of (1) data-driven models (e.g. (Q)SAR) to provide toxicity

predictions and (2) strategies to control the ENM risk and exposure through engineering approaches, multivariate statistical process control and control banding. Work carried out in this WP is summarised in Fig. 1.2. My main role in WP16 was to investigate statistical tools that can give insights into the existing problems in nanotoxicology by identifying nano-toxicity related physicochemical properties and developing models for prediction of potential hazards relevant to ENMs.

(Q)SAR work has been an integral part of not only MARINA but also several other nanosafety projects as it is an important aspect of predictive toxicology to support industry, regulatory and public needs for safe ENMs. In addition to MARINA, this work has made a useful contribution to more recent FP7 projects, NanoReg (NanoReg 2013) and SUN (SUN 2013) funded by the UK government and EU Commission.

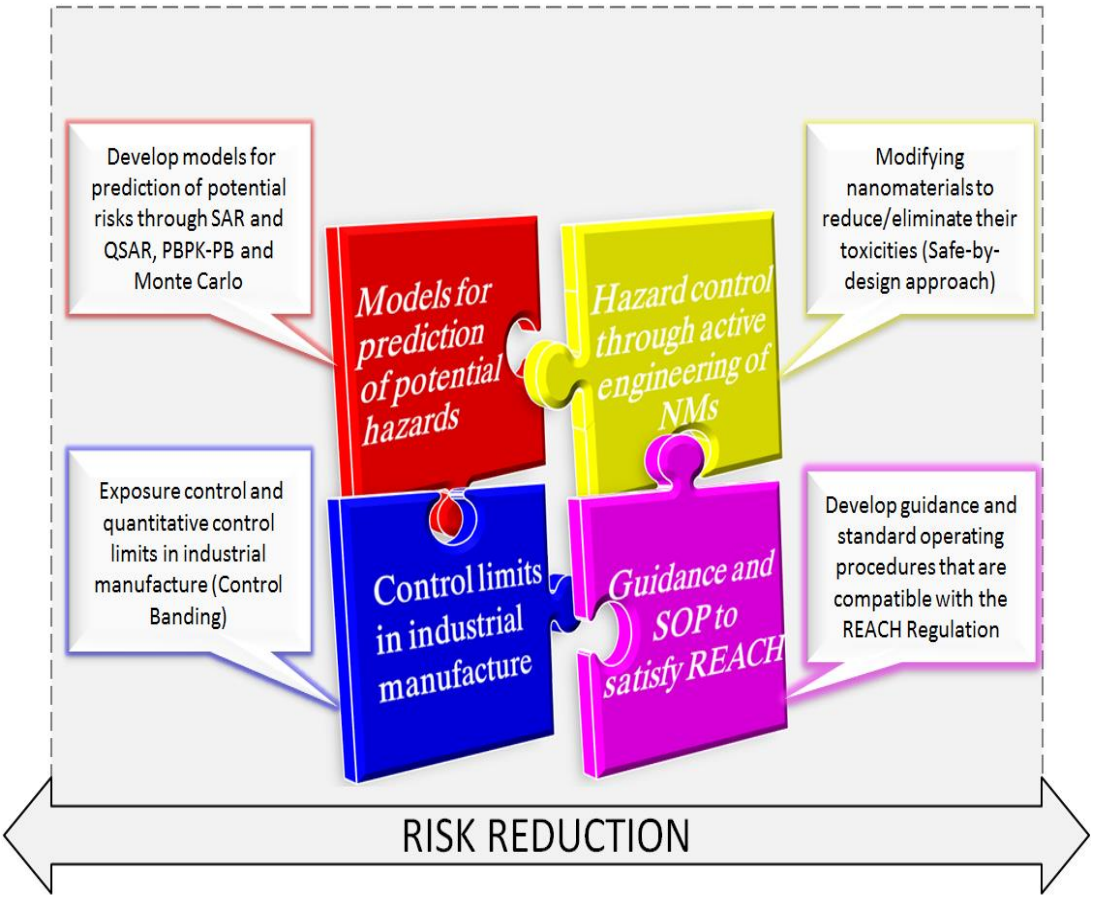

Figure 1.2 Schematic Overview of MARINA WP 16 and WP-specific objectives

1.5 Original Contributions

Computational nanotoxicology modelling is a very new field in nanosafety research. At the initial stage of this PhD work, there was only a few review papers on the implementation of SAR approaches to nanotoxicology (Le *et al.* 2012; Puzyn, Leszczynska and Leszczynski 2009), focusing mostly on the toxicity aspects of nanomaterials. One of the most significant contributions of this study to the knowledge is an important review of this new area of science (Oksel *et al.* 2015). The review paper covers the key components that play an important role in the development of computational models and their practical use for nanotoxicity prediction with a focus on nanospecific needs and knowledge gaps in the field. To the best of my knowledge, this is the most comprehensive review available, addressing not only nano-(Q)SAR research and modelling techniques, but also various aspects of ENM characterisation prior to toxicity testing.

When this study was first started, published data on nanomaterial toxicity was limited and hard to collect due to the lack of common vocabulary terms in use in nanosafety research. To address this difficulty, an annotated bibliography of the primary sources of nano-(Q)SAR data was developed and published (Oksel, Ma and Wang 2015), which can serve as a starting point for those wishing to develop (Q)SAR-like models for nanomaterials.

Another significant contribution to the knowledge concerns the methodology. To my knowledge, the application of evolutionary algorithms (e.g. genetic programming-based decision trees) to nano-(Q)SAR modelling was not previously reported in the literature. This approach generated easily interpretable nano-(Q)SAR models with accuracies equivalent to, or superior to, those of prior modelling studies on the same datasets. It was shown that this method is tolerant of limited data and capable of automatic feature selection as well as modelling both linear and non-linear structure-activity relationships, which are not possible with most of the other tools.

While risk management of ENMs has received significant attention, there is still a research gap in the scientific literature on how to select and implement appropriate risk reduction measures in order to protect nanotechnology workers' health. Another contribution of this work is to support the selection of

the most suitable measures (e.g. based on their efficiency and cost) in order to control and reduce the risk resulting from exposure to potentially hazardous ENMs (Oksel *et al.* 2016 (under review)).

1.6 Thesis Organisation

This thesis is sub-divided into 8 main chapters. Chapter 1 provides an introduction to the aims and structure of the thesis. A critical review of the literature on different aspects of computational nanotoxicology, from characterisation of ENMs for toxicity testing to various toxicity modelling approaches is provided in Chapter 2. Chapter 3 introduces data collection methodology, and more importantly, a variety of nanotoxicity datasets that are useful in developing computational models for toxicity prediction. Chapter 4 presents an application of multivariate data visualisation strategies to explore a set of ENM toxicity and characterisation data. Chapter 5 demonstrates the use of a genetic-programming based decision tree construction tool to develop accurate and interpretable models attempting to relate ENM characteristics to their toxicological outcomes while Chapter 6 takes a more quantitative approach and presents an application of partial least squares (PLS) regression to nanotoxicology data. Overall, Chapters 4 – 6 are focused on prioritising ENMs for toxicity testing, identifying physicochemical properties that drive toxicity and modelling of toxicity endpoints. Chapter 7 takes the issue of risk assessment of ENMs a step further by evaluating existing risk reduction measures that are applicable to nano-scale materials. Chapter 8 reports and discusses the general findings, and provides suggestions for future research.

Chapter 2
Literature Review

There is an increasing recognition that some ENMs may pose a risk to human health and the environment. Moreover, the industrial use of the novel ENMs increases at a higher rate than the data generated for hazard assessment; consequently, many of the ENMs remain untested. The large number of ENMs and their variants (e.g. different sizes and coatings) requiring testing and the ethical pressure towards non-animal testing means that in a first instance, expensive animal bioassays are precluded, and the use of (Q)SAR models as an alternative source of (screening) hazard information should be explored. (Q)SAR modelling can be applied to contribute towards filling the important knowledge gaps by making best use of existing data, prioritising the physicochemical parameters driving toxicity, and providing practical solutions for the risk assessment problems caused by the diversity of ENMs. This chapter covers the core components required for the successful application of (Q)SAR methods to ENM toxicity prediction, summarises the published nano-(Q)SAR studies, and outlines the challenges ahead for nano-(Q)SAR modelling. It provides a critical review of (1) the present availability of ENM characterisation/toxicity data, (2) the characterisation of nanostructures that meet the requirements for (Q)SAR analysis, (3) published nano-(Q)SAR studies and their limitations, (4) in silico tools for (Q)SAR screening of nanotoxicity, and (5) prospective directions for the development of nano-(Q)SAR models.

2.1 Introduction

With the increasing use of ENMs for commercial purposes, human and environmental exposure to ENMs has become more likely. Recent studies have shown that the distinctive nano-characteristics of ENMs not only make them superior to traditional bulk materials, but also may affect their potential toxicity (Arora, Rajwade and Paknikar 2012), and present a challenge for the existing regulatory systems (Falkner and Jaspers 2012). There is a growing number of literature on the potential adverse effects caused by exposure to different types of ENMs (Magrez *et al.* 2006; Jeng and Swanson 2006;

Karlsson *et al.* 2009; Horie and Fujita 2011); however, there are still numerous unanswered questions that complicate the appropriate toxicity evaluation of ENMs.

Toxicological evaluation of ENMs involves many difficulties, such as the availability of a large number and variety of ENMs, the difficulties in categorising ENMs for toxicological considerations, and the fact that even a slight variation in the characteristics of ENMs may also be reflected in the biological response, that dramatically increase the effort required to evaluate the potential adverse effects of ENMs. It seems that the most reasonable approach to obtain toxicity information for the numerous ENMs without testing every single one is to relate the biological activities of ENMs to their structural and compositional features.

The value of using in silico methods, such as the (Q)SAR approach, for toxicity prediction of ENMs was reinforced with European Union's Registration, Evaluation, Authorisation, and Restriction of Chemicals (REACH) regulation that promotes the use of alternative toxicity assessment methods. As the name suggests, (Q)SAR is a computational technique that attempts to predict the biological activity of a compound by relating this activity to a set of structural and compositional properties, such as particle size, size distribution, particle shape, surface area, zeta potential, and crystal structure. The basic idea behind this approach is that different types of toxic effects (e.g. cytotoxic, genotoxic, and inflammatory effects) can be related to measurable or calculable physicochemical descriptors. A schematic representation of the nano-(Q)SAR workflow is given in Fig. 2.1.

This computational approach has many advantages in terms of cost, time-effectiveness, and ethical considerations. Although it has been satisfactorily used to predict the physicochemical properties of NMs, such as solubility (Sivaraman *et al.* 2001; Toropov, Leszczynska and Leszczynski 2007; Toropov *et al.* 2009; Gajewicz *et al.* 2012) and elasticity (Toropov and Leszczynski 2006; Mohammadpour, Awang and Abdullah 2011), the development of reliable (Q)SAR models becomes more complicated when the actual processes and the endpoints of interest are biologically complex.

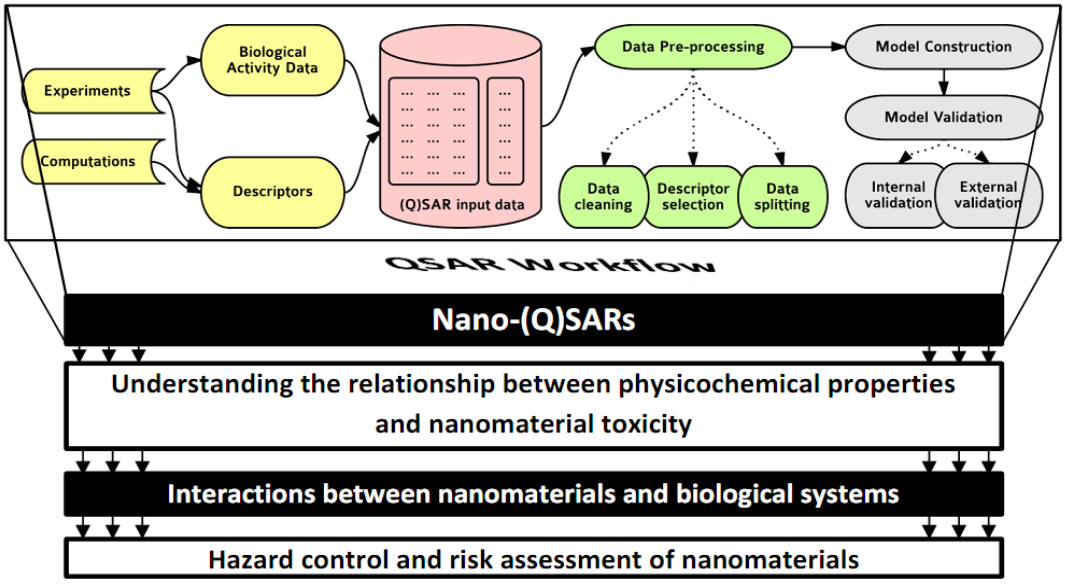

Figure 2.1 (Q)SAR modelling of NM toxicity

Despite all the challenges and open questions, there have been some pioneering studies investigating the use of (Q)SAR models to predict the toxicity of ENMs (Wang *et al.* 2014; Sayes and Ivanov 2010; Fourches *et al.* 2010; Puzyn *et al.* 2011a; Zhang *et al.* 2012; Epa *et al.* 2012; Liu *et al.* 2013b).. Although the initial findings of these nano-(Q)SAR studies are encouraging, there is also a strong need to ensure the reliability of these models to gain the acceptance and confidence of potential end-users including regulatory bodies. Once the main challenges related to the extension of the conventional (Q)SAR approaches to nanotoxicology have been overcome, nano-(Q)SAR models will be able to reach their full performance potential and their outcomes will be more valuable for predicting the toxicity of ENMs.

This chapter focuses on (Q)SAR analysis of ENMs for the purpose of toxicity modelling. It is designed to provide a detailed understanding of the (Q)SAR method used in nanotoxicology research, and present a critical analysis of the nano-(Q)SAR process, the concepts behind it, the appropriate tools to use, and the remaining knowledge gaps in this area. It covers the main components that play an important role in both the development of (Q)SAR models and the practical use of these models for nanotoxicity prediction.

2.2 Nanomaterial Toxicity

Nanotechnology is not an entirely new phenomenon because several natural ENMs, such as clays, have existed in the environment for millennia. Several studies of nanoscale dimensions were conducted in polymer science many years prior to the birth of nanotechnology as a specific scientific field (Paul and Robeson 2008). However, living organisms are assumed to have adapted to naturally occurring nanoparticles (NPs) in their ecosystem but manufactured NPs may be completely new and unprecedented introducing a new set of adverse effects (Sadik 2013). The safety of ENMs falls into a very new field called nanotoxicology. Based on size considerations, these manufactured NMs may have the ability to easily enter into the body, accumulate in tissues, and cause harm (Oberdorster *et al.* 2005). In recent years, some types of ENMs have been shown to be hazardous to human health. It has been reported that carbon nanotubes (CNTs) are capable of inducing reactive oxygen species (ROS) (Sharma *et al.* 2007) and pulmonary effects (Shvedova *et al.* 2005). Toxicological studies have also shown that nanosized titanium dioxide (TiO2) particles have the potential to induce cytotoxic (Saquib *et al.* 2012; Setyawati *et al.* 2012), genotoxic (Trouiller *et al.* 2009; Shukla *et al.* 2011), and inflammatory (Grassian *et al.* 2007; Han, Newsome and Hennig 2013) effects. Another important example of ENM that raises toxicological concerns because of its widespread use in consumer products is nanosilver. Although nanosilver was initially perceived to be rather harmless to human health, recent studies (Hussain *et al.* 2006; Kim *et al.* 2009; Foldbjerg, Dang and Autrup 2011; Asare *et al.* 2012) have provided convincing evidence of toxicity associated with exposure to nanosilver. More detailed information about the potential adverse effects of various ENMs has been provided by several researchers (Magrez *et al.* 2006; Jeng and Swanson 2006; Horie and Fujita 2011; Saquib *et al.* 2012; Holgate 2010; Wani *et al.* 2011; Arora, Rajwade and Paknikar 2012; Sharifi *et al.* 2012).

A toxicological endpoint is the measure of the toxic effect of a substance on human health or the environment, and it determines the harmfulness of a substance. The toxicity of compounds can be evaluated by conducting in vivo, in vitro, and in silico studies. For classical human health hazard assessment

through in vivo testing, several toxicological endpoints are relevant, e.g. acute and chronic dermal, oral or inhalative toxicity as well as skin and eye irritation. Although in vitro assays are commonly preferred to in vivo assays as an initial test because of their time and cost effectiveness, there is also a well-recognised need in the nanoscience community to compare and validate in vitro findings with in vivo observations. In (Q)SAR analysis, it is the specific type of activity, such as cell viability or cytotoxicity, which is going to be modelled and predicted. (Q)SAR models can be built and used for the prediction of all toxicological endpoints as long as sufficient toxicity data is provided as input (Puzyn, Leszczyński and Cronin 2010). Ideally, the biological effects of various compounds with different sizes, structures, and complexities under relevant exposure conditions should be tested with standardised test methods for the successful development of nano-(Q)SAR models.

2.3 Physicochemical descriptors of ENMs

In traditional (Q)SAR analysis, molecular descriptors, which are potentially related to the endpoint of interest, are used to characterise and quantify the physicochemical properties of chemicals. Theoretical descriptors provide a wide variety of physicochemical information and valuable insight into the understanding of the potential relationships between molecular characteristics and biological activity. They can be derived from different theories/semi empirical methods, which may be implemented in commercial software packages. Although more than 5000 descriptors have been proposed and calculated to represent the structure of molecules, most of them are either inapplicable to ENMs or need at least some level of adaptation to be used at the nanoscale. The main problems in the calculation of theoretical descriptors for nanosystems are the complexity and non-uniformity of ENMs, which make the appropriate transformation of the nanostructures into a language for computer representation challenging and extremely time-consuming. Alternatively, the important variables, such as size, shape, and surface charge, can be measured by various experimental techniques and used as descriptors for developing (Q)SAR models. Although the procedure of traditional (Q)SAR analysis is almost standardised, nano-(Q)SAR is still

under development because there is no clear consensus on measurement and modelling standards. The lack of agreement on how to characterise ENMs prior to or during the toxicity tests is widely recognised as one of the main challenges that must be addressed for the successful application of (Q)SAR modelling approach to ENMs. In this section, characteristics that may potentially influence the toxicity of ENMs are identified, and techniques for measuring these toxicity-related parameters are given.

2.3.1 Possible factors affecting the toxicity of ENMs and their measurement

The first step in modelling ENM toxicity is to identify toxicity-related properties that can be used as the potential determinants of adverse effects of ENMs. Because a complete and exact list of parameters influencing the toxicity of ENMs has not yet been established, detailed material characterisation prior to toxicity testing is essential to determine the factors contributing to the biological activities of ENMs and their potential hazards. Although there is still no scientific consensus on the minimum set of relevant nano-characteristics for toxicological evaluation, some particular physicochemical features are included in the majority of recommendations (Powers, Carpinone and Siebein 2012). The size of ENMs is one of the most important characteristics that affects the properties and behaviour of ENMs, and is hence included in the recommendation list by almost all nano-toxicologists. However, as mentioned by Oberdorster *et al.* (2005), the size of the particles is not the only factor that causes changes in the biological activities of materials at the nanoscale. The following characteristics may also be linked to nanotoxicity: size distribution, agglomeration state, shape, crystal structure, chemical composition, surface area, surface chemistry, surface charge, and porosity. Powers, Carpinone and Siebein (2012) investigated the important elements of NM characterisation, and expanded the list reported by Oberdorster *et al.* (2005) to include purity, solubility, and hydrophobicity. In a recent review on the minimum set of physicochemical properties required to characterise NMs, Pettitt and Lead (2013) suggested that, in addition to the parameters that are most likely to have an effect on NM behaviour such as size, surface properties, solubility, and aggregation characteristics,

information about the production process and history of ENMs should also be provided to avoid incorrect interpretation of toxicity data. One of the most comprehensive lists of the important physicochemical characteristics for toxicological studies has been provided by the Organisation for Economic Cooperation and Development (OECD) Working Group on Manufactured Nanomaterials, the OECD WPMN (OECD 2010b). The WPMN suggested a list of physicochemical properties potentially needing to be addressed for characterisation relevant to (eco)toxicity, and devised a testing programme to investigate this. The physicochemical properties mentioned in this guidance are listed in Table 2.1. The term “composition” in Table 2.1 covers chemical identity and molecular structure, as well as degree of purity, impurities, and additives. Another term in this list that is often broadly defined is the “surface chemistry”. Here, it is meant to identify various modifications of the surface (i.e. coatings) and the composition of the outer layer of the NMs. In OECD’s list, there are also many properties, such as dustiness and n-octanol–water partition coefficient, that have not been specified as prerequisites for NM characterisation by other researchers; within the OECD WPMN there is now an agreement that the n-octanol-water partition coefficient is not relevant for NMs. Powers, Carpinone and Siebein (2012) took dustiness as an example and argued that such a measurement for dry NM applications should first be standardised, because the presence of well-established analytical techniques for the measurement of intended properties is essential to express the

in comparable terms; dustiness is not an inherent property but depends on the sample tested. For a detailed description of the potential toxicity-related physicochemical properties shown in Table 2.1, please refer to OECD’s guidance on testing ENMs (OECD 2010b).

Table 2.1 Physicochemical properties and material characterisation

(WR: where relevant; IA: if applicable; WA: where available; AA: as appropriate)

Characterisation (as on the shelf)		**Characterisation (in respective media)**
• Appearance (IA) • Melting point (IA) • Density (IA) • Size, size distribution • N-octanol-water partition coefficient (WR) • Water solubility/dispersibility, hydrophilicity • Solubility/dispersibility in organic solvents, oleophilicity • Auto flammability (IA) • Flammability (IA) • Stability in solvents and identity of relevant degradation products • Oxidizing properties (IA) • Oxidation reduction potential • Explosiveness (IA) • Storage stability and reactivity towards container material • Stability towards thermal, sunlight, metals	• Dissociation constant (IA) • pH (IA) • Agglomeration or aggregation • Crystalline phase • Crystallite and grain size • Aspect ratio, shape • Specific surface area • Zeta potential • Surface chemistry (WA) • Stability and homogeneity (on the shelf, in water and organic solvents) • Dustiness • Porosity, pore and pour density • Photocatalytic activity • Catalytic activity • Radical formation potential	• Composition/purity • Size, size distribution • Agglomeration and aggregation • Zeta-potential • Biophysical properties (AA) (protein binding/corona characterisation, residence times, adsorption enthalpy, conformation changes on binding) • Test item preparation protocol, conditioning, homogeneity and short term stability

2.3.1.1 Particle size and size distribution

The size of ENMs is regarded as one of the most important properties determining the toxicity potential of ENMs. The surface area to volume ratio increases with decreasing particle size. The change in surface-to-volume ratio also affects the surface energy and hence the reactivity of the material. In addition to surface reactivity, the interaction of ENMs with living systems and the uptake and deposition of ENMs within the human body are also affected by particle size (Powers, Palazuelos, Moudgil, & Roberts, 2007). It is generally believed that the risk posed by materials containing nano-sized particles increases with decreasing particle size (Monteiro-Riviere and Tran 2007). Indeed, Gurr *et al.* (2005) showed that the oxidative damage induced by TiO2 particles is size-specific: the smaller the particle size, the greater the oxidative damage induced. Similarly, the toxicity of nanosilver is assumed to be dependent on the particle size. Park *et al.* (2011) compared the cytotoxicity, inflammation, genotoxicity, and developmental toxicity induced by different-sized silver ENMs (20, 80, and 113 nm), and found that the smallest nanosilver particles exhibited higher toxicity than larger particles in the assays. More recently, in an interesting study, Xiu *et al.* (2012) concluded that the toxicity of silver NPs are only indirectly associated with morphological features (i.e. these properties influence the release of silver ions which in turn has an effect on the toxicity). All such findings suggest that the size of particles is a possible factor that may directly or indirectly contribute to the toxicity of chemicals. However, in some cases, no relationship between the toxicity of particles and their sizes is observed (Karlsson *et al.* 2009; Lin *et al.* 2009). There are several techniques that can be used to measure the size of ENMs. Although not a comprehensive list, the most common particle size measurement techniques applicable to ENMs are given in Table 2.2.

The results of different particle size measurement techniques are usually not in agreement because the measurement principles behind each method are different. In general, it is possible to classify the particle size measurement methods applicable to ENMs into three categories: microscopy-based, light scattering-based, and separation techniques (Savolainen *et al.* 2013). Electron microscopy techniques, which are based on scattered (SEM) or

transmitted (TEM) electrons, provide very accurate information and give a clear view of individual and aggregated particles. Therefore, these methods can also be used for polydisperse particle samples. The scanning electron microscopy (SEM) technique provides information about the size, size distribution, particle shape, and morphology, but there is a risk of influencing particle properties during sample drying and contrasting (Bootz *et al.* 2004). SEM and TEM give two-dimensional information on the particles. Unlike electron microscopy techniques, a vacuum environment is not required to obtain images using atomic force microscopy (AFM), which allows the measurement of particle sizes under ambient conditions (Gwaze *et al.* 2007).

Dynamic light scattering (DLS) is based on the Brownian motion of suspended particles in solution and gives the hydrodynamic diameter of the particles measured, which is generally larger than results for dry-measurement diameters. The main advantages of DLS techniques are their simplicity and speed, while their main weaknesses are the high sensitivity to sample concentration and the inability to differentiate between large individual particles and aggregates (Monteiro-Riviere and Tran 2007). Furthermore, DLS cannot be successfully applied to polydisperse suspensions of particles as the intensity of the scattered light is proportional to diameter (D) to the power of six, D^6, meaning that large particles will overshadow smaller ones. Dynamic centrifugal sedimentation (DCS) and analytical ultracentrifugation utilise the difference in sedimentation rates of different sized particles to separate a sample. Tantra *et al.* (2012) emphasised that one of the main disadvantages of DCS is the requirement to know the exact density of the particle including coatings and adsorbed analytes on the surface.

Table 2.2 Particle size measurement techniques

(+) represents advantageous, (–) means disadvantageous.

Method	Parameters measured	Sample required	Particle size range	Additional information
Electron microscopy	Particle size Size distribution Particle shape Agglomeration	Dry	0.3 nm–µm	(+) High resolution (−) Expensive and complex (−) Vacuum is needed (Dhawan and Sharma 2010b)
Atomic force microscopy	Particle size Size distribution Morphology Surface structure Agglomeration	Wet/Dry	1 nm–µm	(+) 3D images (+) Works well in ambient air (−) Particles should be on the surface. (Powers *et al.* 2006)
Dynamic light scattering (DLS)	Particle size Size distribution Agglomeration Zeta potential	Wet	1 nm–6 µm	(+) Cheap and fast (−) Sample polydispersity may distort the results. (Tomaszewska *et al.* 2013)
NP tracking analysis (NPTA)	Particle size Size distribution Agglomeration	Wet	10 nm–2 µm	(+) Particle-by-particle basis (−) Dependence on the settings (Hassellöv and Kaegi 2009)

Centrifugal sedimentation	Particle size Size distribution	Wet	5 nm–10 µm	(+) Accurate and repeatable results (−) Takes long time for small particles to sediment (Laidlaw and Steinmetz 2005)
BET surface area analysis	Particle size Surface area	Dry	5 nm–1 µm	(−) Size distribution is not provided. (Dhawan and Sharma 2010b)
Laser diffraction	Particle size Size distribution	Wet/Dry	40 nm–3 mm	(+) Fast and flexible (−) Dependent on optical parameters (Kübart and Keck 2013)
Mobility analysis	Particle size Size distribution	Dry	2 nm–2 µm	(+) Commonly used for aerosols (−) Interpretation of results may require additional information. (Oberdorster *et al.* 2005)
Acoustic methods	Particle size Size distribution Zeta potential	Wet	20 nm–10 µm	(+)Effective in concentrated suspensions (−) Difficult to interpret the data (Powers *et al.* 2006)

A dry size measurement method is Brunauer–Emmett–Teller (BET) surface area analysis, which calculates the mean particle diameter from surface area measurement based on the assumption that the particles are nonporous and spherical. Additionally, there are several other size measurement methods, including laser diffraction, mobility analysis, acoustic methods, field-flow fractionation (FFF), and fluorescence correlation spectroscopy (FCS), each of which has its pros and cons. Domingos *et al.* (2009) provided a good example of size measurement by multiple analysis methods including TEM, AFM, DLS, FCS, NP tracking analysis (NPTA), and flow field flow fractionation (FIFFF). They confirmed that the particle size measured by DLS is typically higher than those obtained using the other sizing methods. It was concluded that there is no ideal nanoscale measurement technique that is suitable for all sample types. Various factors, such as the nature of the substance to be measured, the constraints of cost and time, and the type of information required, play a decisive role in the choice of the sizing method. Additionally, the structural properties of ENMs, sample preparation, and polydispersity have significant effects on the results of different ENM size measurement techniques.

There are three important criteria that should be met for accurate measurement of particle size: a well-dispersed system, selection of a representative sample, and appropriate selection of the size measurement method considering the nature of the ENM and its intended use (Powers *et al.* 2007). It should also be kept in mind that some methods require dispersion, such as DLS, NPTA, and DSC. The aggregation/agglomeration of particles in dispersions leads to an increase in the measured particle size, as does the formation of corona, when the hydrodynamic diameter is measured. The results from wet measurements may reflect well the biological situation in nano-toxicity studies, depending on the media, because ENMs will actually not be in a dry form when they are in contact with human cells/organs.

Table 2.3 NP mean size measurement results obtained by different size measurement methods

Particle Size (nm)							
Ref.	Thiele, Poston and Brown (2010)				Lee *et al.* (2013)	Akbari, Tavandashti and Zandrahimi (2011)	Borchert *et al.* (2005)
NMs	**Ta**	**$TiSi_2$**	**Ni**	**C**	**SiO_2-7nm**	**Al_2O_3**	**$CoPt_3$**
BET	8	19	35	45	18	27	
TEM	7	13	24	31	19	24	4.86
DLS	316	157	1300		13		
Other						XRD:20; PCS:96	XRD:5; SAXS:4.97
Ref.	Hoo *et al.* (2008)				Supaka (2012)		Boyd, Pichaimuthu and Cuenat (2011)
NMs	**PS-100**	**PS-20**	**PS-20&100**	**PS-20&101**	**CRM-60**	**CRM-100**	**Latex**
DLS	114	23	109	245	73	105	110
AFM	99	16	15–95	16–98	58	58	98
Other					SEM:79	SEM:79	NTA:99

Ideally, the combination of a microscopic technique (e.g. TEM or AFM) and an ensemble technique (e.g. DLS) is appropriate for monodisperse systems, because this can provide a complete picture of the size characteristics in the dry form and suspension. For polydisperse systems, the DLS technique has serious problems, hence it should be replaced or complemented with an alternative size measurement approach. In summary, it is usually useful to combine a single-particle size measurement technique with an ensemble method to obtain a rich dataset of particle sizes and size distributions, especially when a priori knowledge on these parameters is unavailable for the test material. The results of seven studies by different researchers are given in Table 2.3, with the aim of comparing different ENM size measurement techniques. It should also be pointed out that, compared with the average value of the particle size, the size distribution provides a

more realistic representation of particle size information, which is a critical attribute in nanotoxicology. However, measurement of particle size distributions usually provides a large amount of data (e.g. hundreds of size distribution components), which may cause problems in the (Q)SAR analysis (e.g. increased random correlations). Therefore, it is important to find a reasonable way to represent all components of the size distributions with a few variables that still retain all of the information present in the input data. Wang *et al.* (2014) carried out principal component analysis on size distribution data consisting of a large number of particle size distribution measurements to reduce the number of descriptors to a manageable size. This study is a good example of how to handle large size distribution datasets prior to nano-(Q)SAR analysis. Instead of reporting mean particle size values, researchers should also take into account the variations in the size distribution as a whole, because the ENM samples consist of a range of particle sizes, not only a single type of particles.

2.3.1.2 Particle shape

The shape of ENMs is another important feature influencing the biological activities of the particles. The hydrodynamic diameters of spherical and rectangular particles with the same mass, and hence their mobility in solution, vary because of shape effects. Moreover, shape characteristics greatly affect the deposition and absorption kinetics of NPs in a biological environment (Monteiro-Riviere and Tran 2007). The importance of shape in toxicity has been proven for CNTs. Poland *et al.* (2008) showed that long multi-walled CNTs (MWCNTs) are more toxic than short/tangled MWCNTs. The study undertaken by Powers *et al.* (2007) revealed that the antibacterial activity of silver NPs is shape-dependent. In another study, Gratton *et al.* (2008) demonstrated that rod-like (high aspect ratio) NPs are drawn or internalised more efficiently into cells than cylindrical NPs. Although there are several studies investigating and confirming the potential effect of NP shape on toxicity, it is still not possible to draw clear conclusions or define any particular shape inherently “toxic” with current knowledge. Most of the research in this field has focused on toxicological assessment of spherical NPs, while very few have looked at non-spherical NPs or aggregates (Albanese, Tang and Chan

2012). Further research is required on NPs with similar composition but different shape to investigate the role of NP shape in toxicity.

There are several non-dimensional shape indexes that can be used to quantify the shape characteristics of particles, such as sphericity/circularity, aspect ratio/elongation, convexity, and fractal dimensions. The shape index of NPs is usually determined using microscopic methods such as SEM and TEM, which have the ability to simultaneously determine both particle size and shape. Additionally, the ratio of two particle sizes measured by different techniques, such as DLS and TEM/SEM, can be used as a simple expression of particle shape (Hosokawa *et al.* 2007). Because shape characteristics and the distribution of NPs may vary when they are in contact with organisms, shape measurements should also be made for “as-exposed” as well as “as-received” forms. Wang and Ma (2009) defined the shape of a crystal according to the normal distance between each surface of the particle and its geometrical centre. They carried out principal component analysis (PCA) on the shape description dataset for data compression. The calculated surface–centre distances or the resultant principal component values can be directly used as shape indexes of NPs, especially non-spherical NPs, in nano-(Q)SAR. Moreover, these values can also be used as dynamic shape factors to investigate the time and size dependence of shape once this modelling methodology is applied to predict the aggregation/agglomeration behaviour of NPs. If aggregation or agglomeration occurs, the normal distances for some faces may disappear with some new faces, hence new normal distances, appearing. If breakage occurs, some new normal distances will be identified to represent the new faces. Such alternative approaches are useful for nano-(Q)SAR applications because they take into account the dynamic nature of NP shape.

2.3.1.3 Crystal structure (crystallinity)

ENMs with the same chemical composition may have different toxicological properties because of their different atomic arrangements and crystal structure. Jiang *et al.* (2008) investigated the effect of crystallinity on NP activity by comparing the ROS generating capacity of TiO_2 NPs with similar

size but different crystal phases (amorphous, anatase, rutile, and anatase/rutile mixtures). The study found that amorphous samples showed the highest level of ROS activity followed by pure anatase and anatase/rutile mixtures, while pure rutile produced the lowest level of ROS. Nanosilica, which occurs in multiple forms, is another ENM whose toxicity may vary depending on the nature of its crystal structure (Napierska *et al.* 2010).

A widely used technique to obtain information about crystal phases, purity, crystal structure, crystallite size, lattice constants, and defects of NPs is X-ray diffraction (XRD). XRD is a useful tool to characterise nanostructures because it provides non-destructive evaluation of the structural characteristics without the need for exhaustive sample preparation (Edelstein and Cammaratra 1998). Its noncontact and non-destructive features make XRD ideal for in situ measurements (Sharma *et al.* 2012). Measurement in a desired atmosphere is allowed in XRD. This makes XRD advantageous for toxicological characterisation in which the collection of crystal structure data in biologically relevant media becomes an important issue.

Additionally, high-resolution transmission electron microscopy (HR-TEM) and selected-area electron diffraction (SAED) can be used to obtain information about the crystal structure, especially when data acquisition from individual nanocrystals is required. It should be noted that conventional XRD is preferable over TEM for crystallographic investigation of nanostructures because of the sample-damaging and the user-dependent nature of TEM.

2.3.1.4 Surface functionalisation

Surface chemistry is another factor that needs to be considered for the complete characterisation of NPs, because it plays an important role in the surface interactions and aggregation behaviour of NPs in liquid media. Therefore, if the surfaces of ENMs are intentionally functionalised, each chemical species and functional groups on the surface should be identified. The influence of surface coating on the toxicity of Ag-NPs has been investigated by many researchers (Caballero-Díaz *et al.* 2013; Nguyen *et al.* 2013; Zhao and Wang 2012; Yang *et al.* 2011; Silva 2011). The results from Nguyen *et al.* (2013) showed that uncoated Ag-NPs are more toxic than

coated Ag-NPs. However, the coating is not the only factor that reduces the toxicity of Ag-NPs. Changes in the aggregation state and particle size as a result of surface coating may also be important.

Information about how the ENM surface affects the interactions of NPs in a biological environment can be obtained from different techniques, such as electron spectroscopy (X-ray photoelectron spectroscopy (XPS) and Auger electron spectroscopy (AES)), scanning probe microscopy (AFM and scanning tunnelling microscopy (STM)), ion-based methods (secondary ion mass spectrometry and low-energy ion scattering), and other spectroscopic techniques (e.g. IR, NMR, and Raman spectroscopy) (Baer *et al.* 2010). The most important advantage of electron spectroscopy is its high surface sensitivity. XPS is one of the most commonly used techniques for surface analysis (Tougaard 2005). Both XPS and AES can be used to obtain information about the presence, relative surface enrichment, composition, and thickness of coatings.

2.3.1.5 Surface charge

Surface charge is another important characteristic that may affect the toxicity of ENMs. The biological interactions of ENMs, and hence their biological activities, are highly surface-charge dependent. Park *et al.* (2013) analysed the effect of surface charge on toxicity using negatively and weakly negatively charged silica-NPs. They found that negatively charged silica-NPs have a higher level of cytotoxicity than weakly negatively charged silica-NPs. In another study, the core of silicon-NPs was covered with different organic monolayers to obtain different surface charges (positive, negative, and neutral) (Bhattacharjee *et al.* 2010). The study found that positively charged silicon-NPs are more toxic than neutral silicon-NPs, while negatively charged silicon-NPs induced almost no cytotoxicity.

Because it is challenging to directly measure the charge at the surface of particles, zeta potential measurement using dynamic or electrophoretic light scattering is usually used to quantify the surface charge. According to Xu (2008), among the three techniques that can be used to determine the zeta potential (electrophoretic light scattering (ELS), and acoustic and

electroacoustic methods), ELS is preferred for various applications because of its certainty, sensitivity, and versatility. However, classic ELS cannot successfully determine the zeta potential of turbid samples because the light cannot penetrate the sample. Preferably, the sample should be optically clean and nonturbid for accurate measurements. It was also noted in the same study that the accuracy of zeta potential measurements is greatly affected by environmental conditions, such as pH and ionic strength. The pH-dependence of the zeta potential should also be taken into account because changing the pH of a solution may greatly alter the distribution of surface charge.

The current understanding of the relationship between surface charge and toxicity is severely limited, mainly because of the incapability of existing in situ measurement techniques and the environment-dependence of zeta potential measurements (Jiang, Oberdörster, & Biswas, 2009). Because the value of the zeta potential obtained may vary between different techniques and experiments (Glawdel and Ren 2008), multiple tests should be conducted for the best possible accuracy and the results should be reported together with details on measurement conditions (e.g. pH value and sample concentration).

2.3.1.6 Aggregation state

Some NPs have the tendency to form large agglomerates both in the dry form and in suspension. If NPs form clusters, they may behave like larger particles because of their increased hydrodynamic size (Buzea, Pacheco and Robbie 2007). Because agglomeration could affect important physicochemical features, such as particle size and the size distribution, the biological effects of these changes should be identified to avoid incorrect estimation of the toxic potential of ENMs (Dhawan and Sharma 2010a; Jiang, Oberdörster and Biswas 2009).

The aggregation state is often quantified by measuring the size distribution of existing agglomerates. It can be monitored and quantified by microscopic techniques such as TEM, SEM, and AFM. Additionally, DLS can also be used to investigate NP aggregation. However, characterisation of the agglomerate size of NPs in suspensions is very challenging because the degree of aggregation can be influenced by external conditions (e.g. pH,

temperature, and humidity). Ideally, in situ instruments that are capable of measuring the size, shape, and number of all agglomerates in the relevant medium are required to characterise the aggregation state. The particle size information used in early nanotoxicological studies usually refers to the primary size of individual NPs and ignores the effect of aggregation. Although accurate characterisation of the aggregation state prior to nanotoxicity testing is seen as a prerequisite by several researchers (Jiang, Oberdörster and Biswas 2009; Boverhof and David 2010; Von der Kammer *et al.* 2012), there is still no clear consensus on how to characterise aggregation. However, characterising the aggregation shape using fractal dimensions, which provide an index of complexity by measuring the space-filling capacity of an object, may be the way forward (Schaeublin *et al.* 2012).

2.3.2 NP-specific descriptors

Because some properties of ENMs are different from conventional materials, it is very likely that also the toxicity of ENMs could be different and associated to nanophenomena. Therefore, the development of nanospecific descriptors capable of describing the distinctive properties of NPs is one of the main research requirements in the area of computational nanotoxicology. In this section, the different approaches to develop novel NP-descriptors will be presented.

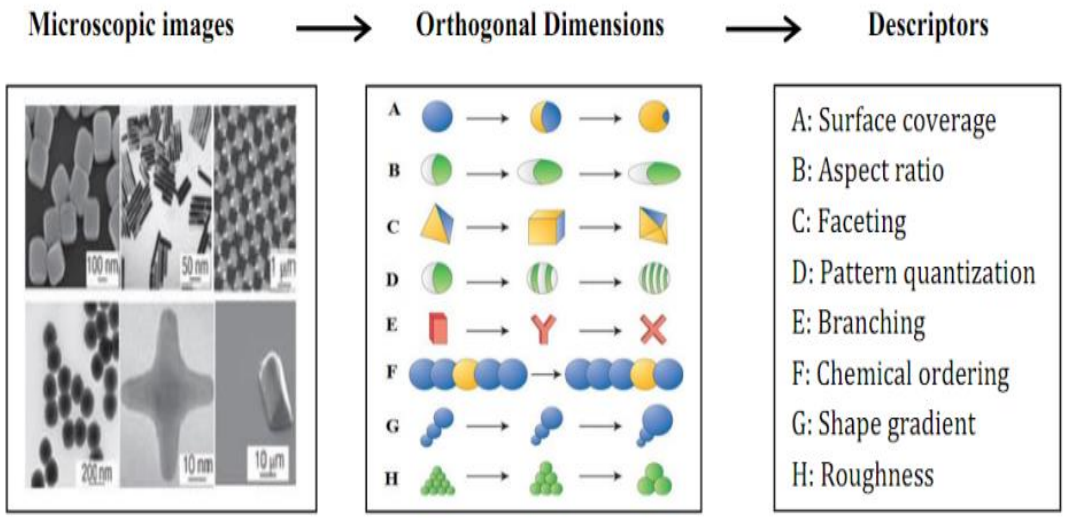

Figure 2.2 Derivation of eight qualitative descriptors based on microscopic images (Glotzer and Solomon 2007)

Glotzer and Solomon (2007) proposed an approach to characterise NPs based on microscopic images. They defined eight orthogonal dimensions that can be used as NP-descriptors to compare the structural similarity of different NPs: surface coverage, aspect ratio, faceting, pattern quantisation, branching, chemical ordering, shape gradient, and variation in roughness (Fig. 2.2). Although the development of new descriptors based on microscopic images is a promising idea, the numerical expression of these eight dimensions is still an unresolved problem.

The idea suggested by Glotzer and Solomon (2007) has inspired other researchers to use microscopic images of NPs for the extraction of structural information. Puzyn, Leszczynska and Leszczynski (2009) proposed to quantify each pixel in SEM, TEM, and AFM images using RGB colour codes or grey-scale representation, and then produce a rectangular array of numbers (Fig. 2.3). They also emphasised that these numerical values of image pixels can be used as new descriptors for encoding the structural properties of NPs.

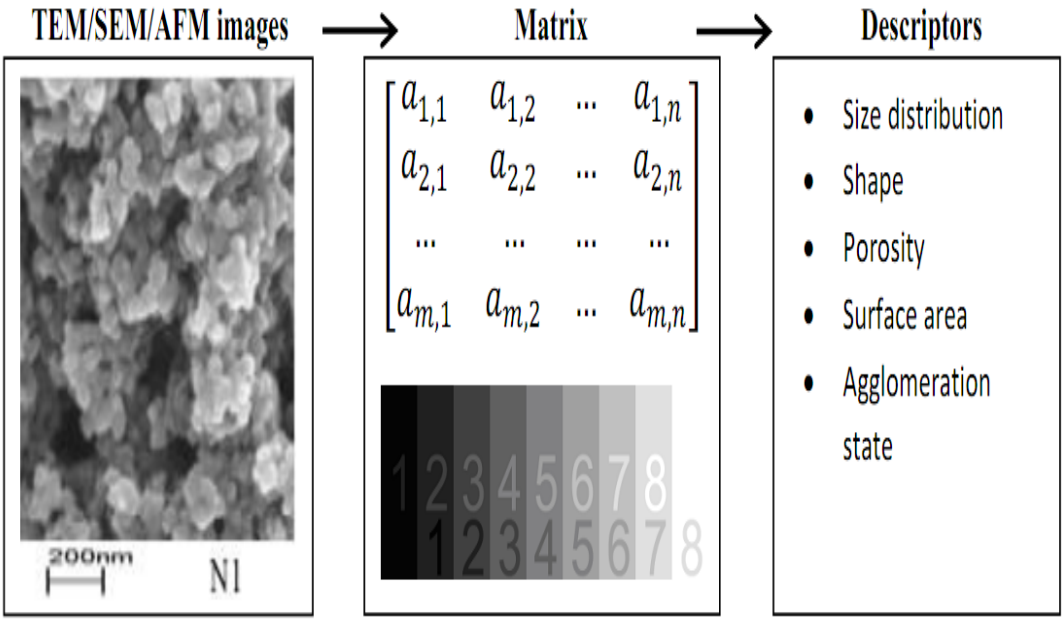

Figure 2.3 Derivation of structural descriptors based on microscopic images (Puzyn, Leszczynska and Leszczynski 2009)

In another study, Xia, Monteiro-Riviere and Riviere (2010) developed a multi-dimensional biological surface adsorption index (BSAI) consisting of five quantitative nanodescriptors: lone-pair electrons, polarity/polarizability, hydrogen-bond donors, hydrogen-bond acceptors, and London dispersion. These five nanodescriptors represent the fundamental forces governing the adsorption process of NPs in a biological environment. In their follow-up study (Xia *et al.* 2011), they performed PCA on five-dimensional nanodescriptor datasets to reduce dimensionality, and obtained a two-dimensional representation of the molecular interaction forces in biological systems and hence facilitated characterisation of the surface properties of ENMs (Fig. 2.4). After obtaining two-dimensional nanodescriptors via PCA, they managed to classify 16 different ENMs into separate clusters based on their surface adsorption properties.

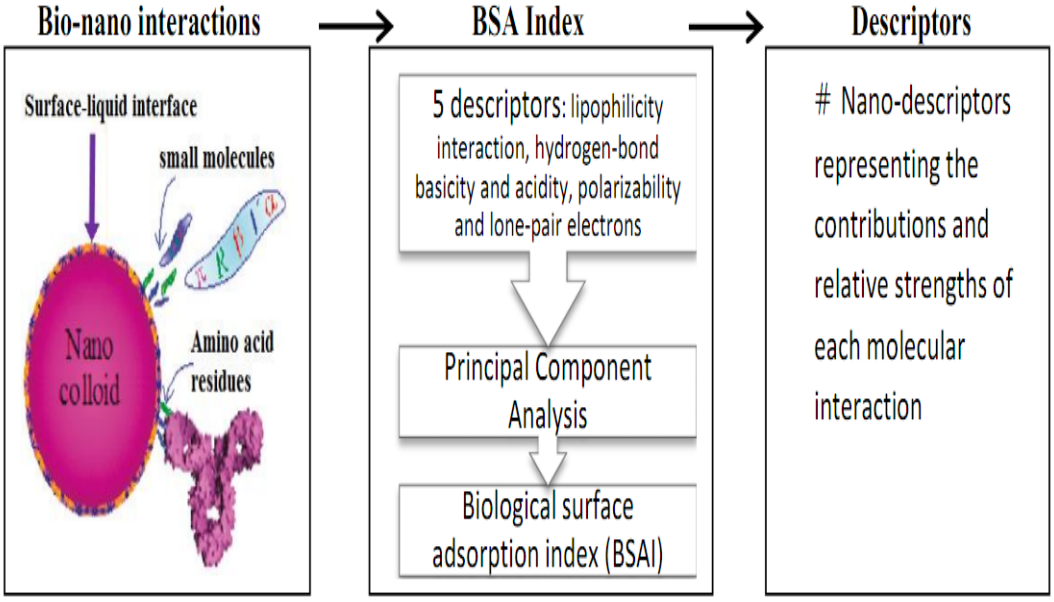

Figure 2.4 Derivation of descriptors that represent the fundamental forces governing the adsorption process of NPs (Xia, Monteiro-Riviere and Riviere 2010)

Burello and Worth (2011a) proposed that different types of spectra (e.g. NMR, IR, Raman, and UV–Vis) can be used as nanodescriptors because they contain fingerprint-like information (Fig. 2.5). The first step is spectral measurement followed by the conversion of the spectra into a numerical

matrix. This data matrix can be seen as spectra-derived descriptors and used for (Q)SAR analysis. It is not entirely a new perspective because spectral information has already been used in a number of studies. The use of IR information for (Q)SAR analysis was shown to be promising by Benigni *et al.* (1999). They compared the IR spectra with several descriptors commonly used in (Q)SAR studies, and found that IR spectra contain unique information that cannot be obtained from molecular descriptors. Zhou *et al.* (2008) used the spectra of multi-walled NTs for characterisation, while Yang *et al.* (2004) attempted to correlate XRD data with photocatalytic performance using the dye decolourisation rate. Clearly, the use of spectra-derived descriptors in (Q)SAR modelling of ENMs is an interesting approach and deserves further investigation.

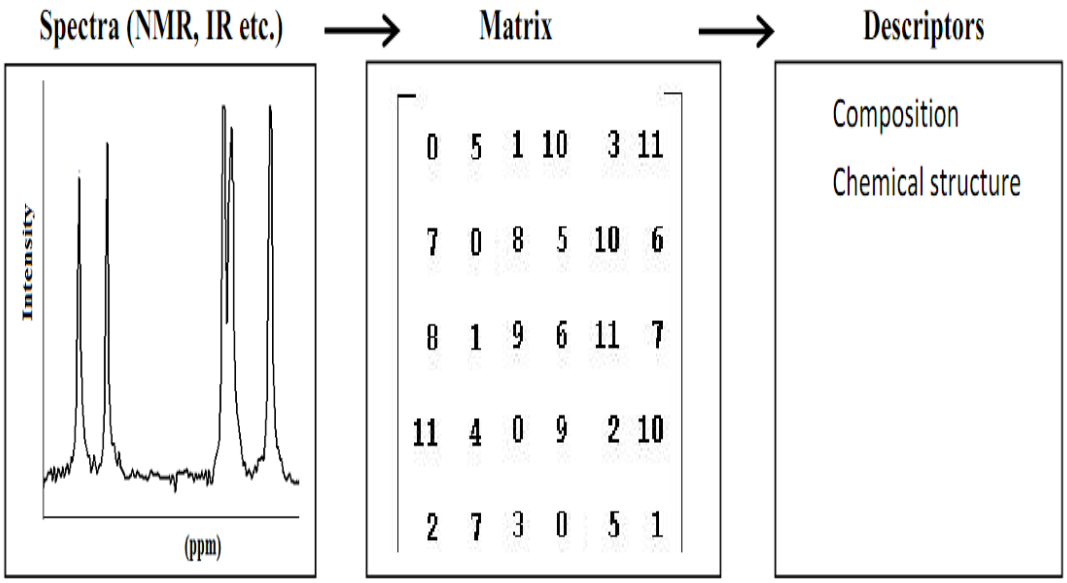

Figure 2.5 Derivation of NP-descriptors based on the spectra of ENMs (Burello and Worth 2011a)

The final properties of materials are related not only to the chemical composition and structure of materials but also to the preparation, synthesis, and processing methods. Le *et al.* (2012) suggested that molecular descriptors characterising physicochemical properties of compounds could be combined with historical descriptors describing the sample preparation and synthesis techniques of materials to develop reliable and predictive models.

Although historical descriptors can be useful for modelling traditional materials, their implementation to nano-(Q)SAR models can be very difficult because they probably have no ability to distinguish between ordinary and nanosized particles. The determination of three-dimensional descriptors that are suitable for nanostructures and NP representation is another promising approach and undoubtedly will be put into practice in the near future. In addition, the development of more sophisticated image analysis approaches (e.g. texture analysis-based methods) would facilitate the rapid extraction of morphological information (e.g. particle size, shape, surface area, and aggregation state) from microscopic images of NPs.

2.4 Nano-(Q)SAR and modelling techniques

A (Q)SAR is a mathematical model that attempts to relate the biological activities or properties of a series of chemicals to their physicochemical characteristics in a quantitative manner (Puzyn, Leszczyński and Cronin 2010). Although the first use of (Q)SAR models is attributed to Hansch (1969), who brought physical organic chemistry and the study of chemical biological interactions together to propose the first (Q)SAR approach, the relationship between chemical structure and biological activity was reported in several earlier studies (Brown and Fraser 1868; Richet and Seances 1893.; Overton 1901). Hansch's (Q)SAR approach has found applications in many disciplines, such as drug design, and chemical and biological science. Moreover, numerous modification of Hansch's approach to (Q)SAR modelling have been developed by many other researchers (Kubinyi 2008).

In (Q)SAR models, it is assumed that the observable biological activity is correlated with the structure of compounds, and this correlation can be expressed in a mathematical equation. The presumed relationship between the activity and structure is expressed with the following form of mathematical equation:

$$y = f\left(x_i\right), \tag{1}$$

where y is the biological activity of the chemical (i.e. toxicity) and $f(x_i)$ is a

function of structural properties. A set of well-characterised compounds with known biological effects is required to obtain this mathematical equation. The structural features of compounds with known biological activities are represented by measured or calculated molecular descriptors. Then, a mathematical model relating the measured activity to the descriptor sets is obtained by regression analysis. The last step is the evaluation of the reliability of the model and its applicability to other compounds. One of the most important steps, which is often omitted, is to define the model's boundaries and limitations to demonstrate how well it will perform when applying to substances that are not used in building the model.

2.4.1 Nano-(Q)SAR research

The research activities focusing on in silico modelling of ENM toxicity are given in Table 2.4. Most of the nano-(Q)SAR studies focused on metal oxide (MO) ENMs because of their common commercial use and high production volume. One of the first attempts to show that computational (Q)SAR can give valuable information about nanotoxicity was reported by Liu and Hopfinger (2008). They used molecular dynamic simulations to investigate the effect of CNT insertion on the cellular membrane structure. Four potential toxicity sources were investigated through membrane interaction-(Q)SAR analysis. Although the result of this study was very informative and encouraging, a proven (Q)SAR model was not established because of the absence of experimental data.

Sayes and Ivanov (2010) assessed the presence of ENM-induced cell damage based on the release of lactate dehydrogenase (LDH) from cells. Six different physical characteristics were measured for each of the selected MO ENMs (TiO_2 and ZnO): primary particle size, size in water and two buffered solutions, concentration, and zeta potential. First, they performed principal component and correlation analysis on the pre-processed dataset to reveal possible correlations between the physical properties and LDH release measurements. Although a strong correlation between some of the physical features were observed, such as particle size and concentration in water, no correlation was found between the measured physical properties and cellular

cell damage in the principal component analysis. Their initial intention was to use the same dataset to develop a regression and classification model. However, they were unable to develop a statistically significant regression model using the TiO_2 and ZnO dataset. The results of classification analysis were better because they managed to produce a classifier with zero resubstituting error. A clear description of the experimental design, ENM preparation, cell culture conditions, and methodology were given in the paper. The inclusion of such knowledge in toxicological research is very important because it greatly improves the interpretability of collected data and enhances its comparability with other studies. The downside of the study is undoubtedly the small number of ENMs and physical descriptors used. It is unrealistic to build a (Q)SAR model with a few ENMs because it does not allow the splitting of the original datasets into training, validation, and test sets. The number of final descriptors used to develop a (Q)SAR model can be less than six, but it is desirable to have a much larger number of initial descriptors, especially in the absence of specific knowledge regarding the relevance of particular properties to nanotoxicity.

Table 2.4 Previously reported nano-(Q)SAR studies

Ref.	NPs	Descriptors	Endpoints	(Q)SAR tool	Criteria met
Sayes and Ivanov (2010)	24 NP susp., 2 MOs	Size measures, conc., zeta pot.	LDH	MLR, LDA	1,2,4
Fourches *et al.* (2010)	44NPs, diverse core	Size, relaxivities, zeta potential	ATP, Red, Apop., Mito	SVM-classification	1,2,3,4
	109NPs, diverse modifier	150 MOE descriptors	Cellular uptake	KNN-regression	1,2,3,4
Puzyn *et al.* (2011b)	17 MO-NPs	12 theoretical descriptors	EC_{50}	MLR-GA	1,2,3,4

Chau and Yap (2012)	105NPs, diverse modifier	679 theoretical descriptors	Cellular uptake	NB, LR,KNN,SVM	1,2,3,4
Zhang *et al.* (2012)	24 MO-NPs	Size, crystallinity, band gap energy, conduction/valance band, dissolution, zeta pot.	MTS, ATP, LDH, DCF, MitoSox, Fluo4, JC1, PI	Regression tree	1,2,4
Epa *et al.* (2012)	31NPs, diverse core	Indicator variables, size, relaxivities, zeta potential	ATP, Red, Apop., Mito	MLR, SLR, feature selection, ANN	1,2,4
	109NPs,diverse modifier	691 theoretical descriptors	Cellular uptake		
Wang *et al.* (2013)	18NPs, MOs and C-based	size, shape, area, porosity, free radicals, reactivity, metal conc. and charge	LDH, Apop., Nec., Proinflammatory, Hemolysis, MTT	PCA	1,2,4
			DiOC6,morph.		
Liu *et al.* (2013a)	44 iron oxide core NPs	Size, relaxivities, zeta potential	ATP, Red, Apop., Mito	NBC,LGR,LDA,NN	1,2,3,4
Liu *et al.* (2013c)	24 MO-NPs	30 molecular descriptors	MTS, ATP, LDH, DCF, MitoSox, Fluo4, JC1, PI	NBC, LR, LGR, LDA, SVM	1,2,3,4
Singh and Gupta (2014)	44 iron oxide core NPs	Size, relaxivities, zeta potential	ATP, Red, Apop., Mito	Ensemble learning (EL)-based techniques	1,2,3,4
	109NPs, diverse modifier	691 theoretical descriptors	Cellular uptake		
	17 MO-NPs	Oxygen percent, molar refractivity, polar surface area	Cytotoxicity (EC_{50})		
	80 MWCNTs	6 topo. and geo. Descriptors	Cell viability		
	48 fullerene derivatives	10 descriptors	The binding affinity		
Kar *et al.* (2014)	109 NPs, diverse modifier	307 theoretical descriptors	Cellular uptake	GFA, MLR, PLS	1,2,3,4

	23 MO NPs	27 NP descriptors (element related, energy/enthalpy, size and surface charge descriptors)	Single- and multi- parameter toxicity assays		
Oksel et al.(under review)	105 NPs, diverse modifier	389 chemical descriptors and 147 chemically interpretable descriptors	Cellular uptake	Genetic programming-based decision tree construction algorithm	1,2,4
	18 MO NPs	29 theoretical descriptors	Cytotoxicity (LC_{50})		
	12 gold NPs	28 descriptors, (experimental parameters, image descriptors and nano-descriptors)	Exocytosis		

In another study, two different experimental nanotoxicity datasets were used to derive a mathematical relationship between the toxicity of ENMs and their physicochemical properties (Fourches *et al.* 2010). The advantage of the data used in this study was the concurrent testing of ENMs under the same conditions. In the first case study, three distinct clusters of ENMs were identified based on their biological activity, and support vector machine (SVM) models with high accuracies were developed. In the second case study, a descriptor quantifying lipophilicity was the most significant predictor of biological activity because it accurately discriminated between ENMs with low and high values of $PaCa_2$ cellular uptake. Overall, it was shown that the (Q)SAR approach can provide useful information for toxicity prediction of new ENMs. The methodology used in this work fulfilled all the principles of the OECD for the validation of (Q)SAR models.

Puzyn *et al.* (2011a) were one of the first few researchers to derive a mathematical equation based on the dataset of cytotoxicity and molecular descriptors. Initially, a set of 12 structural descriptors were quantum-chemically calculated using the semi empirical PM6 method. Among the pool of descriptors, only one structural descriptor (ΔH_{Me+}) representing the enthalpy

of the formation of a gaseous cation with the same oxidation state as that in the MO structure was used to establish the following nano-(Q)SAR model:

$$\log\left(1/EC_{50}\right) = 2.59 - 0.50\Delta H_{\mathrm{Me+}}. \qquad (2)$$

A set of 17 MO-NPs used by Puzyn *et al.* (2011a) can be considered as small from a modelling perspective, but the development of such predictive nano-(Q)SAR models is helpful to encourage new investigations.

Another simple but statistically powerful nano-(Q)SAR model was developed by Epa *et al.* (2012) based on the results of in vitro cell-based assays of ENMs. They used the same dataset as Fourches *et al.* (2010) with minor changes. The difference was that new descriptors encoding the presence or absence of some particular features, such as coating, were added. They managed to build the following nano-(Q)SAR equation based on these dummy variables:

$$\begin{aligned}\text{Smooth muscle apoptosis} = 2.26\left(\pm 0.72\right) - 10.73\left(\pm 1.05\right) I_{\mathrm{Fe_2O_3}} - \\ 5.57\left(\pm 0.98\right) I_{\mathrm{dextran}} - 3.53\left(\pm 0.54\right) I_{\mathrm{surface\ charge}},\end{aligned} \qquad (3)$$

where $I_{\mathrm{Fe_2O_3}}, I_{\mathrm{dextran}}$, and $I_{\mathrm{surface\ charge}}$ stand for indicators (taking values of 1 or 0) for the core material, surface coating, and surface charge, respectively. This was the second quantitative model developed to predict the toxicity of nanostructures. Compared with Eq. (2), this mathematical expression was developed from a more diverse set of data.

Recently, the hypothesis that ENM toxicity is a function of some physicochemical properties was tested by Wang *et al.* (2014). A set of 18 ENMs including carbon-based materials and MOs were used in the study. Different types of cytotoxicity assays were performed, such as LDH, Apoptosis, Necrosis, haemolytic, and MTT, and several structural and compositional properties were measured. Initially, they applied PCA to the cytotoxicity data to combine the toxicity values measured at different doses into a single value that describes all the data points on the dose–response curve. It should be mentioned that, because toxicity is highly dose-dependent,

the toxicological effects are usually evaluated at multiple concentrations in a series of tests, and the results are represented with a dose–response curve. Fig. 2.6 shows examples of the dose–response curves obtained for the 18 ENMs. From this graph, the cell viability is lower in the cells treated with N3 (nanotubes), N14 (zinc oxide), and N6 (aminated beads) than the other ENMs. There are various methods to analyse and compare dose–response curves, such as area under the curve, slope of the curve, threshold values, min/max response, and the benchmark dose approach. In this study, Wang *et al.* (2014) performed PCA to integrate the entire curve, and used the resulting principal components as an overall measure of cumulative response. They concluded that, compared with other approaches, PCA-based representation of the dose–response curves provides more reasonable results when ranking the ENMs according to their hazard potential. Because of the high toxicity level of four particular ENMs (zinc oxide, polystyrene latex amine, Japanese nanotubes, and nickel oxide), nano-(Q)SAR analysis focused on these four ENMs to investigate the potential factors behind their observed toxicity. It was concluded that the physicochemical characteristics leading to the toxicity of ENMs were different, and it was not possible to draw a general conclusion that was valid for all toxic ENMs screened in the study. However, the nano-(Q)SAR method was found to be useful to reveal that some of the measured properties, such as metal content, high aspect ratio, and particle charge, were correlated with the toxicity of different nanosized materials.

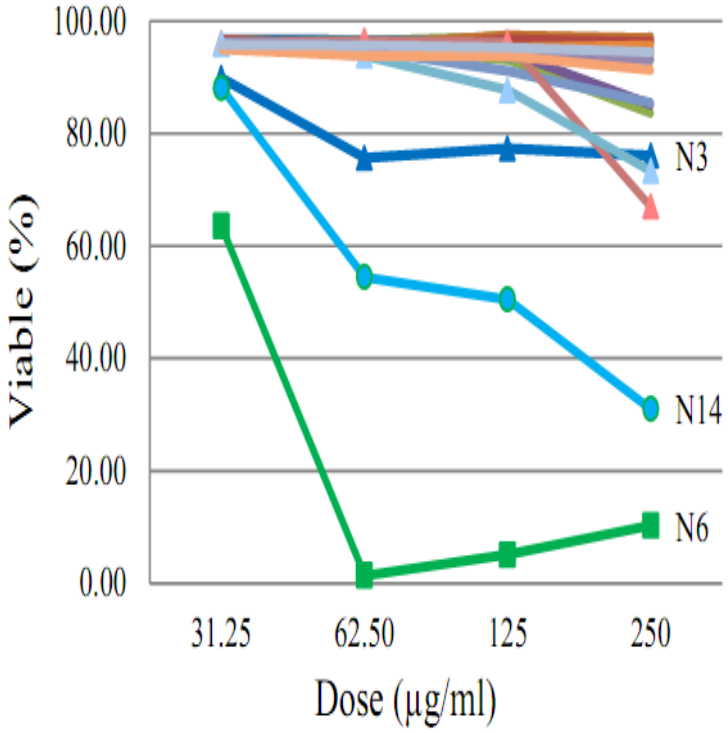

Figure 2.6 Viability results for 18 NMs (Wang *et al.* 2014).

Liu *et al.* (2013b) developed a classification-based (Q)SAR model based on multiple toxicity assays, 44 iron oxide core NPs, and 4 simple descriptors (size, zeta potential, and relaxivities). They suggested that existing nano-(Q)SAR models did not take into account the acceptance level of false negative to false positive predictions. Unlike previously constructed nano-(Q)SAR models, they also investigated the decision boundaries of the nano-(Q)SARs subject to different acceptance levels of false negative/false positive predictions.

In another study, Liu *et al.* (2013d) attempted to relate the physicochemical properties of MO-NPs to their toxicity by developing a structure–activity relationship. A number of classification nano-(Q)SAR models were developed based on a large toxicity dataset of 24 MO-NPs. A set of 30 molecular descriptors were calculated for each NPs, and only two of them (conduction band energy and ionic index) were identified as important molecular descriptors on which the best performing nano-(Q)SAR model was

built. Their conclusion was in a good agreement with the results of Burello and Worth (2011a), who found that the conduction band energy of oxide NPs is related to their toxicity. Similar findings have also been reported by Zhang *et al.* (2012), who indicated that the oxidative stress induced by MO-NPs could be linked to their conduction and valance band energies.

More recently, Singh and Gupta (2014) attempted to build classification and regression nano-(Q)SAR models using ensemble methods such as decision tree forest (DTF) and decision tree boost (DTB). Five different datasets were used to demonstrate and confirm the suitability of these techniques for the (Q)SAR modelling process by comparing the accuracy of the developed nano-(Q)SARs with past studies. It was concluded that the nano-(Q)SAR models constructed had high performance and statistical significance along with superior predictive ability to previous studies.

The common problem in the majority of published (Q)SAR studies is that it is not possible to generalise the results in the absence of explanatory information regarding the underlying reasons for the system behaviour, thus making the usability of these studies limited for compounds outside the study. When the results of (Q)SAR analysis are only valid for the tested compounds, (Q)SAR becomes a data analysis tool with no predictive ability. To ensure the reliability of the established nano-(Q)SARs, researchers should also address model uncertainty arising from experimental error and lack of knowledge. Moreover, most of the existing nano-(Q)SAR studies used small datasets to establish a link between nanostructure and toxicity. Although small datasets can be useful to describe or explain the relationship between NP structure and activity, they may not be very useful for predictive purposes. Table 2.5 summarises the previously reported nano-(Q)SAR studies and compares their methodologies with OECD principles: (1) a defined endpoint, (2) an unambiguous algorithm, (3) the applicability domain, and (4) model validation for stability and predictivity.

2.4.2 Nano-(Q)SAR modelling techniques

The statistical methods that have been used in existing nano-(Q)SAR studies are listed in Table 2.5.

Table 2.5 The statistical methods used in existing nano-(Q)SAR studies

	(M)LR	GA	LGR	NNet	LDA	NB	SVM	NNeig	PCA	Others
Sayes and Ivanov (2010)	✓				✓				✓	
Fourches *et al.* (2010)							✓	✓		
Puzyn *et al.* (2011b)	✓	✓								
Chau and Yap (2012)			✓			✓	✓	✓		
Zhang *et al.* (2012)									✓	RT
(Winkler *et al.* 2014; Epa *et al.* 2012)	✓			✓						EM
(Ghorbanzadeh, Fatemi and Karimpour 2012)	✓			✓						SOM
(Wang *et al.* 2014)									✓	
(Liu *et al.* 2013a)			✓		✓	✓		✓		
(Liu *et al.* 2013c)	✓		✓		✓	✓	✓			
(Shao *et al.* 2013)	✓	✓								
(Kar *et al.* 2014)	✓	✓							✓	PLS
(Singh and Gupta 2014)										EL
(Toropov *et al.* 2013; Toropova and Toropov 2013)										MCO
(Durdagi *et al.* 2008)										PLS
Oksel et al. (under review)										DTs

In principle, a variety of methods that have proven to be effective in classic (Q)SAR modelling, such as statistical methods, neural networks and decision trees, can be applied to nano-(Q)SAR. In practice, however, their direct use in ENM toxicity modelling has difficulties. The major obstacle originates from the availability of data, because some (Q)SAR algorithms require large datasets that are not currently available for ENMs. Considering the current scarcity of nanotoxicity data, it is reasonable to use modelling tools that can make effective use of smaller datasets. In addition, there is still insufficient knowledge about physicochemical descriptors that can predict the toxicity of ENMs. Therefore, current nano-(Q)SAR studies should focus on identifying toxicity-related physicochemical characteristics as well as predicting potential toxicity values. The ease of use (i.e. the ease of model building and interpretation of the results) is another important consideration, particularly in the nano-(Q)SAR world where the ability to interpret the resulting models is the key to understanding the correlation between different forms of biological activity and descriptors. Overall, the following factors have to be considered when selecting nano-(Q)SAR modelling techniques:

- Minimum data requirements. Effective use should be made of limited data without relying on the availability of large datasets.
- Transparency. Models should be transparent (rather than black-box), intuitive, and able to help identify the physicochemical descriptors that are related to the toxicity of ENMs
- Ease of model construction. The technique should be easy to use and easy to implement.
- Nonlinearity. The technique should be able to reveal nonlinear relationships/patterns in the dataset.
- Low overfitting risk. The technique should have low risk of overfitting, which may reduce the generalisation of the model.
- Descriptor selection function. The technique should have the capability of feature selection to exclude redundant descriptors before model building.
- Ease of interpretation. The technique should be able to produce meaningful and interpretable outcomes and explain how the outcomes are produced.

- Low modeller dependency. The technique should have low sensitivity to changes in the model parameters.

Below, some (Q)SAR modelling methods are examined, including feature selection methods, statistical methods, decision trees, support vector machines, neural networks, multi-dimensional visualisation, and knowledge-based expert systems. The focus is on discussing their suitability for nano-(Q)SAR modelling, rather than introducing the individual algorithms.

2.4.2.1 Data visualisation and exploratory data analysis

Exploratory data analysis (EDA) includes a collection of techniques/tools that allow visual exploration of a chosen dataset. It is often carried out in order to identify patterns and extract useful information that is hidden within a given data set. Different data visualisation techniques can be used for visual exploration of multi-dimensional data that describe an item with more than three attributes. They can be used to identify patterns/correlations, to detect clusters/outliers, to visually display relationships between multiple variables (e.g. ENM physicochemical descriptors and toxicity endpoints), to handle limited data sets, and to perform an interactive data analysis with the help of visual features such as colour. For the purpose of data exploration, several techniques can be used to handle multi-dimensional data, such as parallel co-ordinates, heat maps, dimensionality reduction, and clustering methods.

Multi-dimensional data visualisation has many important applications and, in particular, can be considered as an important tool in decision- making processes. In the nanotoxicology community, for example, effective data visualisation will mean the ability to visualise multi-dimensional data to discover correlations between NM physicochemical properties with toxicological effect, that is, to establish what properties nanoscale materials have and how these attributes influence their performance and biological effects. The complexity within nanotoxicology is that no single parameter can describe the properties (e.g. physical, chemical, and toxicological) of ENMs. In fact, there are various features including physical structure, chemical composition, and surface characteristics that have been suggested to

contribute to the biological effects and behaviours of ENMs in different environments. A detailed characterisation including the careful assessment of a wide range of characteristics, is often required to understand the physical behaviour of ENM, to ensure the correct interpretation of the biological activity studies and also to make the inter-comparison of studies possible. However, the complete characterisation of ENMs can lead to the generation of large amounts of data that need to be analysed in detail and well understood. Therefore, there exists a need for a simple but yet effective method of converting multi-dimensional characterisation data (corresponding to multi-variables or features) into a more efficient format that can be visually explored and examined. Such visualisation techniques are necessary in order to get an overall picture of the properties describing individual characteristics of ENMs. This is useful when a large amount of characterisation information is involved. The result of effective data visualisation in nanotoxicology will have the ability to help prioritise ENMs for screening, to identify the key physicochemical parameters that affect toxicity, to provide practical solutions to the risk-assessment-related problems caused by the diversity of ENMs, and to group ENMs (crucial in many aspects, from hazard assessment to knowledge-gap-filling).

Dimensionality reduction techniques, such as PCA and factor analysis, can be used for representing data in a simpler form. PCA is a multivariate statistical tool that searches for patterns and relationships. The method works by taking complex datasets with multiple interrelated variables and reducing them down, with minimal loss of information, to simpler uncorrelated datasets known as principal components (PCs). PCA has the advantage in that it provides a visual aid for identifying homogeneity and differences amongst large datasets, displaying detectable patterns in an unbiased way. It can also be used to replace the large number of compound descriptors by a smaller set of latent variables (e.g. dimensionality reduction). However, the main disadvantage of reducing the dimensionality of descriptor data using PCA-like approaches lies in the difficulty of correctly interpreting the results of dimension reduction analysis since the variables used as input (e.g. latent variables) are not readily interpretable descriptors. Overall, PCA is a useful pattern recognition tool that facilitates understanding trends in data by

reducing complexity. There is no restriction on the ratio of compounds to predictors in the data since PCA can be performed even if the number of variables is higher than the number of objects. However, this linear technique cannot capture the nonlinear patterns as it searches for linear relationships in the data. Together with the interpretability issue mentioned above, the linearity assumption may appear to be the most important limitation of PCA approach in the context of (Q)SAR analysis.

When compared to data visualisation tools, the main disadvantage of the dimensionality reduction methods such as PCA and factor analysis is that some links may be lost during data transformations. On the contrary, direct visualisation techniques (e.g. parallel co-ordinates, radar charts) allow the efficient visualisation of multivariate data points without any information loss.

A heat map is simply a table that has colours in place of numbers. In the case of (Q)SAR analysis, heat maps are particularly useful to prioritise compounds based on toxicity potential and to demonstrate the physicochemical differences between compounds belonging to different activity classes (e.g. toxic and non-toxic). The clustered heat maps display the hierarchy of clusters in the form of a dendrogram and was used to summarise multivariate toxicity outcomes and to display NP cluster membership.

The parallel co-ordinates method is another useful method for visualising multi-dimensional data. Here, N-dimensional space is represented as N parallel lines, typically vertical, and equally spaced. The value of parallel co-ordinates is that certain geometrical properties in high dimensions can be easily transformed into a lower 2D space, which breaks the limitation of traditional dimension representation in the Euclidean space. In parallel co-ordinates, the points used in Euclidean space are represented as series of lines passing through parallel axes, that is, each variable is represented by one parallel axis. Figure 2.7 illustrates the result of transferring a three dimensional point from traditional co-ordinates to parallel co-ordinates.

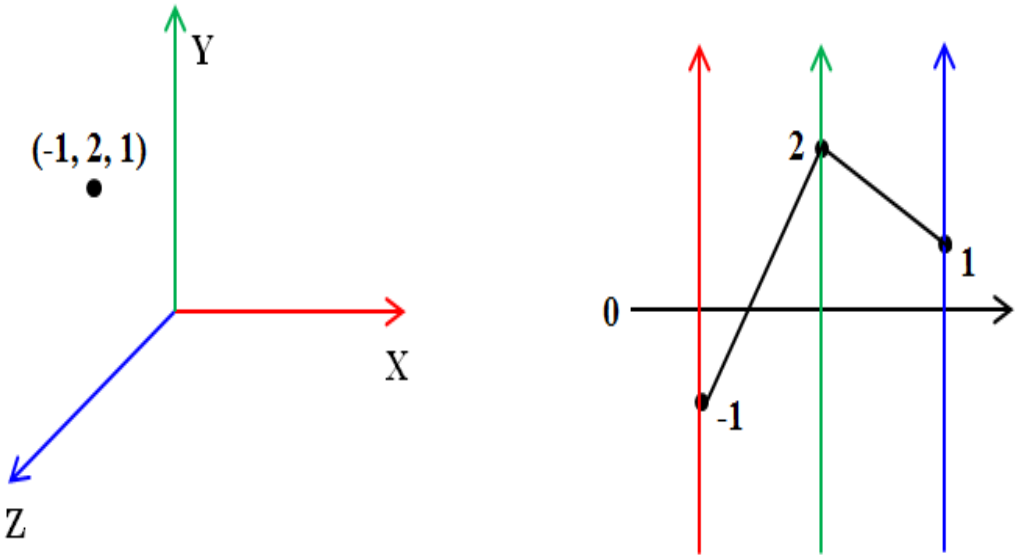

Figure 2.7 A three dimensional point in traditional co-ordinates (left) and in parallel co-ordinates (right)

An interesting feature of parallel co-ordinates is when overlapping lines between adjacent axes form distinct patterns, representing the relation between variables they connect. An advantage of this interactive environment is that it allows the selection of a subset of the plots, thus enabling the operator to highlight the most interesting data, and permuting the axes interactively. The visualisation technique using parallel co-ordinates can transform multi-dimensional data into 2D patterns and make it possible to visualise clusters and outliers of the data. Therefore, it can be used for data clustering and linking analysis. For NP toxicity analysis, it can help identify outliers (e.g. particle samples with high toxicity), and aid in finding corresponding responsible physicochemical descriptors (e.g. for the observed high toxicity).

Although there is a large number of papers about parallel co-ordinates, only a few notable software tools are available to convert databases into parallel co-ordinates graphs. One of the most sophisticated tool for parallel co-ordinates transformation is the C Visual Explorer (CVE) software, which is used in this work.

2.4.2.2 Feature selection methods

A large number of descriptors can be obtained through experimentation and/or computation, but very few carries identical and relevant information that

allows the construction of statistically powerful mathematical models. The aim of feature selection process is to select only the inputs that have an effect on the outputs. In this step, the input variables that have little or no effect on the outputs are excluded from the analysis. There are a wide variety of methods, such as stepwise procedures, genetic algorithms, random forest and clustering methods, utilised for the selection of the most important descriptors. Among the various methods for automatic input feature selection, the genetic algorithm (GA) has shown excellent performance. The GA feature selection approach can be applied together with almost all (Q)SAR model building algorithms. The GA starts from a population of possible solutions (called individuals of chromosomes), which can be randomly generated. Each gene in the first generation of solutions consists of randomly selected descriptors. A (Q)SAR model can be built using the randomly selected descriptors in each chromosome. (Q)SAR models built based on the individuals in the initial population of solutions in this first generation are evaluated using a pre-defined fitness function. Based on Darwin's theory of "survival of the fittest", individuals undergo operations such as mutation and crossover to generate the population of individuals in the next generation. In summary, a GA algorithm has the following essential steps:

(1) Random generation of a set of solutions (the number of solutions can be set by the user) and code into a vector group with fixed length;
(2) Generation of a new set of solutions by the method below, or generation of new solutions to substitute individuals in the current population;

    (2.1) Selection of parent individuals based on the value of fitness function;
    (2.2) Crossover to generate one or several sub-individuals;
    (2.3) Apply mutation operation to some individuals;

(3) Repeat step (2) until one of the stopping criteria is met.

The stopping criteria are reaching the maximum number of generations or time limit, and satisfying the stop criterion for the fitness function. For more

detail, please refer to Liu and Zhou (2007); Reddy, Kumar and Garg (2010); Goodarzi *et al.* (2013); Ma and Wang (2011); Li, Wang and Abebe (2008).

Random forest is another method that can be used to identify the properties that have the most significant influence on the biological activity of interest in (Q)SAR investigations. It has the capability of excluding redundant descriptors by constructing several variations of tree with different sub-sets of descriptors and retaining only the ones that satisfy the pre-defined criteria.

2.4.2.3 Decision trees (DTs)

Automatic generation of decision trees from data is a powerful machine learning technique that can be used as a classification or regression tool for categorical and numerical predictions of biological activity in (Q)SAR studies (Ma, Buontempo and Wang 2008). DTs can be constructed with small, large, or noisy datasets, and then used to detect nonlinear relationships. They have a tree-like structure that splits data points into different classes based on decision rules to categorise and model input data. Various DT generation algorithms are available, and can be broadly classified as those shown in Fig. 2.8. The most significant advantages of DT methods are their capability to automatically select the input variables (i.e. the physicochemical descriptors that contribute to the observed toxicity) and to remove descriptors that are not related to the endpoint of interest. In a previous study, Buontempo *et al.* (2005) demonstrated the use of a genetic programming-based DT generation technique for in silico toxicity prediction. They developed a DT model containing five descriptors selected from a pool of more than a thousand descriptors that has good predictive performance for both training and test datasets. This “knowledge discovery” capability is no doubt valuable to identify the physicochemical descriptors that contribute to the toxic effects of ENMs. Such knowledge has even more benefits for eliminating or minimising the risk of ENMs through engineering approaches (i.e. modification of physicochemical properties that influence the toxicological response through the active engineering of ENMs). Another benefit of DT analysis is its capability to avoid the (Q)SAR model being over-biased towards data in dense areas, which is a problem with some other techniques, such as linear regression and neural networks. Small data cases, i.e. data outside the dense

data area, can also be modelled as branches of a decision tree. An additional advantage of DTs is the ease of their interpretability (Apté and Weiss 1997) and transparency (Ma and Wang 2009). Investigation of DTs for modelling ENM toxicity requires more research, because, in addition to the abovementioned advantages, there are researchers who have voiced concerns about the generalisation ability and predictive power of DTs (Bengio, Delalleau and Simard 2010). DTs (and their extension known as "random forest") have been investigated for (Q)SAR modelling in a number of studies (Sussman *et al.* 2003; Arena *et al.* 2004; Andres and Hutter 2006; Han, Wang and Bryant 2008; Ma, Buontempo and Wang 2008). Further research on DTs should focus on maximising their advantages and overcoming their limitations. An interesting example is random decision forest, and several studies have shown its improved generalisation ability over DTs (Díaz-Uriarte and De Andres 2006; Genuer, Poggi and Tuleau-Malot 2010; Ma and Wang 2009; Teixeira, Leal and Falcao 2013).

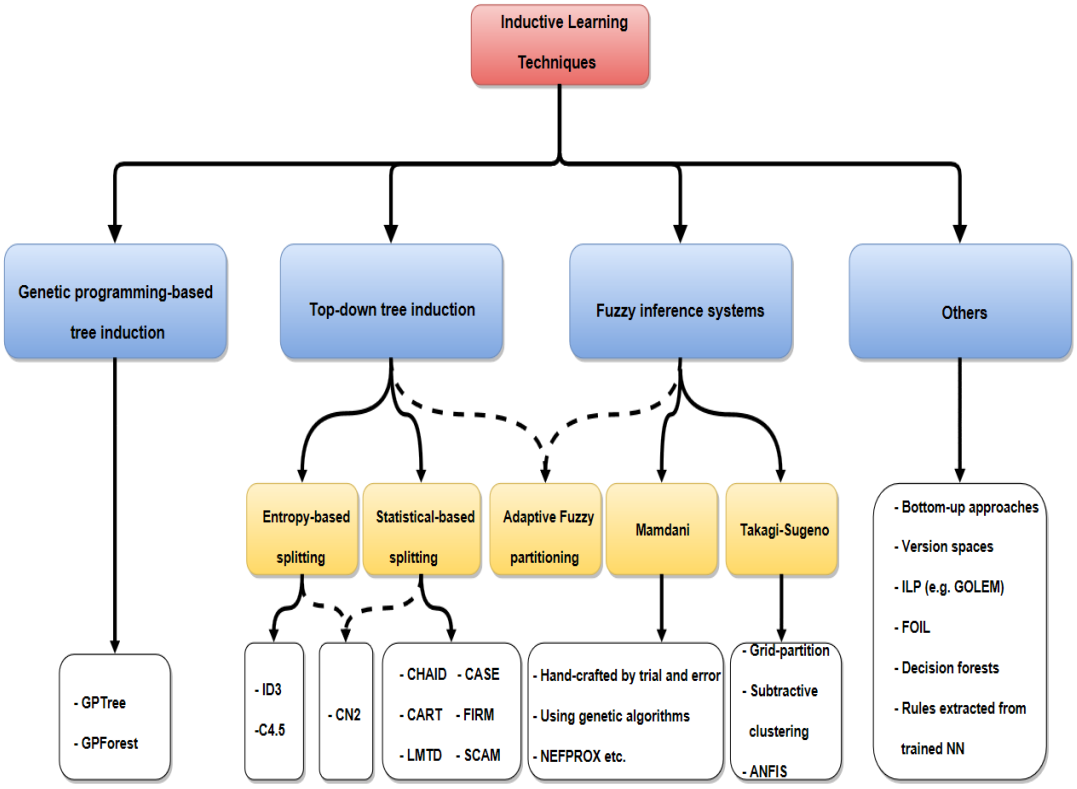

Figure 2.8 Family tree of proposed inductive learning techniques showing a selection of specific implementations of each type.

2.4.2.4 Multiple linear regression (MLR)

MLR is one of the most widely used methods for deriving (Q)SAR models (Leach and Gillet 2007) due to its ease of use and interpretation (Yee and Wei 2012b). However, there are three main factors limiting the use of MLR in nanotoxicity modelling (Shahlaei 2013):

- the linearity assumption: it cannot detect nonlinear causal relationship;
- the restriction on the ratio of compounds to descriptors in the data: the lowest ratio of the number of ENMs to the number of descriptors should be 5:1;
- the dependence of its performance on redundant variables: the presence of correlated input variables and input variables that are irrelevant to the output may lead to poor model performance.

Using MLR in conjunction with a variable reduction technique such as PCA can be useful for filtering out redundant variables and eliminating correlations between input variables (i.e. physicochemical descriptors). Overall, the main advantage of linear models such as MLR over nonlinear models is their transparency. Some information of the relative importance of the physicochemical descriptors can be directly obtained from a linear model by examining the weights, whereas some nonlinear models, such as neural networks, cannot give such direct information.

2.4.2.5 Partial least squares (PLS)

Several statistical methods, such as multiple linear regression (MLR), principal component regression (PCR), and partial least squares (PLS) regression, have been extensively studied in (Q)SAR analysis because of their ease of use and interpretation (Yee and Wei 2012a). PLS is a linear regression method that handles data cases where the number of descriptors is greater than the number of compounds. The PLS method works well when there are several noisy and inter-correlated descriptors, and also allows multiple responses to be simultaneously modelled (Eriksson and Johansson 1996). The usefulness of PLS in (Q)SAR studies, especially when the

descriptors are highly correlated and numerous, has been proven by several researchers (Dunn *et al.* 1984; Cramer *et al.* 1988; Luco and Ferretti 1997; Luco 1999; Eriksson *et al.* 2004; Gu *et al.* 2012). However, this method can only be used for the solution of linear regression problems. To overcome this problem, nonlinear versions of the PLS method have been developed based on different algorithms, such as kernel-based PLS (Rosipal and Trejo 2002), neural network PLS (Qin and McAvoy 1992), and genetic algorithm-based PLS (Hasegawa, Miyashita and Funatsu 1997). These extensions allow nonlinear relationships to be modelled in (Q)SAR studies, which is not otherwise possible with the simple PLS technique.

2.4.2.6 Support Vector Machines (SVMs)

There is increasing interest in the use of SVMs, which can handle both regression and classification problems, as an alternative to linear modelling methods such as MLR and PLS in (Q)SAR studies (Czermiński, Yasri and Hartsough 2001; Mei *et al.* 2005). SVMs can handle many issues that usually affect the performance of other (Q)SAR modelling techniques, such as nonlinear relationships, collinear descriptors, small datasets, and model overfitting (Mei *et al.* 2005). SVMs have good potential for (Q)SAR analysis because of their accuracy and high generalisation capability. On the other hand, the main disadvantages of SVMs are the high sensitivity of model performance to the selection of design parameters (e.g. kernel functions) and the complexity of direct interpretation of SVM decisions. SVMs have been used in numerous studies to construct classification (Czermiński, Yasri and Hartsough 2001; Yao *et al.* 2005; Niu 2007) and regression (Yao *et al.* 2004; Mei *et al.* 2005; Niu *et al.* 2012; Darnag, Minaoui and Fakir 2012) based (Q)SAR models. As previously mentioned, GA-based feature selection can be integrated with SVM in (Q)SAR modelling, as shown in near-infrared chemometrics (Ma and Wang 2011).

2.4.2.7 Artificial Neural Networks (ANNs)

ANNs are algorithms that imitate how the human brain works and computationally simulate human brain activity based on the neural structure of the brain. Although in some cases the poorly understood structure of this

technique affects its practical reliability, successful applications of ANNs in the (Q)SAR world (Jalali-Heravi and Parastar 2000; Habibi-Yangjeh, Danandeh-Jenagharad and Nooshyar 2006; Jalali-Heravi, Asadollahi-Baboli and Shahbazikhah 2008; Ventura, Latino and Martins 2013) keep interest in this method alive. ANNs offer several advantages to (Q)SAR developers, including the ability to deal with the nonlinear nature of structure–activity relationships and large descriptor datasets including unnecessary variables. However, ANNs also have several disadvantages, such as difficulty in interpreting the outcome, selecting the optimum complexity, risk of overfitting, and high sensitivity of the generalisation power to changes in parameters and network topology. In some applications, ANN models are treated as a black-box because of their inability to give deep insight into the encoded relationship between the predictors and predicted outcomes (Guyon and Elisseeff 2003). Other studies have suggested that ANN systems should not still be seen as inexplicable models (Baskin, Palyulin and Zefirov 2009; Sussillo and Barak 2013) because a number of methodologies facilitating the interpretation of model outcomes have been developed (Burden and Winkler 1999; Baskin *et al.* 2002; Guha, Stanton and Jurs 2005). Furthermore, it should be pointed out that, like other modelling techniques, ANN can be used together with GA-based feature selection algorithms to remove redundant variables during the model building process. In addition, some researchers have investigated the use of the sensitivity analysis method for minimisation of the input data dimension and extraction of information about the relative importance of inputs to an output (Zurada, Malinowski and Cloete 1994).

2.4.2.8 Expert knowledge systems

(Q)SAR often refers to data-driven modelling. However, the usefulness of knowledge-based expert systems should not be underestimated, as evidenced by the success of the expert system DEREK of Lhasa Ltd. for toxicity predictions (Greene *et al.* 1999). This expert system draws its knowledge from both literature and databases, and is considered to be one of the most powerful tools for the toxicity predictions of molecules. Considering the gaps and variations in the available ENM toxicity data (i.e. incomplete characterisation of physicochemical descriptors and different measures of

toxicity), knowledge-based expert systems, ideally with some kind of “text data mining” capability that can continuously capture new knowledge appearing in the literature, might be one of the most effective approaches for nano-(Q)SAR.

2.4.2.9 Model validation methods

Irrespective of the method used to construct the (Q)SAR models, the validity of the outcomes of the predictive models should be evaluated both internally and externally. Internal validation is the process of evaluating the prediction accuracy of (Q)SAR models based on the dataset used in the modelling process. The most common internal validation techniques used in (Q)SAR studies are least squares fit (R^2), chi-squared ($\chi 2$), root-mean squared error (RMSE), leave-one-out or leave-many-out cross-validation, bootstrapping, and Y-randomisation (Veerasamy *et al.* 2011). The use of external validation techniques in addition to internal validation methods is increasingly being recommended by researchers (Gramatica 2007; Tropsha 2010; Veerasamy *et al.* 2011) and authorities (OECD 2007b) for the assessment of (Q)SAR model reliability in the best and most trustworthy way. Moreover, it is always beneficial to use more than one validation metric to quantitatively measure the accuracy of the model prediction.

The definition of the applicability domain of the constructed and statistically validated model is the final, but one of the most important, steps in the (Q)SAR model building process. There are several approaches (e.g. geometry, range, distance, and probability density function based approaches) to define the applicability domain region of statistical models based on different algorithms. For more detailed information about the available approaches for defining the (Q)SAR model applicability domain, refer to the review papers of Jaworska, Aldenberg and Nikolova (2005) and Sahigara *et al.* (2012).

2.5 Input data for nano-(Q)SAR and its current availability

In nano-(Q)SAR models, the importance of high-quality and well-described datasets is even more pronounced because the unique properties of ENMs are mostly associated with particular sizes and conditions (Gajewicz

et al. 2012). Ideally, the input data required to build a reliable (Q)SAR model should be (1) obtained from a preferably single and standardised protocol, (2) examined in terms of accuracy and suitability for (Q)SAR analysis, and (3) large enough to allow rational division of the data into training and test sets. Because nano-(Q)SAR is a data-based method, the accuracy of the data determines the quality of the final model. Therefore, it is very important to create a comprehensive nanotoxicity database and make it broadly accessible.

(Q)SAR approach is designed to predict the biological activity of a compound based on its physical and compositional features. To that end, two particular types of data are needed: experimental biological activity data and experimental/computational physicochemical characterisation data. Currently, the most important sources of information regarding the biological activity of ENMs are in vivo and in vitro studies, the results of which can be used as indicators of toxicological effects (i.e. dependent variables) in nano-(Q)SAR analysis. Molecular descriptors can be determined either from experimental data or theoretical calculations. As mentioned in section 2.3, a certain amount of uncertainty exists in both descriptor types.

Figure 2.9 shows the general data collection framework for (Q)SAR studies, together with the issues that directly affect the reliability and suitability of the data collected for modelling purposes. The sufficiency of the data for modelling and the feasibility of developing nano-(Q)SAR models should be evaluated properly, with careful attention being given to:

- the reliability of the data source,
- the quality and quantity of the dataset,
- and the suitability of the data for computational analysis.

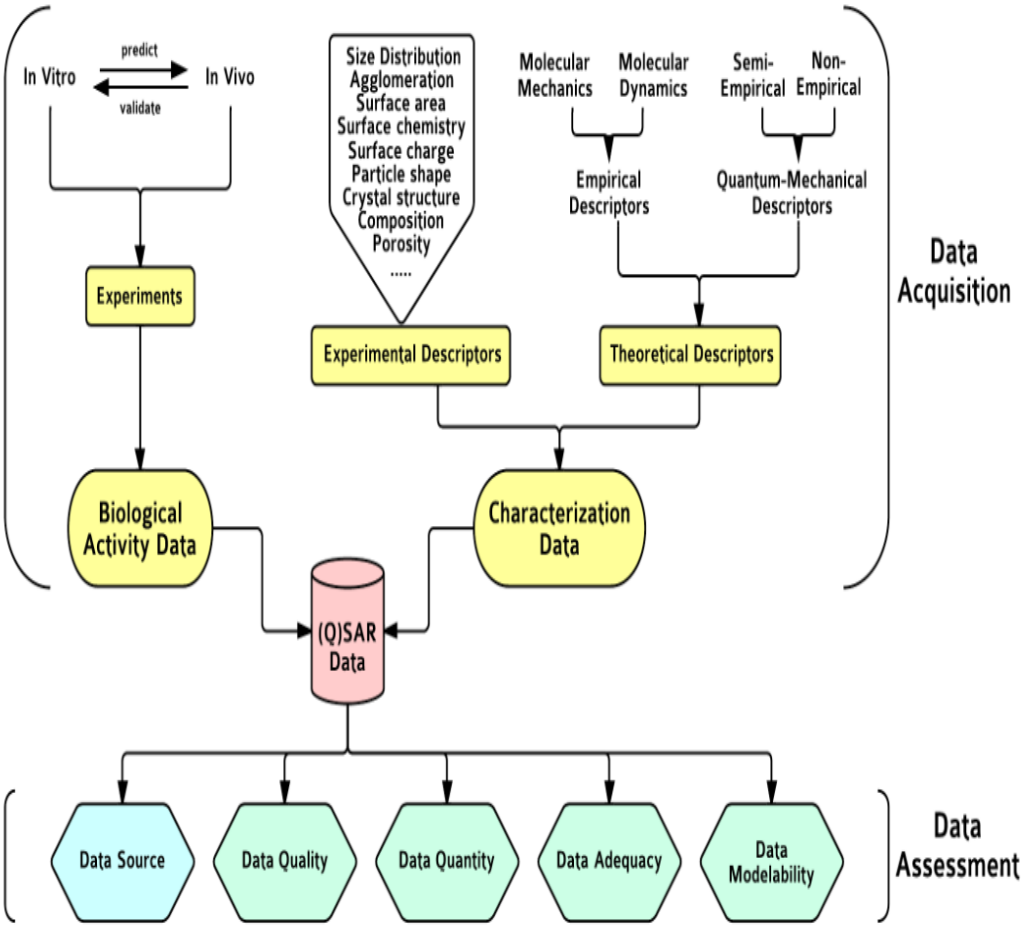

Figure 2.9 Data collection framework for (Q)SAR

One of the unique studies addressing the quality and suitability of the existing research data for nano-(Q)SAR purposes has been conducted by Lubinski *et al.* (2013). They presented a data evaluation framework that places a strong emphasis on the source, quality and quantity of the data, for assessing not only the quality of the data but also its suitability for modelling purposes. In the first part of their study, they provided a set of criteria that are mostly related to the source and quantity of the data, experimental procedures, and international standards followed during the characterisation process and documentation. In the second part, they assessed the quality of a collection of nanotoxicity data by scoring them according to the proposed criteria. The majority (201 out of 342 data points) of the dataset that was collected and scored was evaluated as useful with restrictions for developing (Q)SAR-like models.

In fact, there is now a great amount of data on nanotoxicity. However, the majority of the available data on ENM toxicity comes from studies focusing on a few ENMs, and hence is not useful for modelling purposes. At this point it should be noted that the data obtained by different research groups is often incomparable because of the differences in experimental procedures (e.g. sample preparation, dispersion protocols, assay types, cell types and exposure doses) and ENMs used (e.g. size, shape and surface modifications). Therefore, the data to be modelled should preferably come from the same study/project until standardised testing procedures and specific types of reference materials are available and accepted. Often, the physicochemical properties measured are not directly related to the toxicity of ENMs because characterisation was carried out in the absence of a test medium.

As noted previously, the majority of existing toxicological studies on ENMs are very limited in terms of sample size and the type of compounds involved. However, as listed in Table 2.6, there are some pioneering studies that provide useful data for nano-(Q)SAR modelling purposes. A critical review of the literature data that are particularly suitable for nano-(Q)SAR modelling has been presented in one of our previous papers (Oksel, Ma and Wang 2015), with the available data being provided as supplementary material of this paper. The main objective here was to develop an annotated bibliography of the primary sources of nano-(Q)SAR data. In the initial stages of this project, finding data sources for nano-(Q)SAR investigations was very challenging, due to lack of source of information about where to find systematically gathered data on the biological activity and structural properties of the diverse collection of ENMs. To address this gap, a list of publically available data on nanotoxicity that are particularly suitable for nano-(Q)SAR studies is provided in Table 2.6.

Table 2.6 List of literature data on nanotoxicity that are particularly suitable for nano-(Q)SAR studies

DATASET	NANOMATERIALS	TOXICITY ENDPOINT
Weissleder *et al.* (2005)	109 NMs with the same core but different surface modifiers	Cellular uptake
Durdagi *et al.* (2008)	48 different fullerene derivatives	Binding affinities (pEC50)
Shaw *et al.* (2008)	50 NMs with diverse core structures	ATP content, reducing equivalents, Apoptosis, mitochondrial membrane potential
Zhou *et al.* (2008)	80 surface-modified MWCNTs	Protein binding activities, cell viability, nitrogen oxide generation
Sayes and Ivanov (2010)	42 NMs with two cores (differing in physicochemical features)	Cellular membrane damage (LDH release)
Puzyn *et al.* (2011b)	17 metal oxide NMs	Cytotoxicity (EC50)
Liu *et al.* (2011)	9 metal oxide NMs	Cytotoxicity (PI uptake)
Zhang *et al.* (2012)	24 metal oxide NMs	MTS, ATP, LDH, Mito, Fluo4, JC1 and PI uptake

Wang *et al.* (2014)	18 NMs (carbon-based and metal oxides)	LDH release, Apoptosis, pro-inflammatory effects, haemolysis, MTT, DiOC6, cell
B. Yan (in press)	47 surface-modified gold NPs	Nonspecific protein binding and AChE inhibition
Oh and Park (2014)	12 gold NPs	Exocytosis

Despite all challenges and obstacles, there are now a number of ongoing studies and projects dedicated to improving our knowledge and understanding of ENM toxicity. Thus, one can expect a significant amount of data on nanotoxicology to become available soon. At this stage, there are two issues that need to be dealt with: the development of standardised data sharing formats and the development of property-based ENM toxicity libraries.

There are several reasons why data exchange standards and common terminology are needed in the nanotechnology community, including the diversity of: (1) ENMs (e.g. different cores and surface modifications); (2) test systems (e.g. cell lines, species, etc.); and (3) characterisation methods/conditions. Hence, predefined data formats are necessary to facilitate the storage, maintenance, and exchange of ENM data between different researchers. There are a large number of freely available toxicity databases, most of which are more general in scope and not customised for particular purposes. Commercially available ENM-specific databases are still at the research stage and limited to a few applications. ISA-TAB-NANO introduced by Thomas *et al.* (2013) is a standard NM data sharing format that facilitates the import/export of NM data and enables data exchange between different nanotechnology laboratories and researchers. The ISA-TAB-NANO specification uses four different spreadsheet-based file formats: investigation, study, assay, and material file format. The main features of each file format

are given in Table 2.8. Although the main aim of ISA-TAB-NANO is to facilitate the data exchange between different nanotechnology resources, this data logging system is also useful for accomplishing a broad range of goals, e.g. transparent sharing of NM data and recording of data in a (Q)SAR-ready format.

The OECD WPMN initially launched a database on Research into Safety of Manufactured Nanomaterials in 2009 (OECD 2009). However, it does not provide direct access to data because the overall outcomes and outputs section is usually filled in as “publications”. Furthermore, as interest weaned, the systematic updating has been discontinued and the database put on hold.

NANOhub is a database for managing information about ENMs. It currently hosts several projects, but the access to data is usually restricted to only project participants. The experience of collecting data in NANOhub has been captured in OECD harmonised templates (OHTs) to report regulatory studies for some of the physicochemical endpoints for nanomaterials. These additional templates will also be integrated in the International Uniform Chemical Information Database (IUCLID) under REACH for registration. Another data sharing portal that provides access to ENM characterisation and in vitro toxicity data is caNanoLab (Gaheen *et al.* 2013). The main aim of this data repository is to facilitate the sharing of knowledge on nanomedicine. Similarly, The Nanomaterial Registry (Ostraat *et al.* 2013) is a nanotechnology information resource that has been developed specifically to provide consistent information on the physicochemical characteristics and the environmental/biological effects of NMs.

An alternative approach for collecting nanotoxicity data is to use text mining techniques to develop a customised knowledge repository system. The Nano Health and Environmental Commented Database (NHECD) (Maimon and Browarnik 2010) is a text mining tool that allows automated extraction of information about the effects of ENMs on human health and the environment from scientific papers. However, the current performance of such NM databases using text mining algorithms is not very good because of the nonstandardised recording of ENM information and the difficulties in

extracting numerical data from plots (i.e. a large amount of published data in nanotoxicity is available only in the form of plots). At this stage, it is important to ensure that all data is recorded in a universally agreed format to facilitate the extraction of ENM information from the literature. The existence of specifications for ENM information sharing is also very important from the viewpoint of (Q)SAR modelling, because the establishment of predictive (Q)SAR models requires close collaboration between different disciplines and research groups. The development of an agreed ontology for ENMs and nanosafety research (i.e. a formal representation of nanostructures, biological properties, experimental model systems, conditions, and protocols) will facilitate not only the collection of nanotoxicity data, but also data mining and resource integration efforts.

2.6 Challenges, pitfalls and perspectives in Nano-(Q)SAR research

The nano-(Q)SAR modelling approach has great potential for providing an alternative, fast and cheap way of evaluating the risks of ENMs and predicting their toxicological behaviour in biological systems. However, the scarcity of the systematically gathered data on the biological activity and structural properties of the diverse collection of ENMs is one of the most important factors limiting the performance of (Q)SAR-like modelling methods, as the accuracy of the nano-(Q)SAR model outputs cannot exceed the quality of the data that are used to derive the model itself. According to an OECD guideline (OECD 2007a) on (Q)SAR, the basic criteria that must be fulfilled is the generation of robust and fully validated models, which will allow confidence in the toxicity predictions made. However, there are several barriers preventing the OECD validation criteria from being fulfilled. These barriers are summarised in the following sub-sections.

2.6.1 The need to improve quality of experimental data

Unlike traditional compounds, measuring the physicochemical properties of ENMs in biological medium is not straightforward with current

techniques and tools due to the complex and dynamic nature of bio-nano interactions. From a scientific perspective, ENMs cannot be considered as a homogeneous group, and subsequently this means that getting reliable data is not easy to achieve. Potentially, this leads to a situation in which experimental data gets reported without proper understanding of the associated errors and subsequently the propagation of such errors through the model. Another issue that makes the accurate measurement of physicochemical properties of NPs difficult is the high polydispersity of NPs. To increase the quality of experimental characterisation data, new analytical methods/instruments need to be developed that can deal with the polydispersity and heterogeneity of ENM samples

2.6.2 The need to express nanostructures in a simple but effective format

Another challenge that hinders the computation of classic theoretical descriptors for ENMs is that they are not pure compounds, rather populations of materials with distributions of structures, shapes, sizes, surface properties, and charges. A NP sample can have variations in the physicochemical properties, and hence, cannot simply be considered equivalent to a molecule. This makes the derivation of classic descriptors based on a symbolic molecular representation impossible. Therefore, it is of critical importance to (realistically) transform nanostructures into a language for computer representation that are sufficient to distinguish between different sizes, shape etc. forms of a same NP.

2.6.3 The need to have practical guidelines

The development of reliable and predictive nano-(Q)SAR models is not straightforward due to the lack of practical guidelines and standardised validation metrics for the construction and validation of the nano-(Q)SAR models. In addition to guidance on what data to measure, and how and where to measure the data, it is also important to continue the development of standardised data reporting formats in nanotoxicology to facilitate consistent reporting of the outcomes of nanotoxicity studies, which will greatly facilitate

data collection, database development, data mining, and resource integration efforts in the field of nanotoxicology.

2.6.4 The need to standardise and harmonise activities for the purpose of regulation

In order to implement nano-(Q)SAR, it is vital to demonstrate to regulators, and industry, that these models are scientifically valid and that clear explanations on how to use such models for making decisions are made (Mays, Benfenati and Pardoe 2012). Once this is achieved, our next step is to ‘‘harmonise activities’’, e.g. by forging internationally agreed document standards and guidelines. Guidelines of relevance should include the provision of detailed guidance in relation to the practicalities on the use of nano-(Q)SAR, e.g. detailing how to identify acceptability criteria, how to generate adequate and relevant descriptors (Patlewicz, Chen and Bellin 2011). There is a widespread regulatory and scientific interest in developing intelligent and cost-effective hazard screening tools. In particular, REACH is promoting the use of alternative toxicity assessment methods including (Q)SAR. These computational models offer the advantages of higher speed and lower costs, having been seen as ‘‘an enabler’’ in bringing new chemicals to commercialisation. The reliability of these models with regard to ENMs, however, is still an open question.

2.7 Concluding Remarks

(Q)SAR models have been successfully used by engineers, and physical and medical chemists to predict hazardous properties of molecules for over 50 years. Although the adaptation of the (Q)SAR approach to nanotoxicology has been encouraged by many investigators (Burello and Worth 2011a; Puzyn and Leszczynski 2012), there are still several barriers that need to be overcome to establish predictive, reliable, and legally acceptable nano-(Q)SAR models.

To sum up, a critical review of the literature on the application of computational approaches to better understand and predict ENMs’ toxicity has led to the following conclusions:

- One of the main issues that complicates the adaptation of computational toxicity approaches to nanotoxicology is the scarcity of consistent and high-quality experimental data. Moreover, finding the useful nanotoxicity data sources for computational studies is very challenging due to confidential issues, non-systematic reporting in nanotoxicology and lack of guidelines on where to find data. To address this limitation, the primary sources of nano-(Q)SAR data have been summarised in this chapter (e.g. Section 2.5) and a detailed description of the publically available nano-(Q)SAR datasets will be provided in Chapter 3.
- Although predictive modelling tools receive considerable attention in the field of nanosafety, it is also equally important to make use of exploratory data analysis methods (e.g. visualisation and clustering tools) to provide biological insights into diverse types of nanotoxicity data, to support effective interpretations of the results of more sophisticated statistical investigations, to group ENMs based on their hazard potential, and thus to provide practical solutions to the risk-assessment-related problems caused by the diversity of ENMs. To address these needs, the use of data visualisation and clustering tools will be introduced in Chapter 4. More specifically, Chapter 4 focuses on multi-dimensional data visualisation tools that are useful to represent complex nanotoxicity data in a visually appealing and easily understandable form, to group ENMs with similar biological activities together, and to identify highly concerned ENM classes.
- The ability to predict the toxicity of ENMs through computational approaches is of great help in the assessment and reduction of risks associated with ENMs. However, the most commonly used (Q)SAR modelling methods work best with large data sets that are currently very limited for ENMs. The nano-(Q)SAR tools available at present should be able to make use of limited data (e.g. no restriction on the ratio of compounds to descriptors), identify physicochemical descriptors that influence biological responses (e.g. rank descriptors based on their relative importance) and

produce interpretable outcomes. As the available nanotoxicity data is far from ideal for modelling purposes, the choice of nano-(Q)SAR tools used in this study (e.g. decision tree and partial least squares) was made by considering the nature of the existing data (e.g. limited datasets, collinear input data) and desired outcomes (e.g. easily-interpretable models). Chapter 5 describes the application of a genetic programming-based decision tree construction tool (GPTree) to nano-(Q)SAR modelling while Chapter 6 demonstrates the use of partial least squares regression in nanotoxicity modelling.

Chapter 3
Data Collection

As a part of this study, a large amount of nanotoxicity data have been accumulated from the available literature and completed/ongoing EU projects, and via private communication channels. Overall, 12 different sets of nanotoxicity data have been collected. Table 3.1 provides a summary of the data assembled for the analysis. A detailed description of the data collection methodology is presented in section 3.1 while 3.2 describes the datasets that have been used in this study.

3.1 Data collection methodology

Published literature from 2005 to 2015 was searched for studies on toxicity of ENMs using the Web of Science database. The following keywords have been used to identify the relevant studies: nanoparticle or nanomaterial toxicity, nanotoxicology, nano + ecotoxicology, nano + biological activity, nanoparticle or nanomaterial characterisation, structure-activity relationship analysis, (Q)SAR, nano-(Q)SAR. The bibliographies of the identified articles were searched for further relevant studies.

All data generated from MARINA project partners on toxicity and ENM characteristics relevant to toxicity have been collected by IOM and made available to project partners via the MARINA database. The further project search on CORDIS with the relevant keywords revealed a large number of EU-funded projects on nanosafety. The scientific findings from these projects were also inspected to find out whether they obtained data that may be useful for the development of nano-(Q)SAR models, which resulted in the collection of one additional dataset from the NANOMMUNE project, with the courtesy of Lang Tran and Peter Richie from IOM.

Table 3.1 Datasets collected

Ref	ENMs No.	Toxicity Endpoint	Characterisation
Wang *et al.* (2014)	18	LDH release, Apoptosis, pro-inflammatory effects, haemolysis, MTT, $DiOC_6$, cell morphology assay	size, surface area, morphology, metal content, reactivity, free radical generation and zeta potential
Shaw *et al.* (2008)	50	ATP content, reducing equivalents, Apoptosis, mitochondrial membrane potential	core composition, coating type, surface modification, size, relaxivities and zeta potential
Puzyn *et al.* (2011b)	17	Cytotoxicity (EC_{50})	12 different quantum-mechanical descriptors
Weissleder *et al.* (2005)	109	Cellular uptake	theoretical descriptors
Liu *et al.* (2011)	9	Cytotoxicity (PI uptake)	a set of 10 descriptors
Gajewicz *et al.* (2014)	18	Cell Viability	18 quantum-mechanical, 11 image-based, 3 experimental descriptors
Oh and Park (2014) and Bigdeli, Hormozi-Nezhad and Parastar (2015)	12	exocytosis in macrophages	10 combinatorial, 12 image-based, 6 experimental descriptors
Zhang *et al.* (2012)	24	Single- and multi-parameter toxicity assays	27 NP descriptors including element related energy, enthalpy, size and charge.
Sung IK (private communication)	14	Cell Viability	Size, Zeta potential, XRF, TGA loss
Zhou *et al.* (2008) and B.Yan (private communication)	83	Protein binding activities, cell viability, nitrogen oxide generation	theoretical descriptors
Marina Project	9	In vitro assays	experimental descriptors
Nanommune Project	18	In vitro assays	core, coating, 2 sizes and zeta potential

3.2 Description of datasets collected

One of the most comprehensive nanotoxicology studies ever performed was carried out by Weissleder *et al.* (2005). They tested the cellular uptake of 109 NPs with the same core (cross-linked iron oxide) but different surface modifiers in five cell types ($PaCa_2$, HUVEC, U937, GMCSF and RestMph). Of the five cell lines, only $PaCa_2$ (human pancreatic cancer cell line) and HUVEC (human umbilical vein endothelial cells) showed surface chemistry sensitive responses. The raw data generated by Weissleder *et al.* (2005) have been examined below in the context of their ability to be used for developing nano-(Q)SARs:

- **Material group:** The data are associated with (magnetic) iron oxide NMs.
- **Homogeneity:** The data are homogeneous as they contain no other than super paramagnetic iron oxide core NPs.
- **Sample size:** The dataset is large and contains more than a hundred NPs, which are decorated with different small molecules. The dataset is large enough (in terms of the number of compounds being included) to develop and validate computational models.
- **Toxicity endpoints:** Cellular uptake of NPs in five different cell types
- **Characterisation:** Although the authors stated that all materials were characterised by size measurements, relaxometry, amine content and mass spectrometry, the characterisation data were not presented in the paper or supplementary document. The main reason why this dataset is useful for (Q)SAR analysis, despite the limited information on the physicochemical characteristics of NPs, is that it enables the computation of the theoretical descriptors based on the chemistry of the surface modifying molecules, as all of the screened NPs have the same pre-dominant core. Two different descriptor datasets were separately used as input data in modelling part of this study. Firstly, a total of 690 1D and 2D descriptors was calculated using DRAGON 6 software (Mauri *et al.* 2006). After removing those descriptors with little variation across the nanoparticles, 389 chemical descriptors were retained.

Secondly, a pool of 147 chemically interpretable descriptors was obtained from David Winkler via private communication (Epa *et al.* 2012).

In another study, Shaw *et al.* (2008) determined the biological activity of 50 different NPs with diverse metal cores under 64 different sets of conditions (four doses × four cell types × four assays). They performed four replicates for each toxicity measurement and expressed the results in terms of standard deviations (Z scores). The raw data collected by Shaw *et al.* (2008) are examined below in the context of their ability to be used for developing nano-(Q)SARs:

- **Material group:** The data are associated with metal core NPs, especially iron oxide based NPs (FexOy core).
- **Homogeneity:** The data are reasonably homogeneous as the great majority of NPs included contain the iron oxide core.
- **Sample size:** The dataset is large in terms of the number of compounds (50) and toxicity endpoints screened.
- **Toxicity endpoints:** Biological activity of NPs assessed by a profile of 64 features
- **Descriptors:** The authors reported seven different qualitative and quantitative descriptors for most of the screened NPs: core composition, coating type, surface modification, size, relaxivities (R1 and R2) and zeta potential. Although the number of measured (physicochemical) properties is limited, it is still possible to gain some useful information about what factors are likely to govern the toxicity of the ENMs.

In 2008, Zhou *et al.* (2008) created a library containing 83 multi-walled nanotubes (MWNTs) with known biological activities. They tested the toxicity of these decorated nanotubes using six different toxicity endpoints (four protein binding activities, cell viability and nitrogen oxide generation). The raw data generated by Zhou *et al.* (2008) and collected from Bing Yan via private

communication are assessed below to determine their suitability for developing nano-(Q)SAR models:

- **Material group:** The data are associated with multi-walled carbon nanotubes.
- **Homogeneity:** The data are very homogeneous as the designed library contains 80 surface-modified multi-walled carbon nanotubes.
- **Sample size:** The dataset obtained is large in terms of the number of compounds (80) and biological endpoints tested.
- **Toxicity endpoints:** Protein binding activities, cell viability and nitrogen oxide generation
- **Descriptors:** The dataset allows the computation of the theoretical descriptors based on surface-modifying organic molecules. A total of 623 1D and 2D descriptors was calculated using DRAGON 6 software (Mauri *et al.* 2006). After removing those descriptors with little variation across the nanoparticles, 412 chemical descriptors were retained.

The dataset used by Puzyn *et al.* (2011b) includes the in vitro toxicities of 17 different metal oxide NPs against the bacterial species Escherichia coli. The authors gathered the toxicity data for 10 different metal oxide NPs in their laboratory and combined them with the toxicity data taken from their previous study (Hu *et al.* 2009). The raw data collected by Puzyn *et al.* (2011b) are examined below in the context of their ability to be used for developing nano-(Q)SARs:

- **Material group:** The data are associated with metal oxide NPs.
- **Homogeneity:** The data are homogenous and include a panel of 17 metal oxide NPs that are widely used in industrial applications.
- **Sample size:** The sample size of data is not huge but large enough to investigate the relationship between the structure of a set of NMs and their in vitro cytotoxicity.
- **Toxicity endpoints:** Cytotoxicity in bacteria
- **Descriptors:** The authors calculated a pool of 12 different quantum-mechanical descriptors based on the electronic (structural) properties of 17 metal oxide NPs.

In 2011, Liu *et al.* (2011) measured the in vitro toxicity of nine different metal oxide NPs: Al_2O_3, CeO_2, Co_3O_4, TiO_2, ZnO, CuO, SiO_2, Fe_3O_4 and WO_3. Of these nine NPs, only three of them (ZnO, CuO and SiO_2) were identified as being toxic according to the results of the plasma membrane integrity assay. The raw data generated are assessed below in the context of their ability to be used for developing nano-(Q)SARs:

- **Material group:** The data are associated with metal oxide NPs.
- **Homogeneity:** The data are homogenous.
- **Sample size:** The sample size of the data is small as it only covers nine different compounds.
- **Toxicity endpoints:** Cytotoxicity assessed by measuring plasma-membrane leakage via Propidium Iodide (PI) uptake (i.e. an indicator of plasma membrane damage)
- **Descriptors:** The authors provided a set of simple constitutional descriptors (e.g. number of metal and oxygen atoms, atomic mass of the nanoparticle metal, molecular weight of the metal oxide, group and period of the NP metal, atomisation energy) and a few experimental descriptors (e.g. NP primary size, zeta potential, isoelectric point and different concentration measures) which can be used as an input variables in nano-(Q)SAR analysis. These characterisation data, although far from ideal and complete, can help to develop classification-based (Q)SAR models.

In another nanotoxicity-related study, Zhang *et al.* (2012) assessed the toxicity of 24 different metal oxide NPs in a set of single-parameter (i.e. MTS, ATP and LDH) and multi-parameter (Fluo-4, JC1, PI, MitoSox and DCF) toxicity assays. The TEM images of NPs are given in Fig. 3.1. The raw data generated by Zhang *et al.* (2012) are evaluated below in the context of their ability to be used for developing nano- (Q)SARs:

- **Material group:** The data are associated with metal oxide NPs.
- **Homogeneity:** The data are homogenous and contain metal oxide NPs only.

- **Sample size:** The sample size of the collected data is sufficiently large in terms of the number of ENMs and toxicity endpoints studied.
- **Toxicity endpoints:** Cellular viability (assessed by single-parameter assays), oxidative stress (assessed by multi-parameter assays) and acute toxicological responses
- **Descriptors:** The characterisation part of this study is relatively detailed as the authors performed the following physicochemical characterisation studies:
    - Measurement of the primary size and shape of NPs by TEM,
    - Measurement of hydrodynamic sizes by dynamic light scattering (DLS),
    - Measurement of band gap energies by ultraviolet–visible (UV-Vis) spectroscopy,
    - Measurement of metal dissolution by inductively coupled plasma-mass spectrometry,
    - Measurement of zeta-potential and point of zero zeta-potential by zeta analyser,
    - Computation of conduction and valence band energies.
    - Additionally, in a follow-up study, Liu *et al.* (2013c) determined a set of 30 descriptors capturing the physicochemical properties of NPs.

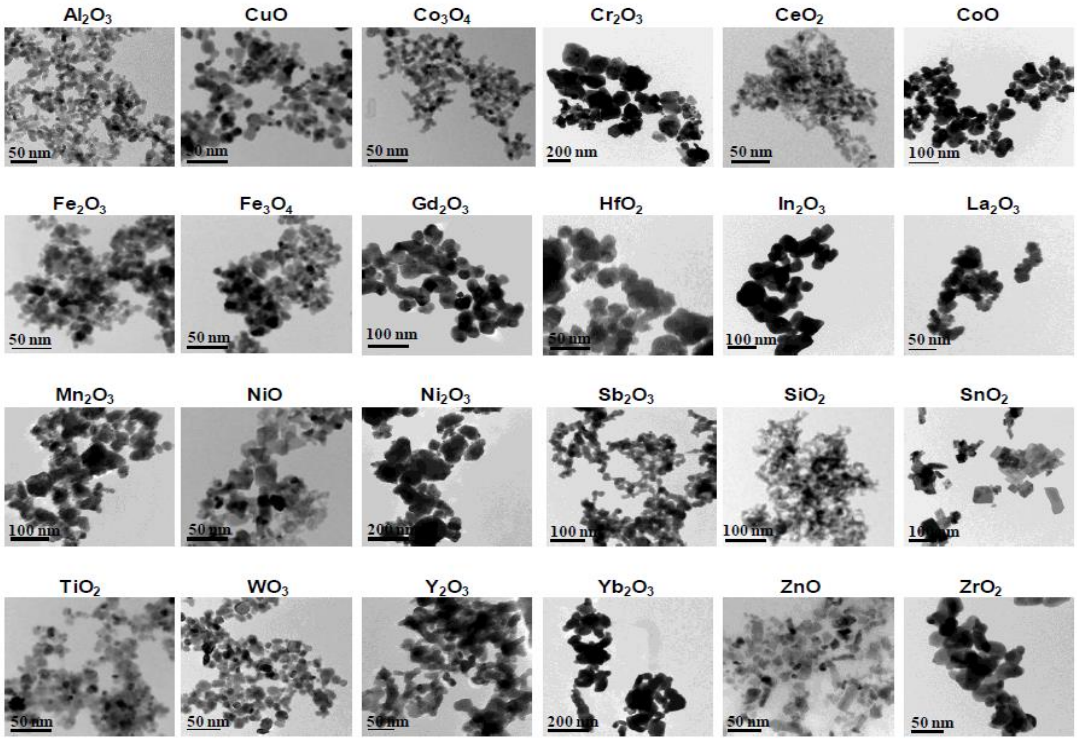

Figure 3.1 TEM images of 24 NPs investigated by Zhang *et al.* (2012)

The research conducted by Wang *et al.* (2014) has been revealed to be one of the most useful datasets for nano-(Q)SAR modelling. The authors selected a panel of 18 ENMs with varying structures and conducted a set of in vitro cytotoxicity assays, including LDH release, Apoptosis, Necrosis, viability, MTT and haemolytic effects. The SEM and TEM images of NPs investigated in this study are given in Fig. 3.2. The raw data generated by Wang *et al.* (2014) are examined below in the context of their ability to be used for developing nano- (Q)SARs:

- **Material group:** The data are mostly associated with metal (oxide) NPs, as the majority (i.e. 11 out of 17) of the compounds screened are metal-based NPs.
- **Homogeneity:** The dataset can be considered as slightly heterogeneous, as it contains different types of ENMs (e.g. metal oxide NPs and carbon-based NMs).
- **Sample size:** The dataset is limited in terms of the number of compounds included (i.e. 18 ENMs), but it is still useful to test the hypothesis that ENM toxicity is a function of some structural or compositional features.

- **Toxicity endpoints:** Acute in vitro toxicity
- **Descriptors:** The particle characterisation section of this study includes the measurement of several physicochemical properties (e.g. particle size and size distribution, surface area, morphology, metal content, reactivity and free radical generation). This is one of the most comprehensive characterisation dataset available in literature.

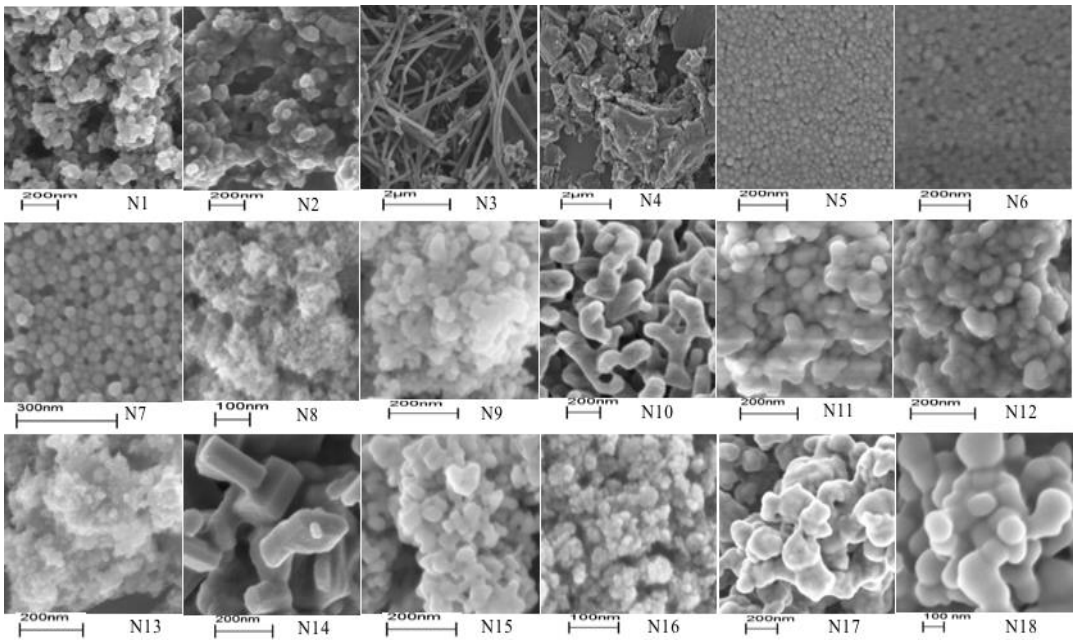

Figure 3.2 SEM and TEM images of the 18 NPs

Oh and Park (2014) examined the role of surface properties in the exocytosis of gold NPs (GNPs) in macrophages. They reported the exocytosis rates of 12 GNPs expressed as the % of GNPs leaving the macrophage, and a set of 6 experimental descriptors including zeta potential, hydrodynamic diameter, and maximum wavelength both prior to and after protein coating (Oh and Park 2014). The TEM images of GNPs with different sizes and coatings are given in Fig. 3.3. The raw data generated by Oh and Park (2014) are examined below in the context of their ability to be used for developing nano-(Q)SARs:

- **Material group:** The data are associated with GNPs.
- **Homogeneity:** The data are homogenous and contain GNPs only.

- **Sample size:** The sample size of the collected data is small in terms of the number of GNPs (i.e. 12) studied.
- **Toxicity endpoints:** Exocytosis rates of GNPs in macrophages.
- **Descriptors:** The characterisation part of this study includes 6 experimental measurements such as zeta potential, hydrodynamic diameter, and maximum wavelength both prior to and after protein coating. Additionally, Bigdeli, Hormozi-Nezhad and Parastar (2015) extracted 12 nano-descriptors (e.g. size, surface area, aspect ratio, corner count, curvature, aggregation state, and shape) from TEM images of GNPs and calculated 10 descriptors such as charge densities, adjusted aspect ratio, charge accumulation values, spectral size, spectral surface area, spectral aspect ratio and spectral aggregation by combining TEM extracted image descriptors with experimental parameters.

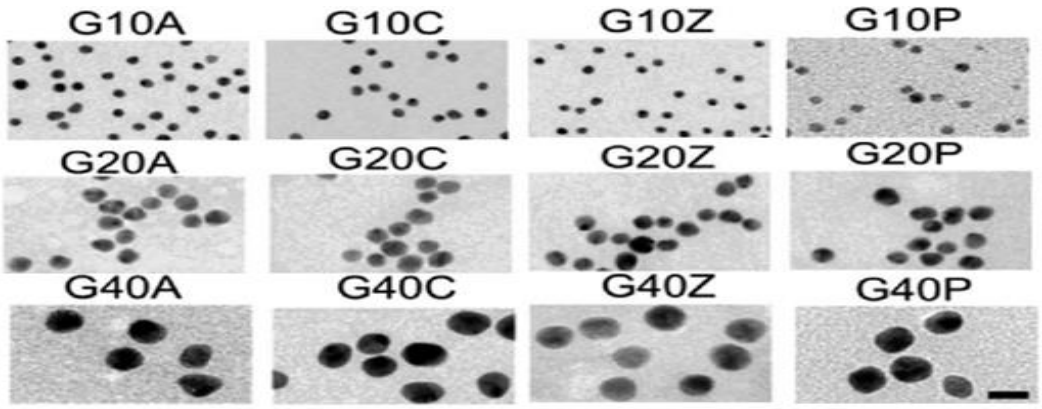

Figure 3.3 TEM images of GNPs

In another study, Gajewicz *et al.* (2014) measured the cytotoxicity of 18 metal oxide NPs to human keratinocyte cell line using the CytoTox-Glo cytotoxicity assay and calculated LC50 values for all NPs. TEM images of the18 metal oxide NPS are shown in Fig. 3.4. The dataset also includes 29 descriptors (quantum-mechanical, image-based and experimentally measured descriptors) representing the structural features of 18 metal oxide NPs. The raw data generated by Gajewicz *et al.* (2014) are

examined below in the context of their ability to be used for developing nano- (Q)SARs:

- **Material group:** The data are associated with metal oxide NPs.
- **Homogeneity:** The data are homogenous and contain metal oxide NPs only.
- **Sample size:** The sample size of the collected data is not large in terms of the number of NPs studied but sufficiently large in terms of the number of descriptors calculated/measured.
- **Toxicity endpoints:** Cell viability
- **Descriptors:** The characterisation part of this study includes 16 quantum-mechanical descriptors, 11 image-based descriptors and 2 experimental measurements

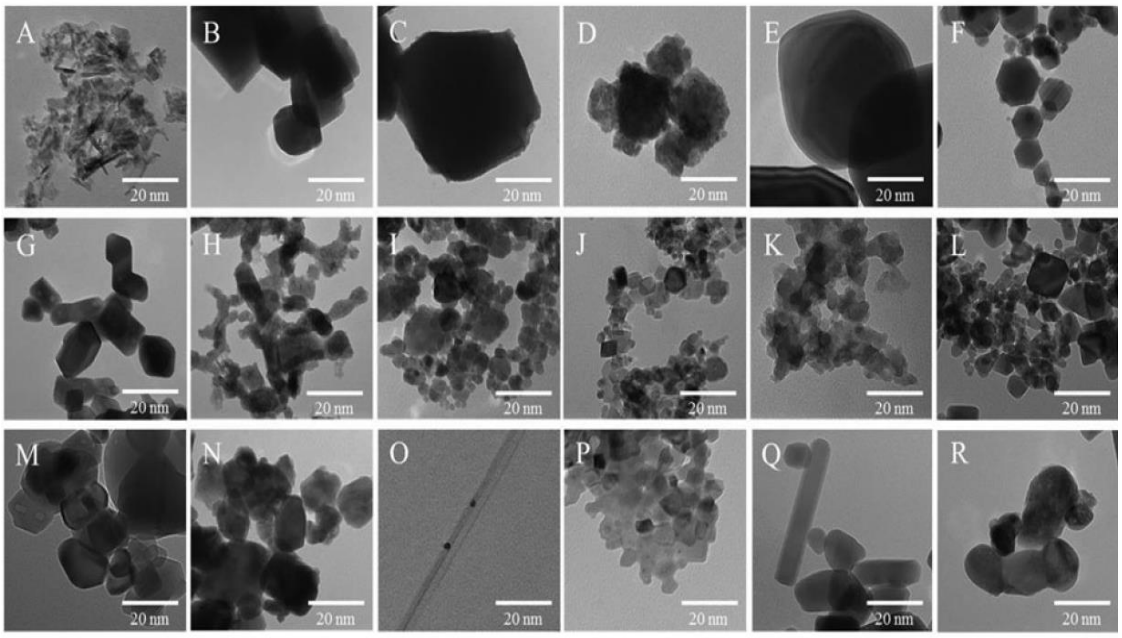

Figure 3.4 TEM images of the NPs analysed in this study

The next dataset obtained from Sung IK via private communication. It includes 14 TiO_2 NPs with varying properties (e.g. size, shape, purity, charge etc.). This dataset includes the cell viability of BEAS-2B cells after exposure to the 14 TiO_2-based NPs and six different physicochemical properties (e.g. size measurements, shape, metal content, zeta potential and TGA loss) of

these NPs. The raw data obtained from Sung IK (private communication) are examined below in the context of their ability to be used for developing nano-(Q)SARs:

- **Material group:** The data are associated with TiO_2 NPs.
- **Homogeneity:** The data are homogenous and contain TiO_2 NP_s only.
- **Sample size:** The sample size of the collected data is small in terms of the number of NPs studied and the number of descriptors measured.
- **Toxicity endpoints:** Cell viability
- **Descriptors:** The characterisation part of this study includes 5 quantitative (e.g. TEM size, DLS size, metal content, zeta potential and TGA loss) and 1 qualitative (e.g. shape) measurements.

NANOMMUNE was a 3-year EU-funded project launched on September 1st, 2008. The NANOMMUNE dataset is collected form IOM via private communication. It includes a number of in-vitro toxicity assay results such as ROS generation (available for 5 NMs), cell viability (available for 7 NMs) and cytokine release (available for 7 NMs). However, only Apoptosis assay results measured at four different doses are available for a relatively large number of NMs (i.e. 18 NMs). TEM images of the 18 NMs included and tested in this project are given in Fig. 3.5 while the raw data are examined below in the context of their ability to be used for developing nano- (Q)SARs:

- **Material group:** The data are associated with four different metal oxide core NMs (iron oxide, titanium dioxide, zinc oxide and cerium oxide) with different sizes, shapes and coatings.
- **Homogeneity:** The dataset is homogeneous as it contains metal oxides only
- **Sample size:** The dataset is limited in terms of the number of compounds (i.e. 18) and descriptors included.
- **Toxicity endpoints:** Apoptotic cell death

- **Descriptors:** The characterisation part of this study includes NM core type, coating type, particle size (TEM and DLS) and zeta potential measurements

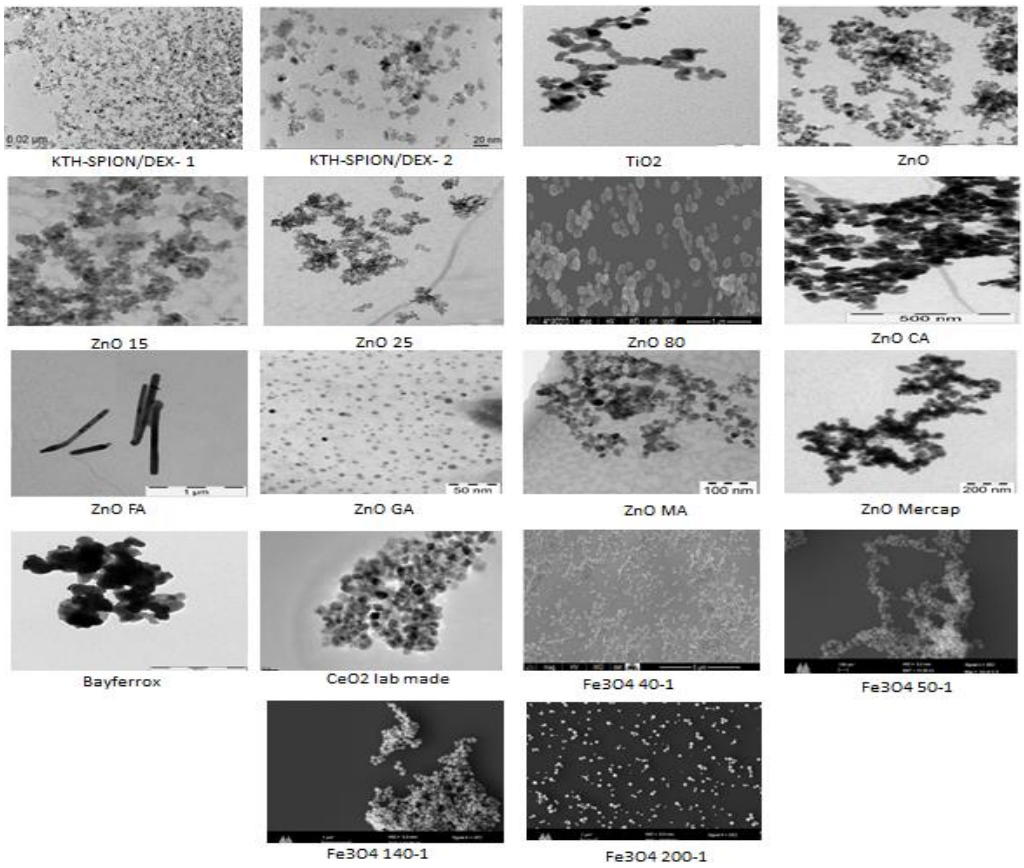

Figure 3.5 TEM/SEM images of the NPs analysed in the NANOMMUNE project

MARINA was another EU FP7 project dedicated to establish the risk management methods for ENMs. A panel of 9 NPs were tested in this project in terms of their toxicological properties. TEM images of the NPs screened are shown in Fig. 3.6, while the raw data are examined below in terms of its suitability for modelling studies:

- **Material group:** The data are associated with metal oxides and carbon nanotubes.
- **Homogeneity:** The dataset can be considered to be heterogeneous as it contains metal oxide- and carbon-based NMs

- **Sample size:** The sample size of the collected data is very small in terms of the number of NPs; limited in terms of the number of descriptors but very large in terms of the toxicity endpoints studied.
- **Toxicity endpoints:** Several in vitro toxicity assays (e.g. LDH release, ELISA, Neutral red assay and Resazurin) performed in different cell lines (e.g. HDMD, RAW 264.7, MHS, Calu-3 human epithelial cells) and ecotoxicity assays.
- **Descriptors:** Characterisation file consists of data from a number of different sources that have been working on the bank of NMs for MARINA. Some of the data has been generated in the MARINA project, other data have been harvested from other projects or sources (e.g. ENPRA, JRC-nanohub). Although the length of characterisation table is very long as it includes several attributes such as particle size, elemental composition, surface characteristics, dissolution, reactivity and so on, there are several gaps (i.e. missing values) in the table. In other words, only a few measurements (e.g. particle size and surface area) are available for all 9 NPs.

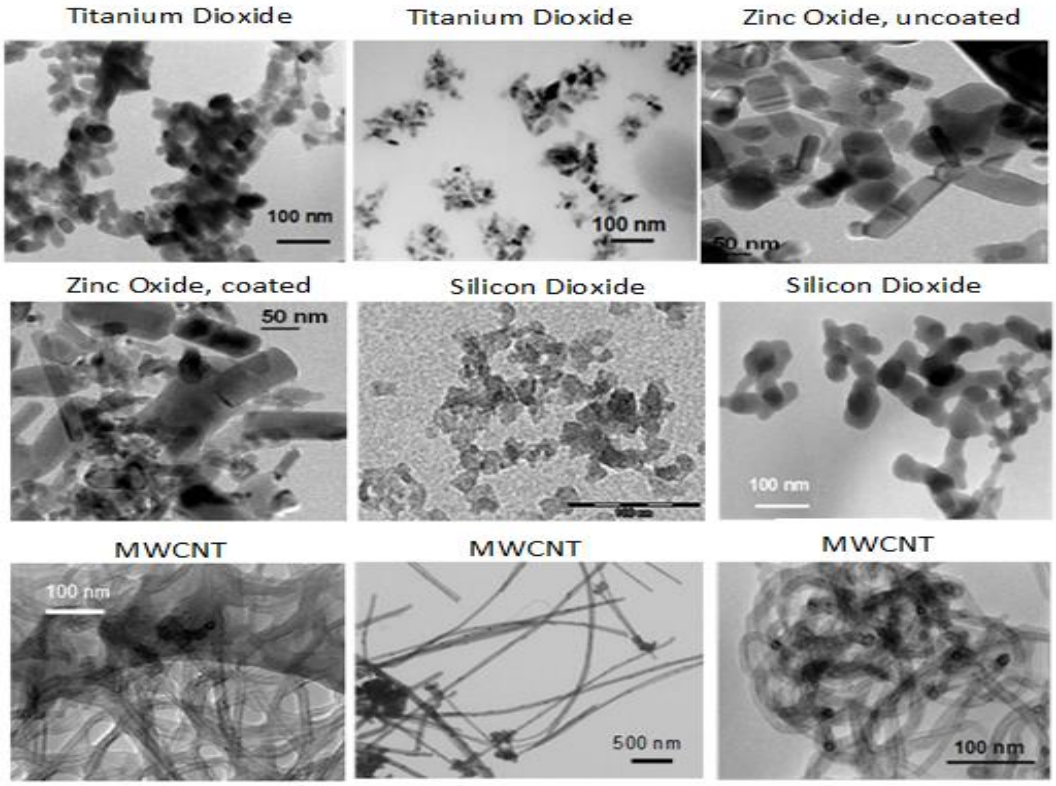

Figure 3.6 TEM images of the 9 NPs analysed in the MARINA project

3.3 Concluding Remarks

The nano-(Q)SAR modelling approach has great potential for providing an alternative, fast and cheap way of evaluating the risks of ENMs and predicting their toxicological behaviour in biological systems. However, the main factor currently limiting the performance of the predictive nanotoxicity models is the data reliability referring to the accuracy and completeness of existing nanotoxicology studies. Combining the existing datasets in order to create more comprehensive datasets required by the in silico approaches might be the solution that first comes to mind, but in many cases this is not practical due to the differences in toxicity assays, cell lines, experimental conditions, exposure times/doses and metrics used to measure toxicity in different studies. Since (Q)SAR is a data-driven method, the presence of systematically gathered data on the biological activity and structural properties of the diverse collection of ENMs is one of the most important prerequisites for reliable model building. To address this limitation and expand the potential for the application of computational methods in nanotoxicity modelling, a set of 12 datasets on ENM toxicity and characterisation were collected and are presented in this chapter. Although the nanotoxicity datasets collected are the largest ones among related works, some of them are not very suitable for the development of predictive models. Therefore, exploratory data analysis (Chapter 4) is employed to better understand the data gathered and to select the most suitable datasets for the development of robust and interpretable nano-(Q)SAR models.

Chapter 4

Exploratory Visualisation of Multivariate Data in Nanotoxicity

Multi-dimensional data visualisation is an approach that allows visual exploration of high dimensional data sets in a lower-dimensional display. It significantly contributes to better understanding of the more complex statistical procedures and resulting models in relation to the dataset. Therefore, before moving onto more sophisticated data modelling procedures, multi-dimensional visualisation tools were employed for data exploration and turning raw data into meaningful information to support predictive model development. More specifically, two different multi-dimensional data visualisation tools, heat maps combined with hierarchical clustering and parallel co-ordinate plots, were employed to visualise large-scale nanotoxicity data, to rank and prioritise ENMs by toxicity level and to reveal the relationship between descriptors and biological activity. This chapter presents a series of case studies and reports the results of multi-dimensional data visualisation tools to visually explore the nature of the data gathered.

4.1 Introduction

Multi-dimensional data visualisation has many important applications and, in particular, can be considered as an important tool to summarise and visually explore the important characteristics of the dataset being analysed. The result of effective data exploration in nanotoxicology will mean the ability to better understand the nature of data gathered, to help prioritise ENMs for screening, to group ENMs based on their hazard potential, and thus to provide practical solutions to the risk-assessment-related problems caused by the diversity of ENMs.

Our focus in this chapter is on using two common data visualisation tools including parallel co-ordinate plots and cluster heat maps to gain insight into datasets collected and presented in the previous chapter. The focus here is to identify high priority ENMs that are of high concern to human health and the environment, to identify those physicochemical properties that potentially contribute to the toxicity of ENMs, and to select the most suitable datasets

that will be employed for the development of accurate and interpretable nano-(Q)SAR models.

4.2 Methodology

Each data set is analysed separately, using multi-dimensional data visualisation techniques that are particularly useful for graphical displays (Fig. 4.1). As a first step, multi-dimensional toxicity data were scaled to have a mean of 0 and standard deviation of 1. After this data normalisation step, pre-scaled toxicity data were projected onto a heat map, which was then combined with a clustering algorithm to place the NPs into groups based on their toxicity potential. Heat map graphical displays were used here as exploratory visuals to compare toxicity of NPs measured using different assays under different experimental conditions while agglomerative hierarchical clustering was employed to group NPs according to their toxicity level and parallel co-ordinate plots were employed for rapid assessment of correlations between descriptors and toxicity endpoints. In clustering analysis, Euclidean distance was used to obtain distance matrix while the hierarchical cluster algorithm was used for clustering. Parallel co-ordinate plots were used to support correlation analysis. In a parallel co-ordinate plot, crossing segments indicate a negative (inverse) correlation while parallel segments (e.g. not intersecting) lead to highly positive correlations.

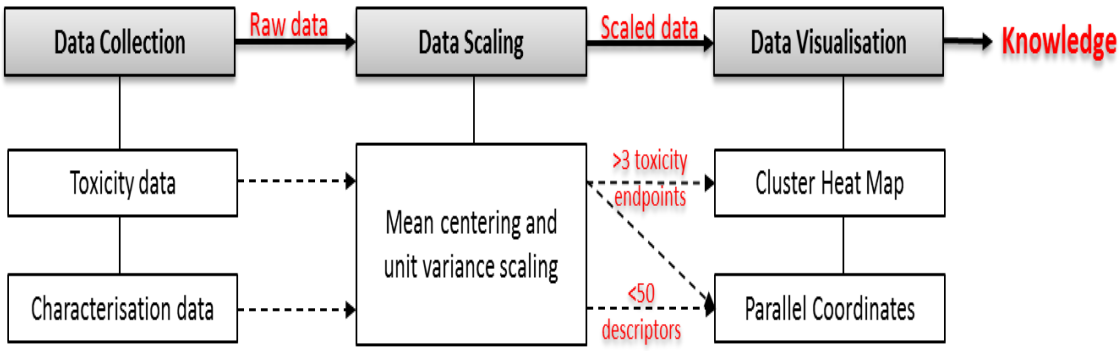

Figure 4.1 Visual data exploration steps

4.3 Results of visual exploratory data analysis

4.3.1 Case Study I - Wang datasets

In the first case study, data resulting from a series of toxicity tests and characterisation methods (Wang *et al.* 2014) were used. Initially, viability and MTT assay results showing the percentage of viable cells were subtracted from 100, in order to reflect the percentage of the dead cells (i.e. low values are correlated with low toxicity and high with high toxicity). Then, toxicity data was normalised according to the control values, negative and positive control:

$$\text{toxicity}\ (\%) = \frac{\text{experimental value} - \text{negative control}}{\text{positive control} - \text{negative control}} \text{x}100$$

Toxicity relative to untreated control was used where the positive control was not available. In the next step, the data were scaled by subtracting the mean value of each variable from the data and multiplying the resulting values by the inverse of standard deviation. This scaling step was performed in order to bring all of the variables into proportion with one another. Then, a heat map of toxicity data combined with hierarchical clustering was constructed using the R software package (Team 2014).

Fig. 4.2 displays the clustering result in a heat map as a row dendrogram. Hierarchical clustering does not require a pre-defined number of classes but it allows one to cut the hierarchy at some points (e.g. pre-specified value of similarity or dissimilarity). One possible cut of the dendrogram as shown in Fig. 4.2 (pink dashed line) resulted in the formation of 4 clusters (i.e. Aminated PLB, Zinc oxide, Nanotubes and others). Examination of clustering results with heat map representation of toxicity values revealed that three particular NPs (i.e. (Aminated PLB, Zinc oxide and Nanotubes) were distinguished from the rest due to their relatively high toxicity potential. The remaining NPs did not exhibit high levels of toxicity. Interestingly, amine-modified polystyrene NPs showed significantly higher toxicity than carboxyl-modified and un-modified polystyrene NPs. Among metal oxide NPs tested, Zinc oxide was the most toxic NPs and Nickel oxide exhibited a modest increase in cytotoxicity, while the remaining metal oxides (i.e. alumina, titanium dioxide, silicon oxide and cerium oxide) did not show any toxic responses. Between the three

carbon-based ENPs screened (i.e. Nanotubes, Fullerene and Carbon black), only Japanese nanotubes showed high toxicity. Additionally, the toxicity of Alumina NPs showed a modest increase with decreasing particle size (e.g. $Alumina_{7nm}$> $Alumina_{50nm}$ >$Alumina_{300nm}$). Another interesting finding was that the aminated sample showed a high level of apoptotic cell death at the lowest dose (e.g. APO.1) while a significant reduction in Apoptosis was observed at higher doses. The reverse of this trend was observed for zinc oxide NPs. This finding confirms that toxicity is highly dose-dependent and hence, toxicological effects should be evaluated at multiple concentrations to reveal differences in toxicity that might otherwise lead to wrong conclusions.

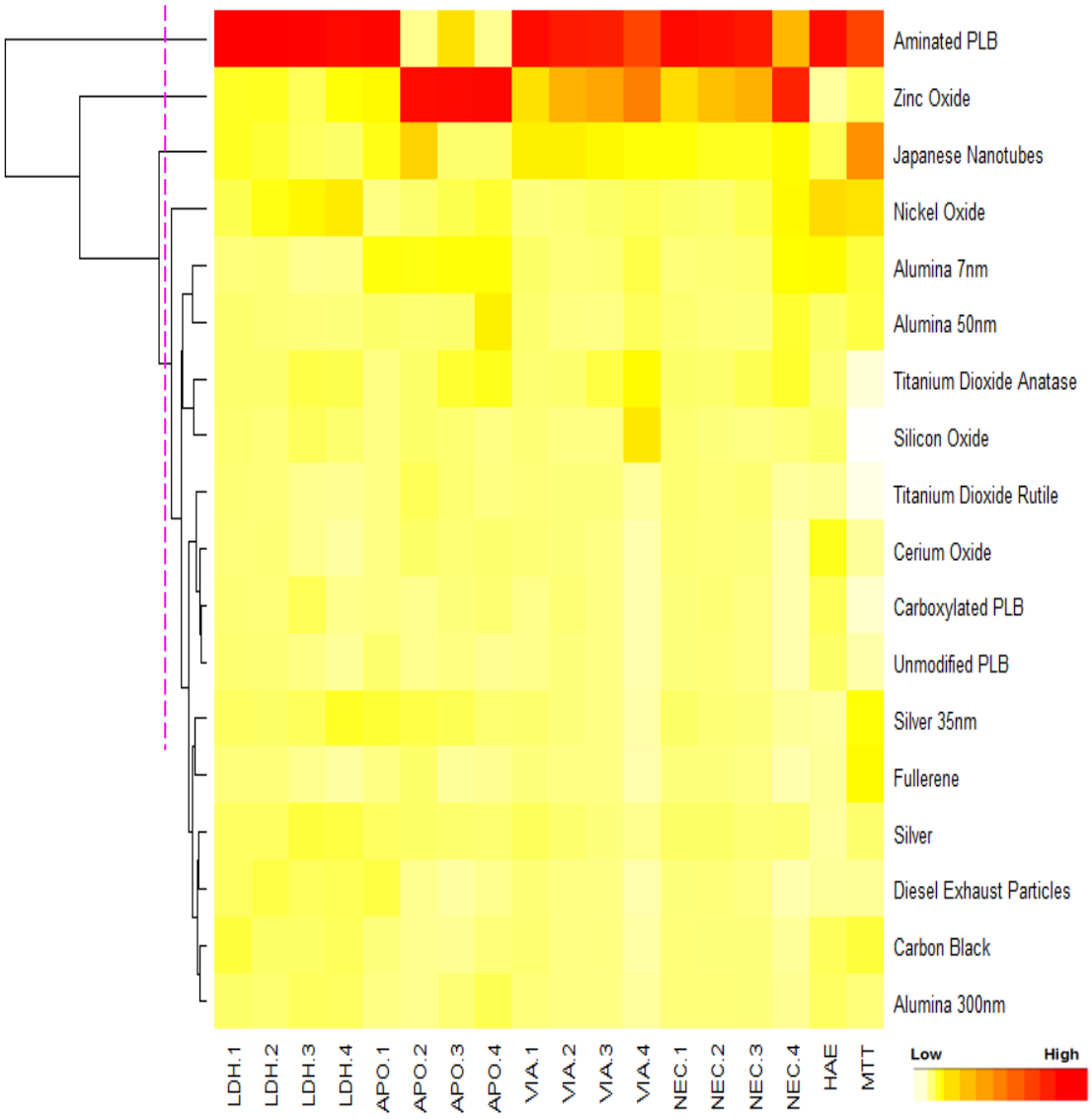

Figure 4.2 A cluster heat map displaying auto-scaled toxicity values of 18 NPs

(Toxicity endpoints included: LDH releases, Apoptosis (APO), Cell Viability (VIA), Necrosis (NEC), Haemolysis (HAE) and MTT test)

A correlation matrix heat map with all the pairwise correlations between in vitro toxicity tests is given in Fig. 4.3. It was created using only toxicity data and re-ordered according to the Pearson Correlation coefficients using a hierarchical clustering order. Clearly, Apoptosis and Necrosis results showed very high correlation while their pairwise correlation with LDH release assays was very low. The lowest correlation values were obtained between toxicity assays conducted at different doses (e.g. dose 1 vs dose 4) which confirmed the dose dependency of toxicity.

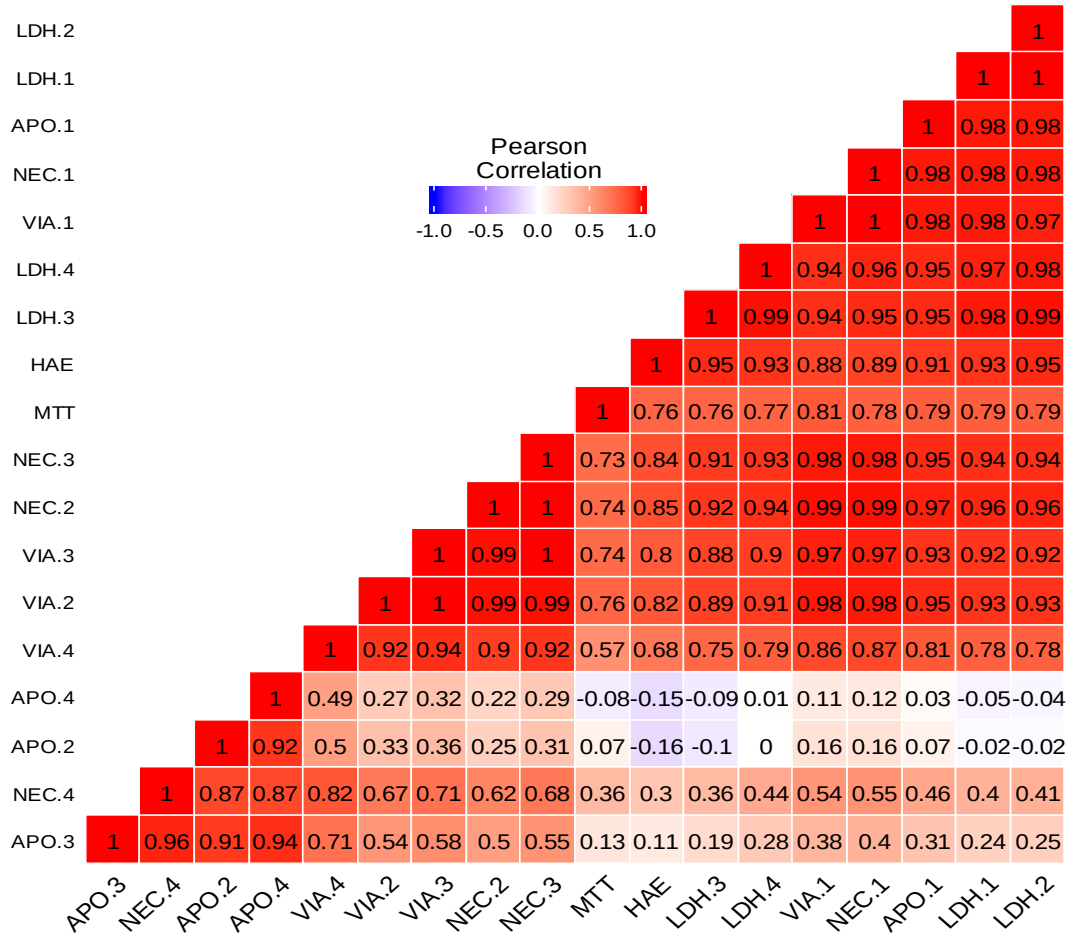

Figure 4.3 A heat map displaying the pairwise correlations between toxicity assays

(Toxicity endpoints included: LDH releases, Apoptosis (APO), Cell Viability (VIA), Necrosis (NEC), Haemolysis (HAE) and MTT test)

As a second step, C-Visual Explorer (CVE) was used as a tool to create parallel co-ordinate plots of the multivariate data. The results of multi-dimensional visualisation using parallel co-ordinates on cytotoxicity data and characterisation data are displayed in Figs. 4.4 and 4.5, respectively. The results associated with three particular NPs (e.g. Aminated PLB (N6), Zinc oxide (N14) and Japanese Nanotubes (N3)), that were shown to have mid-high toxicity in at least one of the toxicity assays via heat map visualisation, were highlighted in yellow, blue and green, respectively.

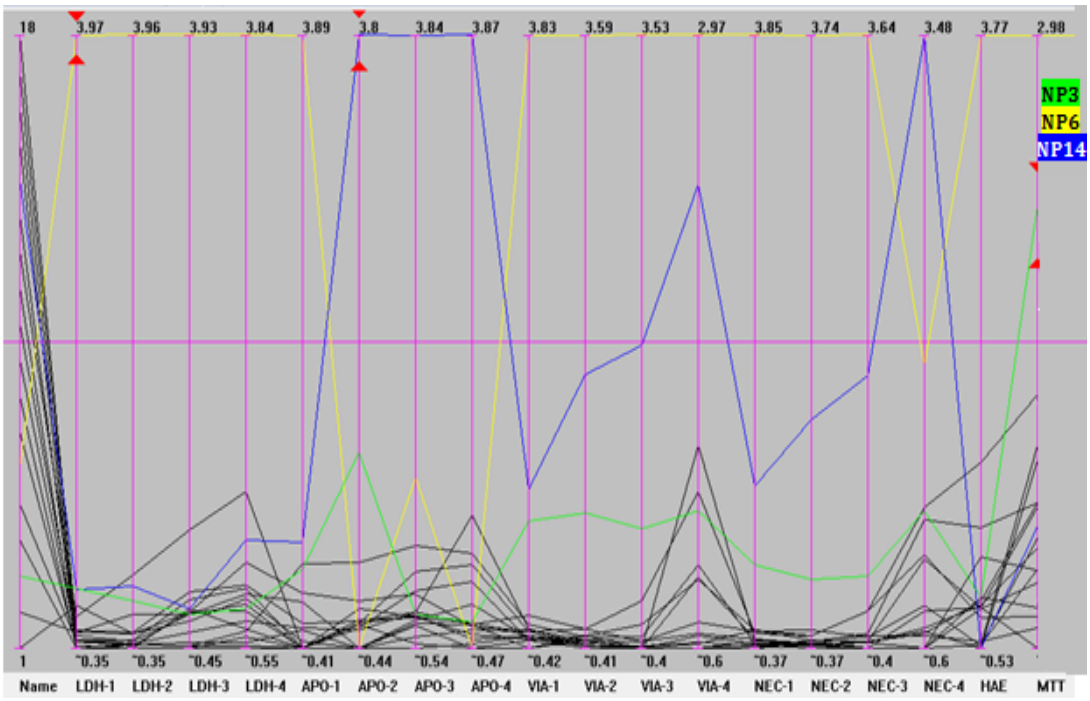

Figure 4.4 A parallel co-ordinate plot of the toxicity data

(Toxicity endpoints included: LDH releases, Apoptosis (APO), Cell Viability (VIA), Necrosis (NEC), Haemolysis (HAE) and MTT test)

As mentioned earlier, Fig.4.4 shows the parallel co-ordinate plot of the toxicity data. If once considers the dense area as the lower toxicity envelope, then any deviation from this area may be considered in the realm of higher toxicity. Similar to heat map visualisation results, the parallel co-ordinate plot shows that the aminated PLB (N6 in yellow) and zinc oxide (N14 in blue) had the highest toxicity values in nearly all assays, followed by nanotubes (N3 in green) that had medium to high toxicity values in viability and MTT assays.

As a third step, ENM characterisation data, excluding BET and DTT data that were not available for all samples, were plotted in the parallel co-ordinate plot, each descriptor was represented by a parallel line, and each data row was displayed as connected line segments. Here, special attention was given to identify the properties contributing to the high toxicity of three particular NPs that were shown to have high toxicity. The parallel co-ordinate plot of physicochemical descriptors available for 18 NPs is given in Fig. 4.5. The following conclusions were drawn:

- high toxicity of NP14 was likely to be related to its high Zn content;
- toxicity of NP3 was driven by many factors including aspect ratio, volume weighted mean ([4,3]), uniformity, D(0.5) and D(0.9);
- no meaningful correlation was observed between the toxicity of NP6 and its physicochemical characteristics.

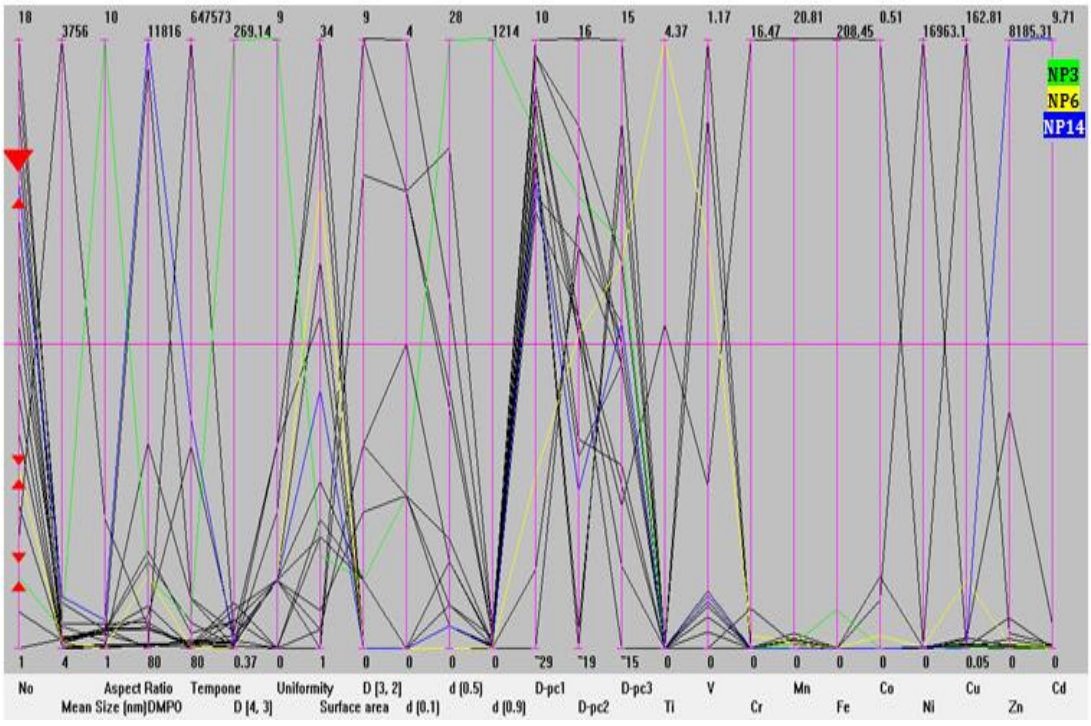

Figure 4.5 A parallel co-ordinate plot of the characterisation data

(Characterisation data includes aspect ratio and mean size measured by SEM, Oxygen-centred free radical generation measures using DMPO and Tempone H, size distribution data replaced by 3 principal components D-pc1, D-pc2 and D-pc3, and seven other size properties (mass diameter, uniformity, specific surface area, surface area mean diameter and three mass diameters) analysed by Mastersizer, and water soluble concentration of ten heavy metals (Ti, V, Cr, Mn, Fe, Co, Ni, Cu, Zn and Cd)

Further investigation of descriptor results associated with three polystyrene beads yielded to the conclusion that the differentiation of toxic aminated beads from other two non-toxic beads was not possible based on characterisation data (i.e. no clear differentiation between unmodified, aminated, and carboxylated latex beads based on the measured characteristics). As the measured properties were unable to explain the high toxicity of the aminated sample, other possible reasons that explain toxicity specific to the aminated beads, were suspected. According to Wang *et al.* (2014) the toxicity of the three NPs can be explained by their difference in surface properties. They reported measured zeta-potentials for N6 (e.g. 37.8, 37.5, and 40.3), for N5 (e.g.−36.2, −38.8, and −36.8), and for N7 (e.g. −54.9, −55.3, and −58.6). Results clearly showed that N6 had positive zeta-potential values, while N5 and N7 had negative zeta-potentials. They concluded that the large positive charge of N6 potentially contributed to its observed high toxicity (despite its structural similarity to N5 and N7). Further modelling investigations were undertaken on this dataset (Chapter 5 and 6) to model the properties that influence the toxicity of NPs.

4.3.2 Case Study II - Shaw dataset

The second dataset explored consisted of four descriptors representing the structural properties of 51 NPs (Shaw *et al.* 2008). Although the original dataset included 51 NPs, data associated with 19 NPs were removed when exploring structure-activity correlations due to missing characterisation values. As a first step, a heat map of toxicity data available for 51 NPs was constructed and combined with hierarchical clustering using the R software package (Team 2014) and given in Fig. 4.6.

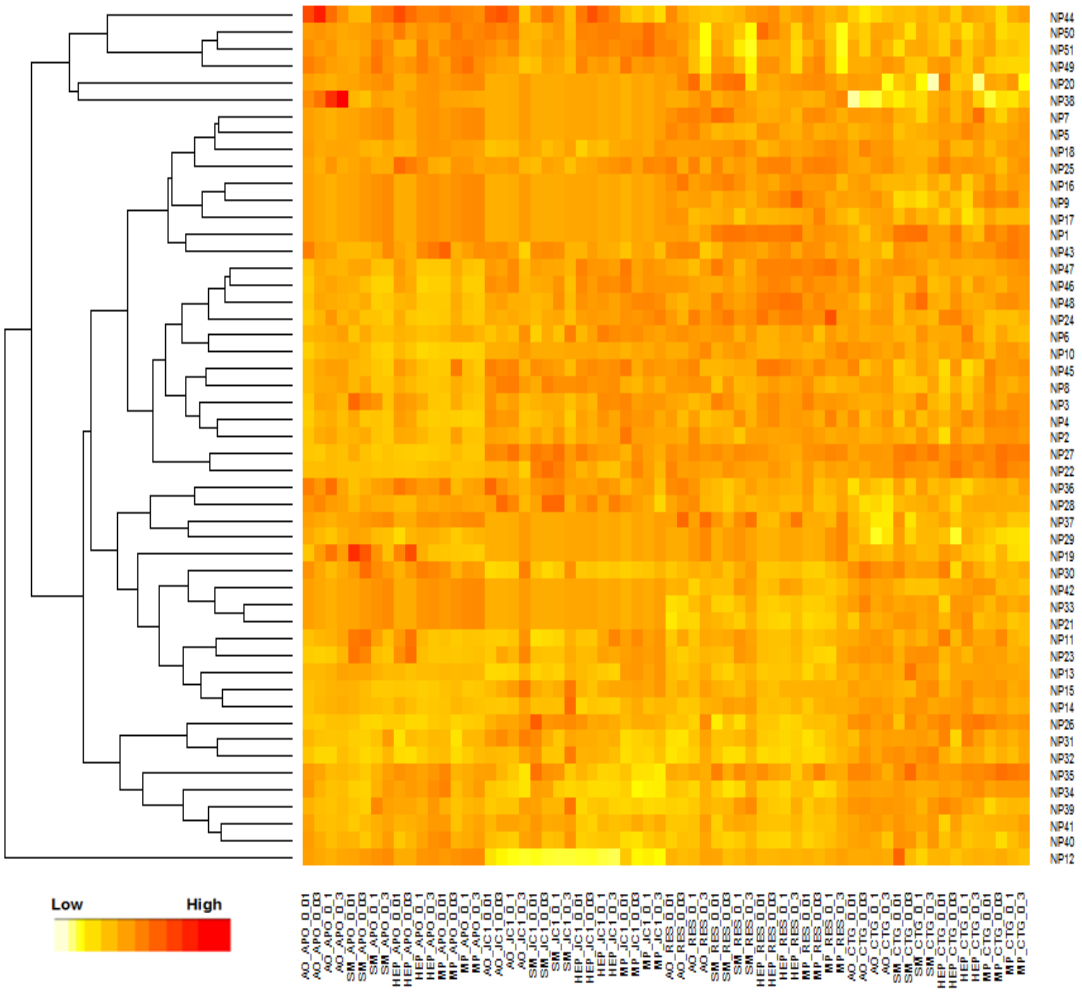

Figure 4.6 A cluster heat map displaying toxicity values of 51 NPs (toxicity endpoints included: Apoptosis (APO), mitochondrial potential (JC1), reducing equivalents (RES) and ATP content (CTG) tested under 16 different conditions, e.g. four doses x four cell types)

The most significant finding from the heat map given in Fig. 4.6 was that CdSe-core Quantum dots (NP49, 50 and 51) showed a high level of toxicity, especially in Apoptosis assays. It was indeed reported in the literature that Cd-containing Quantum Dots are capable of killing cells in culture and hence, there is a significant chance that they are harmful to human health (Hardman 2006). Examination of the 6 NPs located in the high-toxicity cluster revealed that they significantly differed in NM type (e.g. PNP, Qdots and CLIO), core type (e.g. Fe2O3, CdSe and Fe2O3) and coating type (e.g. PVA, PEG, Cross linked dextran), suggesting that toxicological effects are not caused by one characteristic but rather a combination of several parameters.

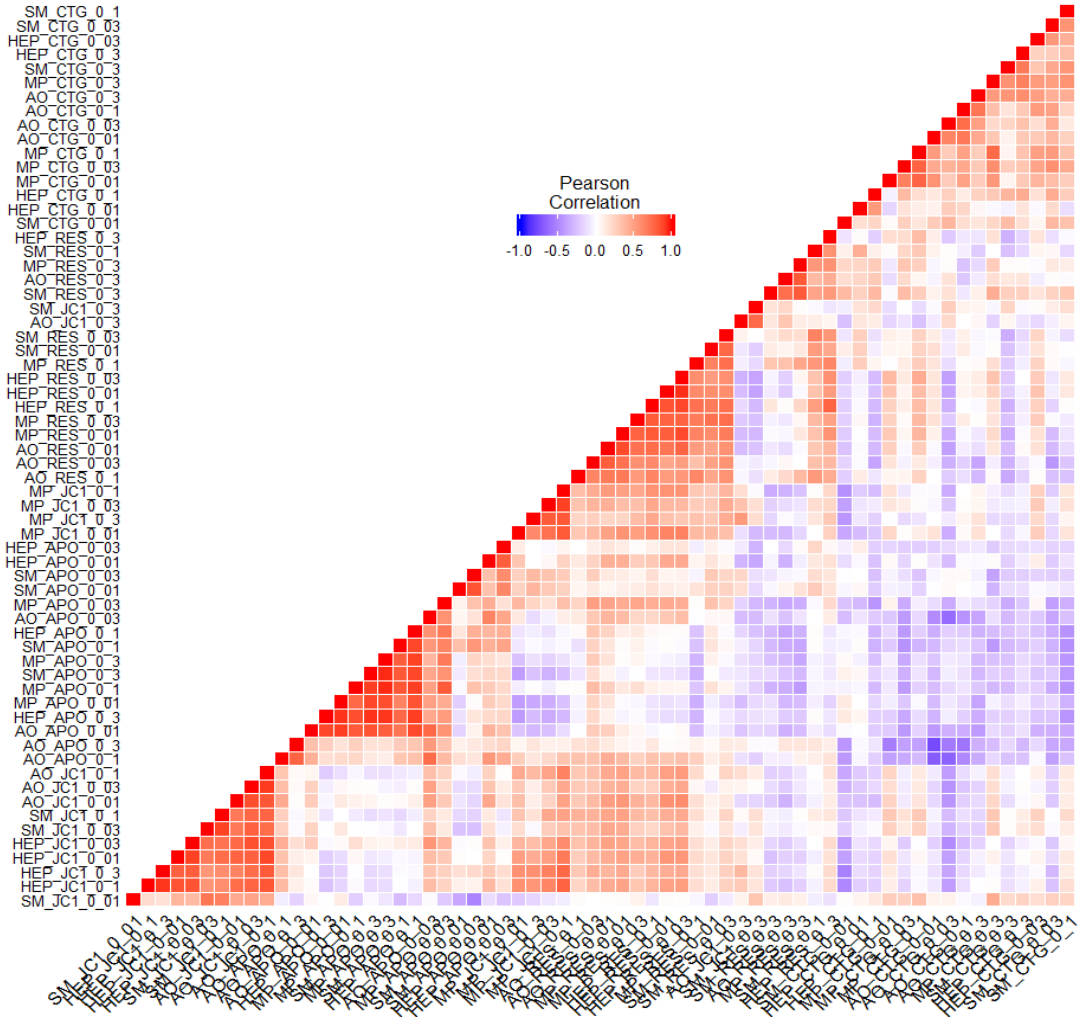

Figure 4.7 A heat map displaying the pairwise correlations between toxicity endpoints measured under 64 different conditions

A correlation matrix heat map with all the pairwise correlations between toxicity tests performed under 64 different conditions is given in Fig. 4.7. Mitochondrial membrane damage (JC1) and Apoptosis results (APO) were ranked the highest, suggesting that they were more representative of the complete toxicity data.

Then, as a next step, the scaled data containing four quantitative descriptors available for 31 NPs (e.g. 20 NPs were excluded due to missing characterisation values) were plotted together with mean toxicity values in a parallel co-ordinate plot (Fig. 4.8). The aim here was to visually assess the correlations between descriptor variables and toxicity values and to identify interaction effects in the data.

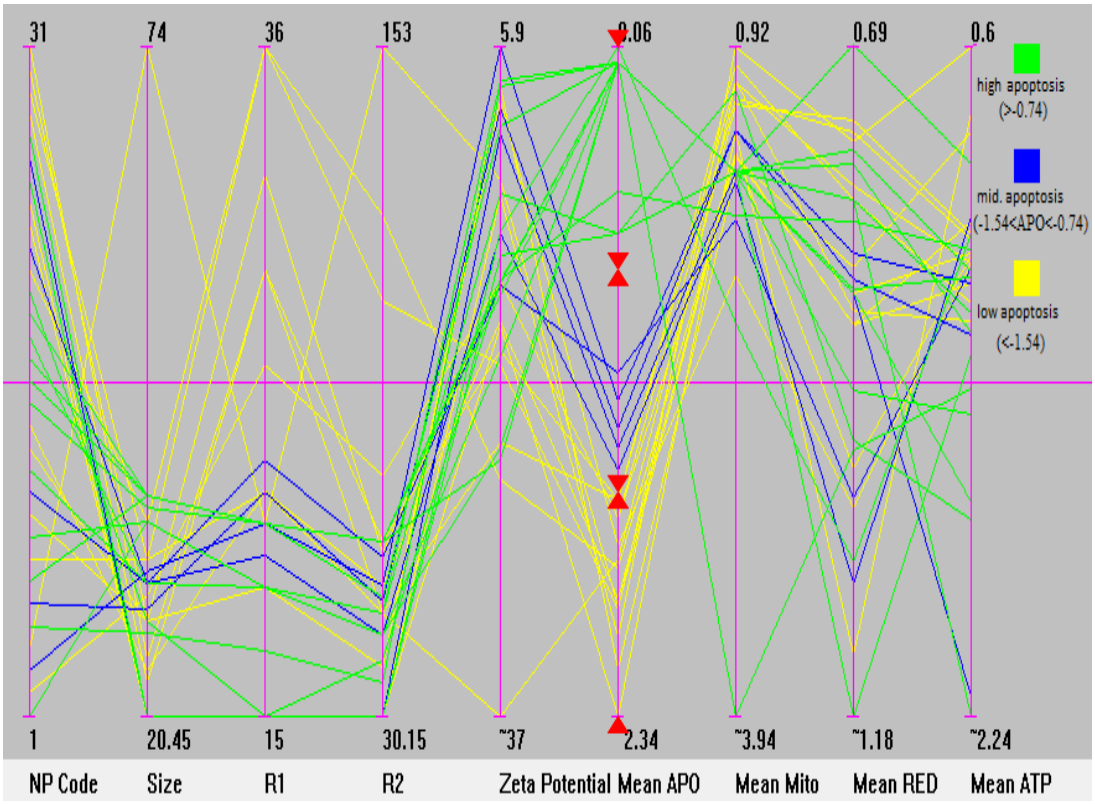

Figure 4.8 A parallel co-ordinate plot of the data collected by Shaw et al. (2008)

(Descriptors: Size, Relaxivities and Zeta potential; Toxicity Endpoints: Apoptosis (Mean APO), mitochondrial potential (Mean Mito), reducing equivalents (Mean RED), ATP content (Mean ATP)). The mean Apoptosis data (z scores) is divided into three categories; low (<-1.54), medium (-1.54<APO<-0.74) and high (>-0.74).

Initially, the mean Apoptosis data (z scores) were divided into three categories; low (<-1.54), medium (-1.54<APO<-0.74) and high (>-0.74). Each category was coloured differently to support clustering and correlation analysis. For example, low values of Apoptosis were highlighted in yellow; medium values were highlighted in blue and high values were highlighted in green. These colour codes can help in understanding the possible relationship between Apoptosis results and structural descriptors. As can be seen from the colour-coded parallel co-ordinate plot, the most obvious correlation was observed between R1 (relaxivity) and Apoptosis values. Clearly, the R1 values were inversely related to the Apoptosis assay results (e.g. low values of R1 lead to high Apoptosis). Additionally, a slight correlation was observed between R2 and Apoptosis results. The remaining two descriptors, size and

zeta potential showed no noticeable correlations with apoptotic effects. These initial findings were in great agreement with the modelling study performed by Epa *et al.* (2012) who found a significant correlation between Apoptosis results and R1 values. However, as illustrated in Fig. 4.8, the effect of relaxivity values differs depending on the type of toxicity assay. For Apoptosis, the higher the R1, the lower the toxicity whereas for mitochondrial membrane potential, the higher the R1, the higher the mitochondrial damage. In terms of activity-activity relationship, the most prominent correlation was observed between Apoptosis level and ATP content. The parallel co-ordinate plot shows ATP level decreases with an increase in the number of apoptotic cells. Further modelling studies were performed to quantify the observed relationship between relaxivity values and toxicity outcomes (Chapter 6).

4.3.3 Case Study III - NANOMMUNE dataset

The dataset collected from the NANOMMUNE project includes in vitro detection of Apoptosis induced by a panel of 18 NMs together with a number of quantitative and qualitative descriptors (e.g. NM core type, coating type, particle size and zeta potential). Three different indicator variables for core material, zeta potential and particle shape were added to the characterisation for future modelling purposes:

• Core material indicator variable: Feature encoded in this way was the nature of the nanoparticle core (+1 for iron oxides, -1 for zinc oxide and 0 for others such as TiO_2 and CeO_2).

• Zeta potential indicator variable: Feature encoded in this way was the magnitude of the zeta potential (+1 for values>+10, 0 for values between -10 and +10, -1 for values<-10).

• Particle shape indicator variable (1 for spherical particles, -1 for other shapes).

As the toxicity is highly dose-dependent, the toxicological effects are usually evaluated at multiple concentrations in a series of tests, the results of which are represented with a dose-response curve. Figure 4.9 shows the dose-response curves obtained for 18 NMs tested in this study. Toxicity data

was normalised according to positive (e.g. Fas antibody) and negative (untreated cells) control values as described in Section 4.3.1. After data normalisation relative to control values, all negative values were treated as zero. In the next step, toxicity data were scaled by subtracting the mean value of each variable from the data (e.g. mean centring) and multiplying the resulting values by the inverse of standard deviation (e.g. unit-variance scaling). This scaling step (i.e. standardisation) was performed to bring all of the variables into proportion with one another.

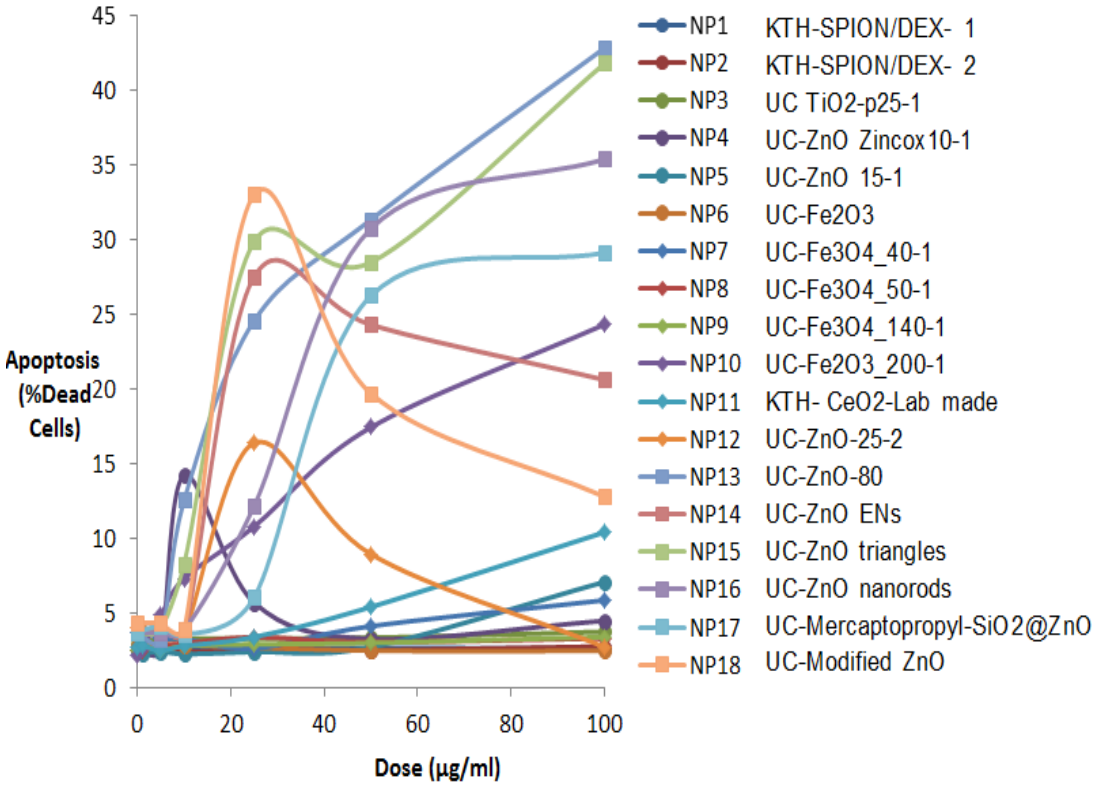

Figure 4.9 Dose-response curve for 18NMs investigated

As a next step, a heat map of toxicity data combined with a hierarchical clustering was constructed using the R software package (Team 2014) and given in Fig. 4.10.

As can be seen from the re-ordered dendrogram shown in the heat map of the toxicity data, six particular NPs (five ZnO-based NPs and Fe2O3-based nanocubes) were grouped in the high toxicity cluster, suggesting that they induced a high level of apoptotic activity. However, two uncoated ZnO NPs

located in the bottom of the heat map, (e.g. NP5 and NP12) induced significantly lower Apoptosis rates in cell culture. This finding confirms that although the core material type has an important role in determining toxicity, surface properties can greatly affect different dimensions of biological activity.

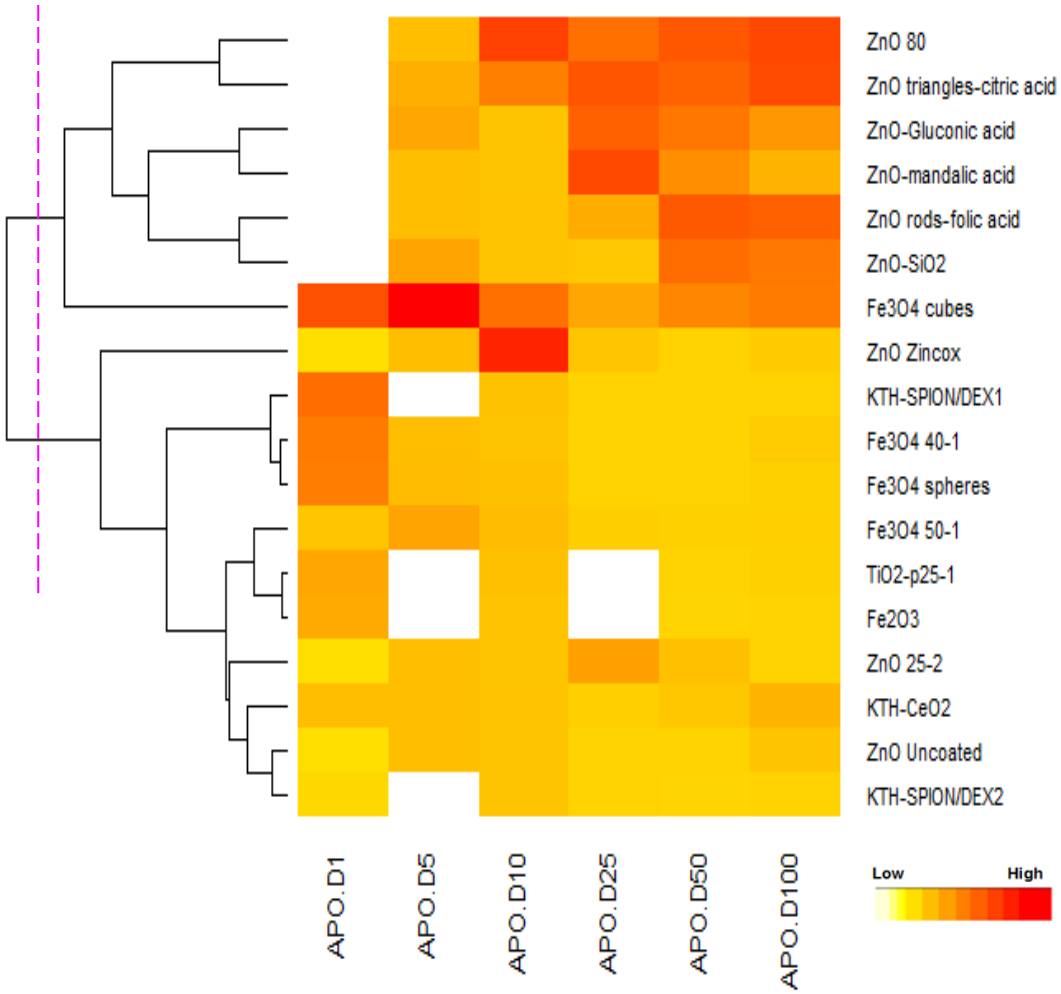

Figure 4.10 A cluster heat map displaying the level of apoptotic cell death after different doses of exposure to 18 NMs

In order to convert Apoptosis results measured at different doses into a single cumulative toxicity index, PCA was performed on the entire set of toxicity data and a single principal component explaining > 98% variance of the data was obtained. As a next step, toxicity data represented by one principal component were displayed together with the characterisation data in a parallel co-ordinate plot to qualitatively identify the correlations (Fig. 4.11). In this plot, low toxicity values (e.g. Cluster 1) were highlighted in green while

medium (e.g. Cluster 2) and high toxicity (Cluster 3) values were shown in yellow and blue, respectively. As can be seen from the colour-coded parallel co-ordinate plot given in Fig. 4.11, the most obvious correlation was observed between the main compound code and toxicity values. It seemed that NPs with -1 core material code (zinc oxide) tended to have relatively high toxicity (blue cluster). The remaining descriptors, particle sizes and zeta potential, showed no obvious correlation with toxicity level. Further modelling studies on this dataset have been performed and reported in Chapter 6.

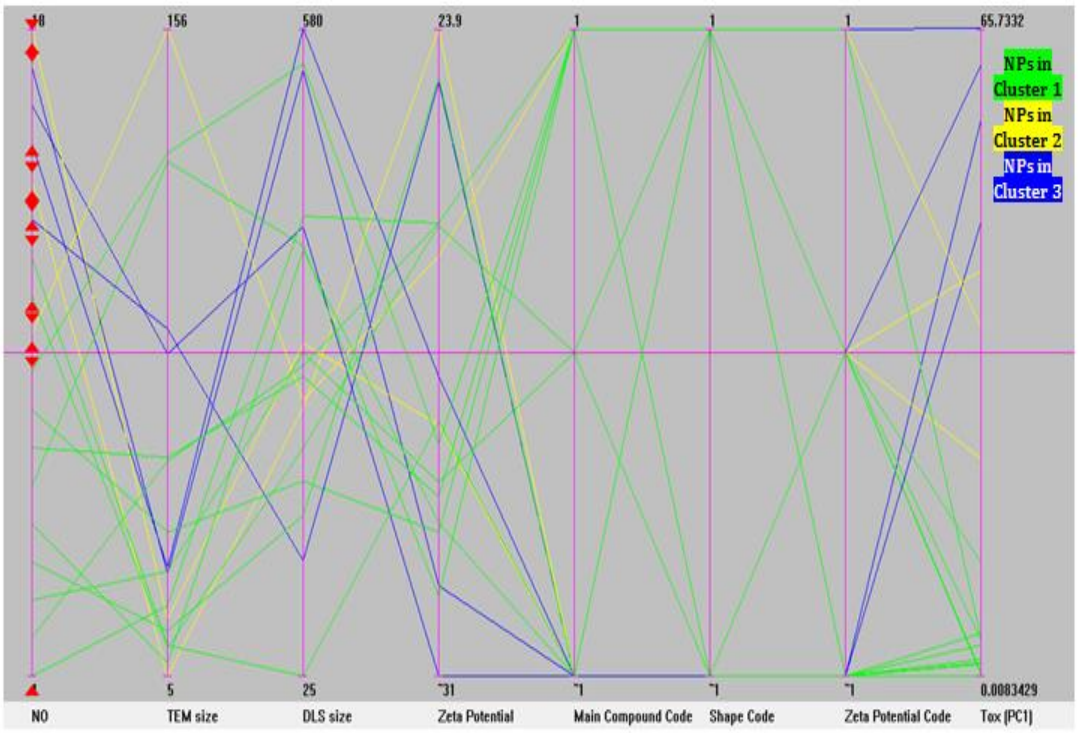

Figure 4.11 A parallel co-ordinate plot of the NANOMMUNE data

(NO: Number of NPs, Descriptors: TEM Size, DLS Size, Zeta potential and 3 indicator variables; Toxicity Endpoint: PCA-based toxicity categories).

4.3.4 Case Study IV - Liu dataset

The fourth dataset analysed consists of ten descriptors representing the structural properties of 9 NPs and toxicity results measured at 7 different concentrations (Liu *et al.* 2011). Initially, a heat map was generated using the scaled toxicity values of 9 NPs and combined with a dendrogram to illustrate the arrangement of clusters (Fig. 4.12). Examination of clustering results

revealed that high toxicity of ZnO at higher concentration and high toxicity of CuO at lower doses led to their discrimination from the rest of the NPs. In order to find out the structural reasons behind the relatively higher toxicity of these two metal oxide NPs, characterisation data were displayed in a parallel co-ordinate plot (Fig. 4.13) and descriptor values associated with these two particular NPs (i.e. ZnO and CuO) were highlighted in yellow.

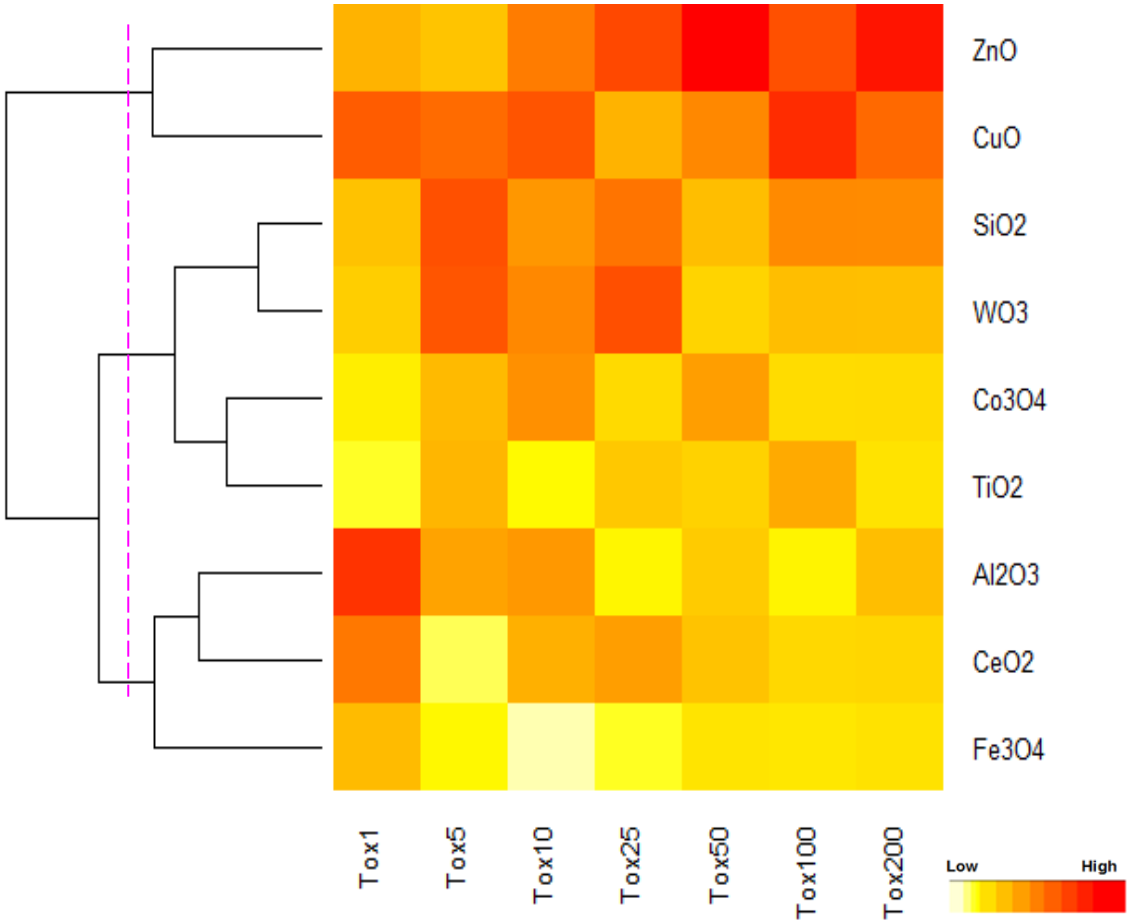

Figure 4.12 A cluster heat map displaying toxicity values of 9 NPs measured at 7 different doses

As can be seen from the colour-coded parallel co-ordinates graph given in Fig. 4.13, the most significant and meaningful factor potentially contributed to the high toxicity of ZnO and CuO was the atomisation energy of the metal oxides (EMeO). No other obvious and meaningful correlations between structural features and toxicity were observed.

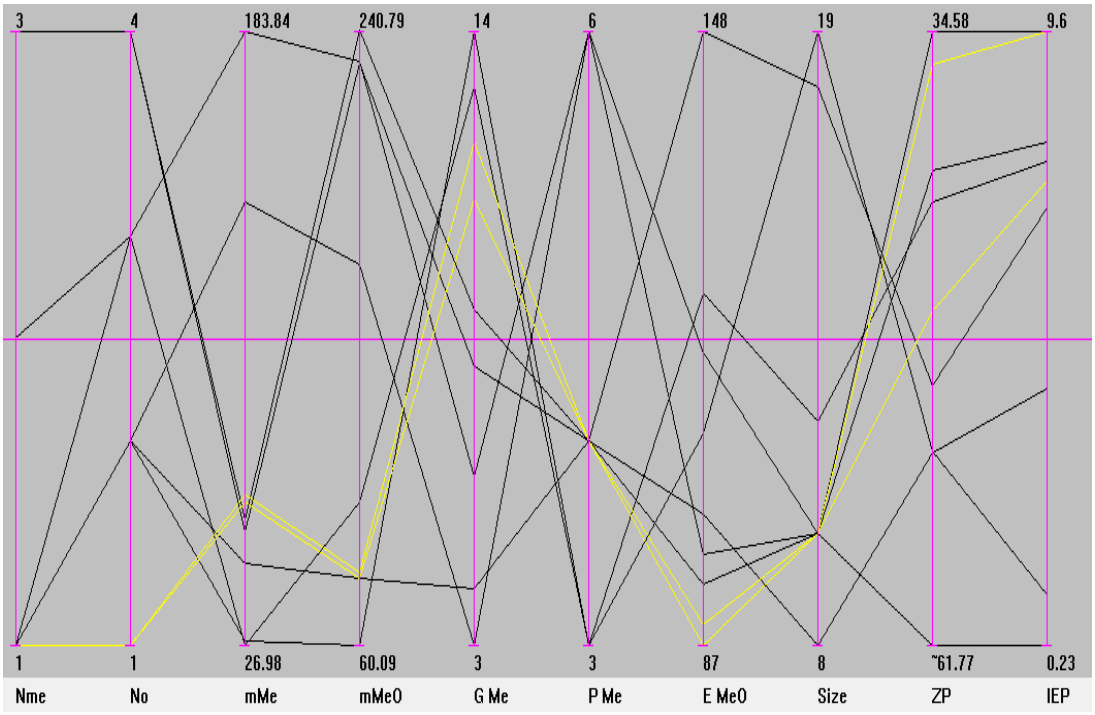

Figure 4.13 A parallel co-ordinate plot of the characterisation data.

(Nme and No: number of metal and oxygen atoms; mMe: atomic mass of the nanoparticle metal; mMeO: molecular weight of the metal oxide; G Me and P Me: group and period of the nanoparticle metal; E MeO: atomisation energy of the metal oxide; size: nanoparticle primary size; ZP: zeta potential (in water at pH = 7.4); IEP: isoelectric point).

4.3.5 Case Study V - Zhou dataset

This dataset consists of a set of 83 CNTs with known biological activities (e.g. four protein binding activities, cell viability and nitrogen oxide generation) (Zhou *et al.* 2008). As a first step, the biological activity values were scaled to have a mean value of 0 and a standard deviation of 1. Then, the scaled data was used to form the heat map given in Fig. 4.14. The red colour represents high biological activity whereas the yellow colour stands for low biological activity.

As can be seen from the heat map representation of biological activity data, surface modified CNTs behave similarly in protein binding assays. Particularly, a significant correlation was observed between CA, CT and HB protein binding assays, while BSA binding seemed to be less correlated. Another interesting observation was that CNTs decorated with different organic molecules showed significantly different cytotoxicity (WST1 assay)

and immune response (NO generation), confirming the influence of surface characteristics of CNTs on their toxicity.

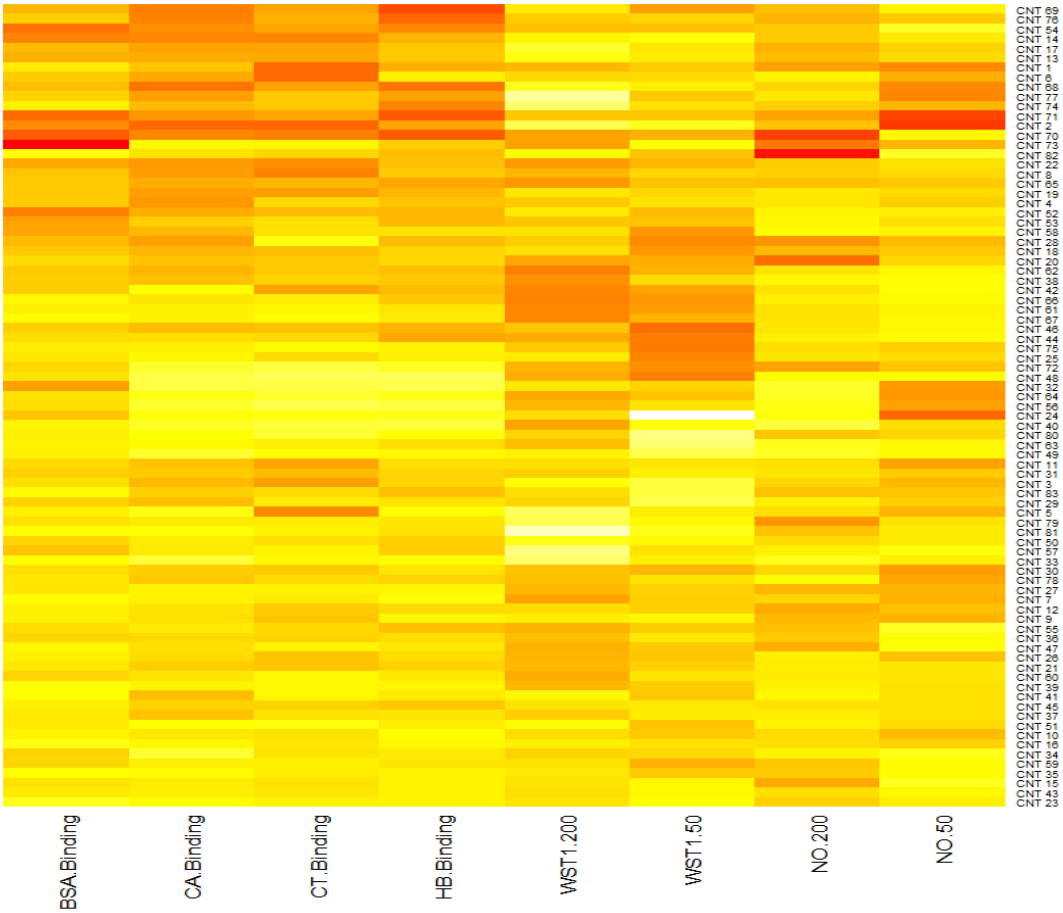

Figure 4.14 A cluster heat map displaying biological activities of 83 CNTs. (BSA, CA, CT HB protein binding activities, WST1 Cell viability assay and nitrogen generation).

4.3.6 Case Study VI - MARINA dataset

This dataset consists of 9 NMs (e.g. 6 metal oxide NMs and 3 multi-walled carbon nanotubes) was investigated for cytotoxicity by two different toxicity assays (i.e. Neutral red assay and Resazurin) in four different cell lines. After data normalisation relative to control values, a heat map of toxicity data was constructed using the R software package. Figure 4.15 and 4.16 show a combined dendrogram and heat map plot of cytotoxicity rates measured by Neural Red and Resazurin assay, respectively.

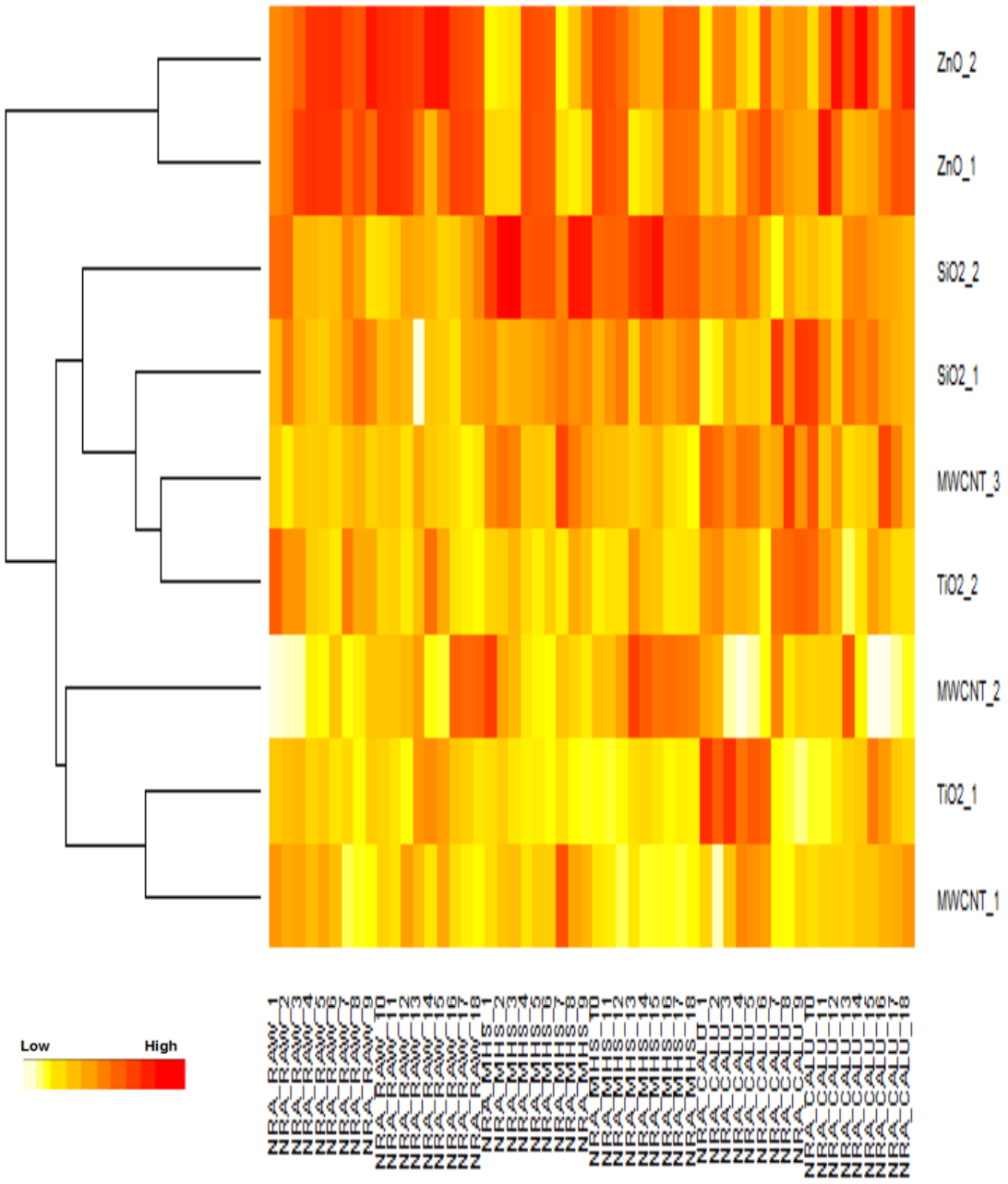

Figure 4.15 A cluster heat map displaying cytotoxicity results as assessed by Neural Red assay in four different cell lines (HDMD, RAW 264.7, MHS, Calu-3) at six different doses (2.5, 5, 10, 20, 40 and 80 µg/ml) and 3 different time points (24, 48 and 72 hours)

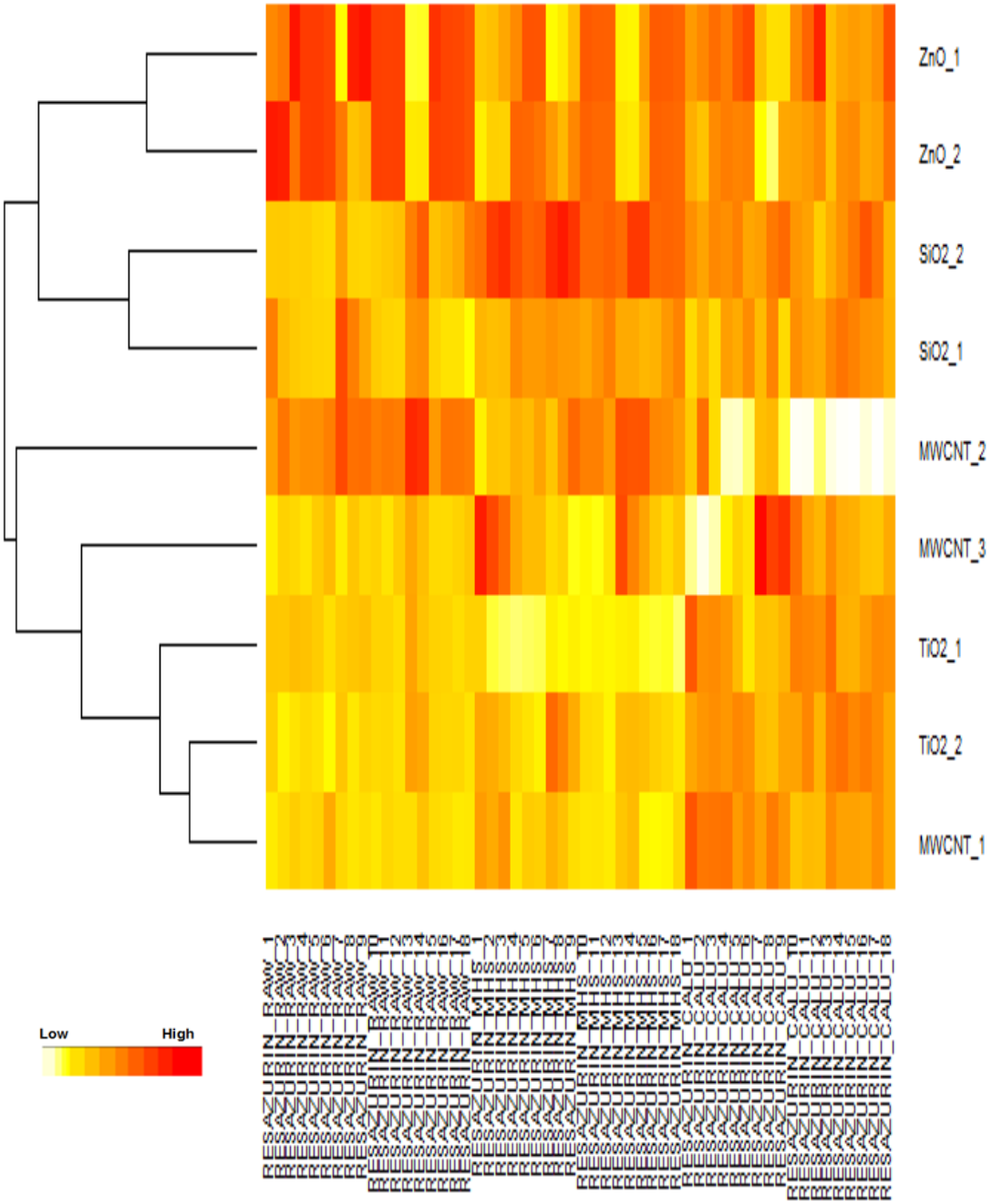

Figure 4.16 A cluster heat map displaying cytotoxicity results as assessed by Resazurin in four different cell lines (HDMD, RAW 264.7, MHS, Calu-3) at six different doses (2.5, 5, 10, 20, 40 and 80 µg/ml) and 3 different time points (24, 48 and 72 hours)

It was clearly shown in Fig. 4.15 and 4.16 that the most toxic metal oxide NPs tested were ZnO NPs in both assays followed by SiO_2 samples while TiO_2 NPs showed the lowest cytotoxicity. Interestingly, MWCNTs differing in length induced different levels of cytotoxicity, with the shortest MWCNTs (MWCNT1) being the least toxic. The results of parallel co-ordinate analysis was not reported here as there was no added value in this case, due to the high number of toxicity endpoints tested and very limited number of descriptors available.

4.3.7 Case Study VII - Gajewicz dataset

This dataset consists of 20 descriptors (e.g. 18 quantum-mechanical descriptors and 2 experimental measurements) representing the structural features of 18 metal oxide NPs and measured cytotoxicity of the same set of NPs to human keratinocyte (HaCaT) cell line (Gajewicz *et al.* 2014). The heat map visualisation of biological activity data was not relevant here since only one toxicity term (e.g. LC_{50} values) was available for each NPs tested. To investigate the structure-toxicity profile of 18 metal oxide NPs, logarithmic inverse of LC_{50} values were plotted together with theoretical and experimental descriptors in a parallel co-ordinate plot in Fig. 4.17. In this plot, NPs with low toxicity were highlighted in blue while high toxicity NPs were highlighted in yellow to help identify the possible relationship between toxicity results and structural descriptors. The most significant correlation was observed between formation enthalpy of metal oxides (Delta_Hf) and toxicity values. There was also a positive correlation between electronegativity and toxicity. These initial findings are very important to demonstrate the applicability of SAR analysis to model NM toxicity. Further modelling investigations are undertaken on this dataset (Chapter 5 and 6) to model the properties that influence toxicity of metal oxide NPs.

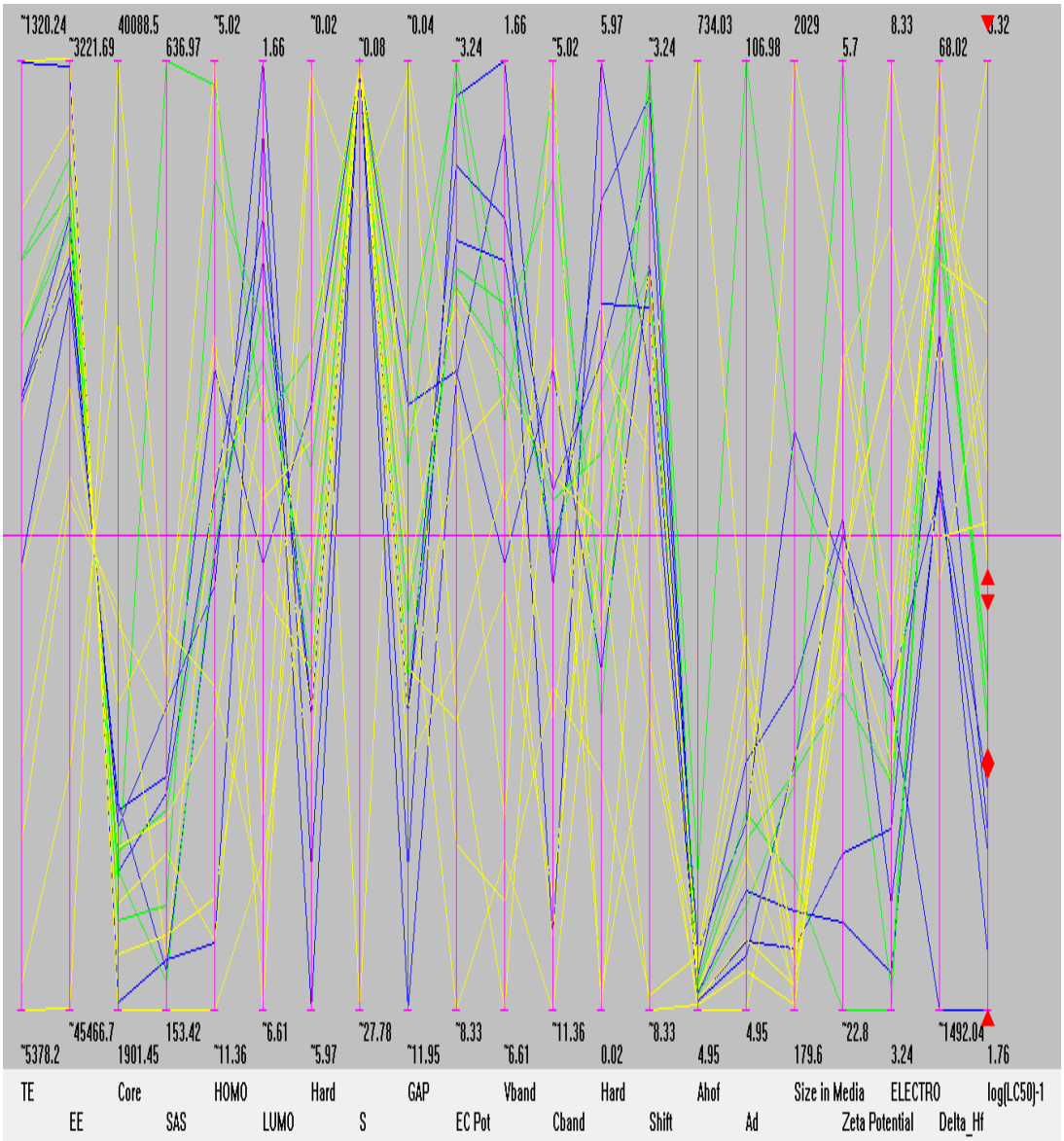

Figure 4.17 A parallel co-ordinate plot of the theoretical and experimental descriptors plotted together with LC50 values.

(TE: Total energy, EE: Electronic energy, Core: Core–core repulsion energy, SAS: Solvent accessible surface, HOMO: Energy of the Highest Occupied Molecular Orbital, LUMO: Energy of the Lowest Unoccupied Molecular Orbital, Hard: Chemical hardness, S: Total softness, GAP: HOMO-LUMO energy gap, EC Pot: Electronic chemical potential, Vband: Valance band, Cband: Conduction band, Hard: Parr and Pople's absolute hardness, Shift: Schuurmann MO shift alpha, Ahof: Polarizability derived from the heat of formation, Ad: Polarizability derived from the dipole moment, Size in media, Zeta potential, ELECTRO: Mulliken's electronegativity, Delta_Hf: Standard enthalpy of formation of metal oxide nanocluster).

4.3.8 Case Study VIII - Sung dataset

This dataset consists of 14 TiO_2-based NPs (e.g. different size, shape and charge) investigated for cell viability in BEAS-2B cells. The characterisation data includes 6 quantitative measurements representing Ti content, Cu content, TEM size, DLS size, zeta potential and TGA loss of these NPs. Similar to previous case studies, the heat map visualisation of biological activity was not relevant here since only one toxicity value (e.g. cell viability measured at 150 ppm) was available for each NPs analysed.

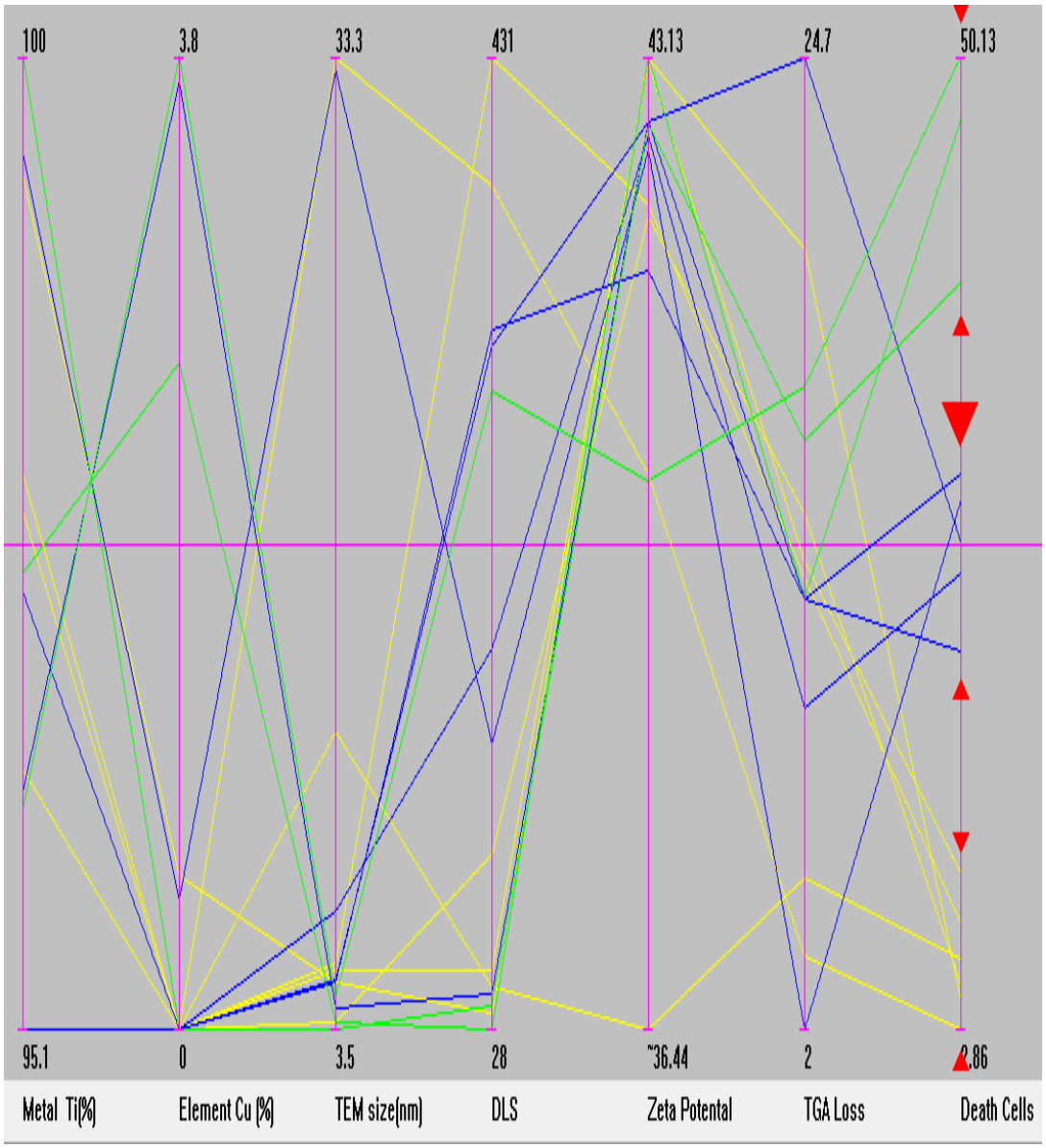

Figure 4.18 A parallel co-ordinate plot of the theoretical and experimental descriptors plotted together with LC_{50} values

To investigate structural factors that were responsible for different toxicity profiles of 14 TiO_2-based NPs, cell death values were plotted together with experimental descriptors in a parallel co-ordinate plot in Fig. 4.18. NPs with low toxicity were highlighted in yellow while high toxicity NPs were highlighted in blue. The composition of the metal core (e.g. Ti and Cu content) was observed to be the most influential factor affecting cell viability. Additionally, the higher values of Cu content seemed to cause an increase in the percentage of dead cells. Moreover, low values of particle size and high values of zeta potential seemed to increase the level of cell death.

4.3.9 Case Study IX - Puzyn dataset

This dataset contains the *in vitro* toxicity of 17 different NPs and a pool of 12 different quantum-mechanical descriptors based on the electronic properties available for 16 metal oxide NPs (Puzyn *et al.* 2011a). Again, the heat map visualisation of toxicity data was not relevant here since only one toxicity dose term (e.g. EC_{50} values) was available for each NPs analysed. To investigate structure-activity relationships, the parallel co-ordinate plot given in Fig. 4.19 was drawn. To make visual assessment easier, descriptors associated with low-toxicity NPs (e.g. EC_{50} values less than 2.3) were coloured in blue while the characteristics of highly toxic NPs (e.g. EC_{50} values greater than 2.95) were highlighted in yellow. Consequently, the most obvious correlation was observed between one of the calculated descriptors, Delta HMe, and EC_{50} values: low values of this descriptor seemed to cause an increase in toxicity. It was concluded that this structural descriptor, formation enthalpy of metal oxides, was one of the potentially important descriptors for estimating the EC_{50} values of metal oxide NPs. This finding is in agreement with the literature, where it has been demonstrated that this descriptor can be used as an effective predictor of the cytotoxicity (Puzyn *et al.* 2011a).

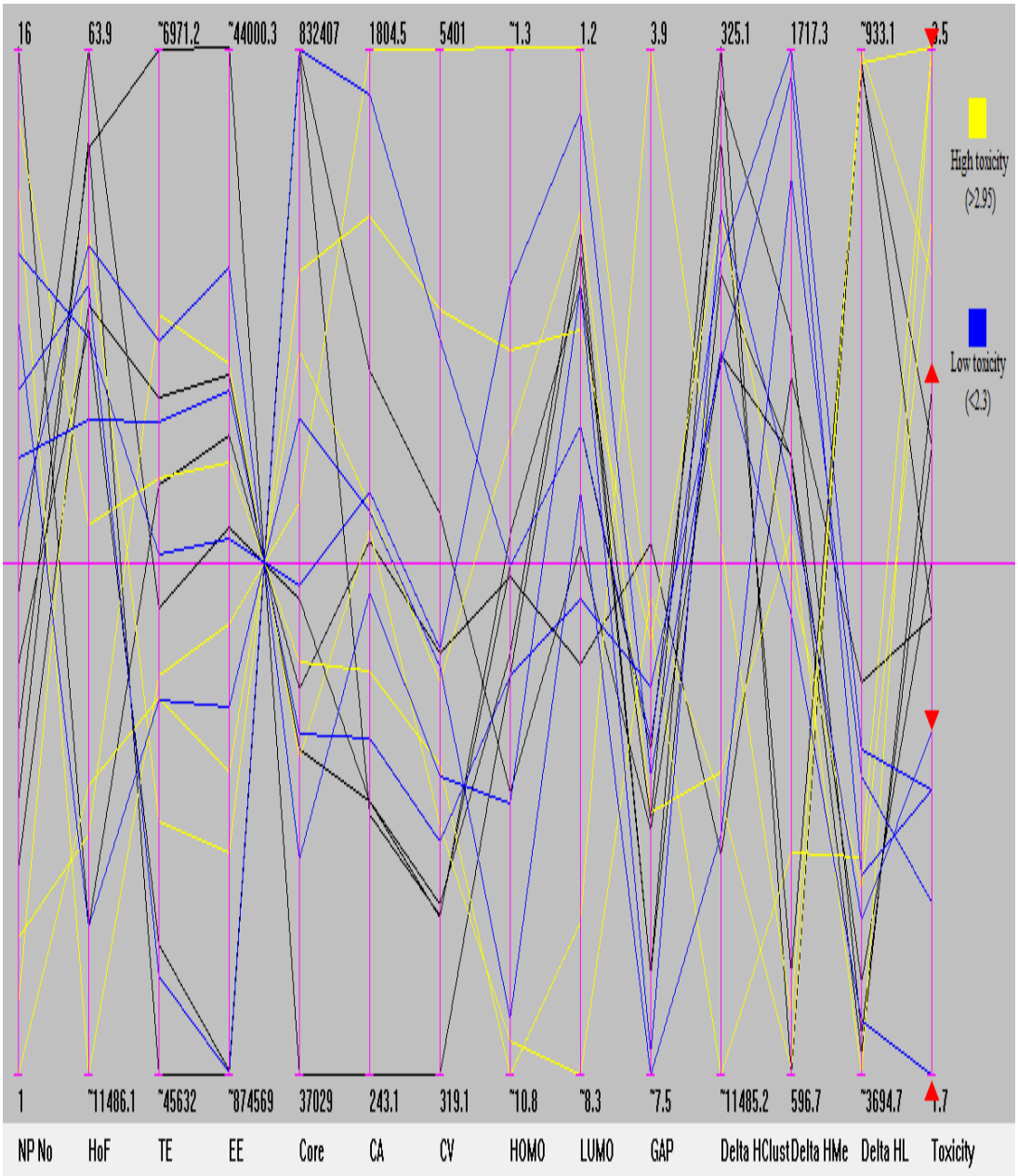

Figure 4.19 A parallel co-ordinate plot of the theoretical descriptors plotted together with EC50 values representing toxic behaviour of 16 NPs

(HOF: Standard heat of formation of the oxide cluster, TE: Total energy, EE: Electronic energy, Core: Core–core repulsion energy, CA: Area of the oxide cluster calculated based on COSMO, CV Volume of the oxide cluster calculated based on COSMO, HOMO: Energy of the Highest Occupied Molecular Orbital, LUMO: Energy of the Lowest Unoccupied Molecular Orbital, GAP: HOMO-LUMO energy gap, Delta HClust: Enthalpy of detachment of metal cations Men+ from the cluster surface, Delta HMe: Enthalpy of formation of a gaseous cation, Delta HL: Lattice energy of the oxide).

4.3.10 Case Study X - Zhang dataset

This dataset contains categorical toxicity data belonging to 23 different NPs and a pool of 27 descriptors representing elemental properties, energy/enthalpy, particle size and surface charge of the same set of NPs (Zhang *et al.* 2012). Initially, the parallel co-ordinate plot given in Fig. 4.20 was drawn to visually display the causal relationships between NPs' descriptors and the toxicity endpoint. For the ease of visual assessment, descriptors associated with NPs that showed no toxicological effects (e.g. class 1) were highlighted in blue while the descriptors describing properties of high toxicity NPs (e.g. class 2) were shown in yellow. The most obvious correlation was observed between one of the calculated descriptors, NP conduction band energy (EC), and toxicity clusters: high values of this descriptor seemed to cause a decrease in toxicological effects. Careful examination of the parallel co-ordinate plot below revealed that the first molar ionisation energy of metal and (e.g. DeltaHIE 1+) particle size measures (e.g. d, d2) were positively related to toxicity while ionic index (Z2/r) and atomisation energy (EAmz) of metal oxides seemed to be inversely related to toxicological outcomes. This result can be considered as a strong indication of the presence of a structure-toxicity relationship within the given dataset. Further investigations were carried out to model the causal relationships between NP' descriptors and the toxicological effects (Chapter 5).

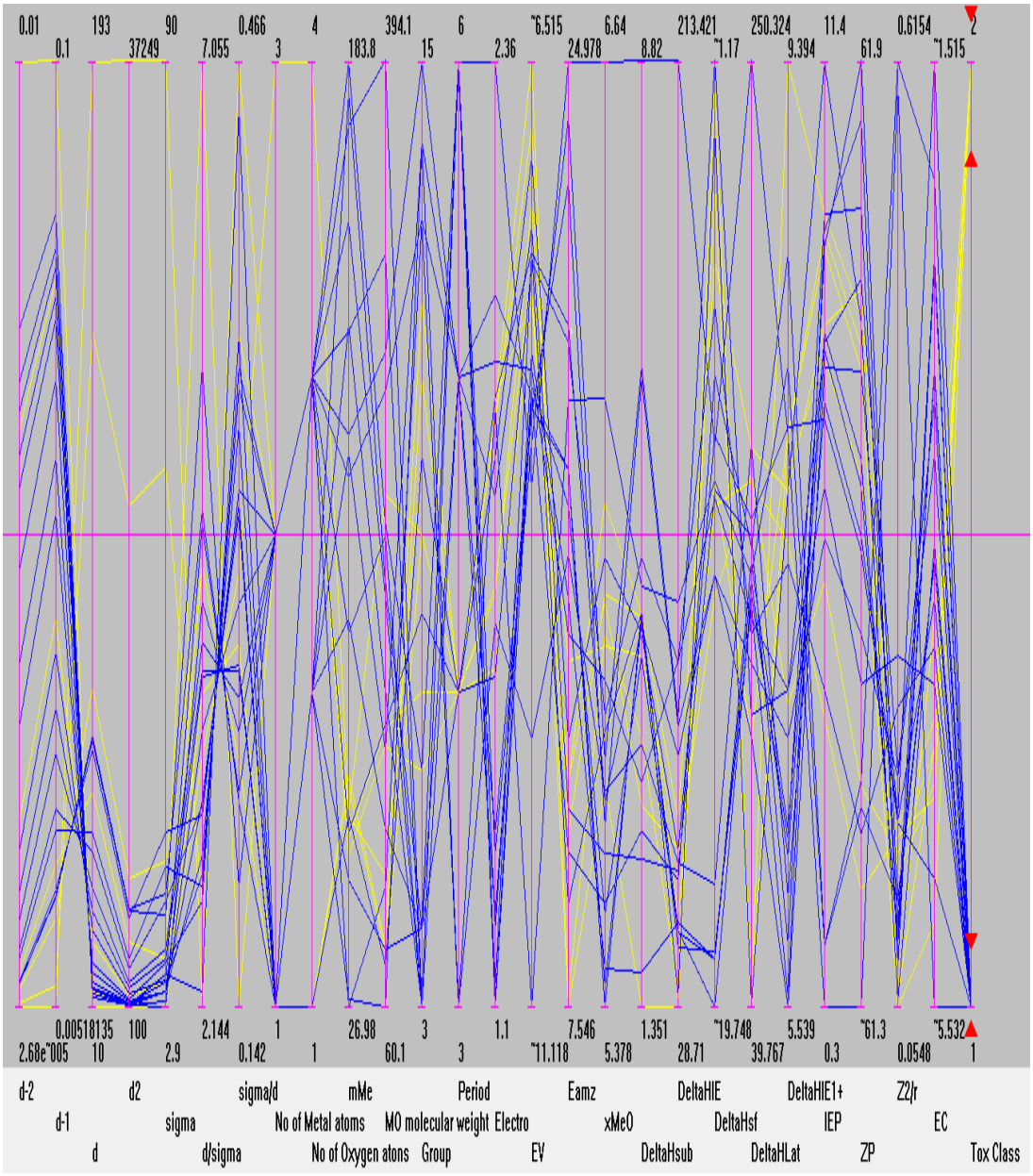

Figure 4.20 A parallel co-ordinate plot of a pool of descriptors associated with low-toxicity (shown in blue) and high-toxicity (shown in yellow) NPs

(Four particle size descriptors based on different orders of average particle size (d-2, d-1, d d2), sigma: standard deviation, d/sigma: mean/standard deviation ratio, sigma/d: coefficient of variation, numbers of metal and oxygen atoms, nMe: atomic mass of metal, MO molecular weight: metal oxide molecular weight, group and period of metal, EV: NP energy of valence band, EAmz: metal oxide atomisation energy, xMeO: metal oxide electronegativity, DeltaHsub: metal oxide sublimation enthalpy, DeltaHIE: metal oxide ionisation energy, DeltaHsf: metal oxide standard molar enthalpy of formation, DeltaHLat: metal oxide lattice enthalpy, DeltaHIE 1+: first molar ionisation energy of metal, IEP: NP isoelectric point, ZP: NP zeta potential in water at PH of 7.4, Z2/r: ionic index of metal cation, EC: electrophilicity)

4.3.11 Case Study XI - Oh and Park dataset

Fig. 4.21 shows the parallel co-ordinate plot of 16 descriptors and exocytosis rates of 12 gold NPs (Oh and Park 2014). NPs were divided into three categories based on their exocytosis levels in macrophage and each category was coloured differently to support correlation analysis. For example, low values of exocytosis were highlighted in yellow; medium values were highlighted in blue and high values were highlighted in green. Evidently, high values of three particular descriptors (charge accumulation B, charge density B and zeta potential B) were related to an increase in the exocytosis rates of 12 GNPs. This finding confirms that the descriptors that depend on the charge of NPs have an influence on exocytosis behaviour. Further modelling studies have been carried out to model the observed relationship and the results are reported in Chapters 5 and 6.

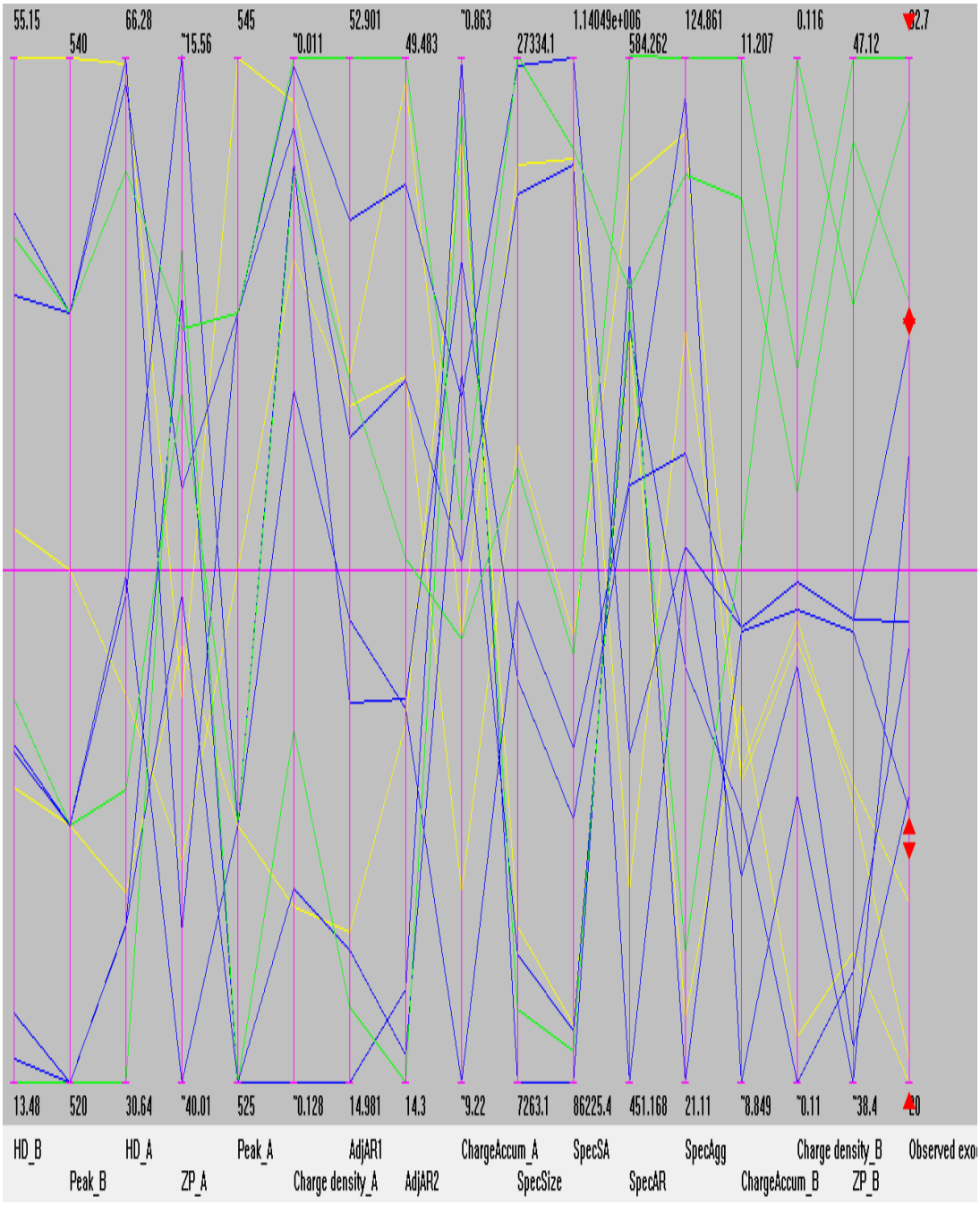

Figure 4.21 A parallel co-ordinate plot of a pool of descriptors and exocytosis rates of 12 gold NPs

(HD_A/B: hydrodynamic diameter before/after coating, ZP_A/B: zeta potential before/after coating, Peak_A/B: maximum wavelength before/after coating, Charge density_A/B: charge density before/after coating, ChargeAccum_A/B: charge accumulation before/after coating, AdjAR1 and 2: adjusted aspect ratios, SpecSize: spectral size, SpecSA: spectral surface area, SpectAR: spectral aspect ratio and SpecAgg: spectral aggregation)

4.3.12 Case Study XII - Weissleder dataset

This dataset consists of 109 iron-oxide based NPs investigated for cellular uptake in various cell types and more than three hundred theoretical descriptors calculated based on surface modifiers (Weissleder *et al.* 2005). Fig. 4.22 shows the logarithmic cellular uptake values of each NPs in five different cell lines (PaCa2, HUVEC, U937, GMCSF and RestMph). Clearly, the cellular uptake values of 109 iron-oxide based NPs measured in the same cell differ significantly. Additionally, a significant change was also observed in cellular uptake among cell types (e.g. HUVEC, MPH, PaCa2 etc.). Parallel co-ordinate representation of descriptors is not used here as the number of descriptors are too high to visually identify correlations (i.e. when the number of dimensions is high, the lines get cluttered and obscures the inherent structure in the data) (Luo *et al.* 2008). Further modelling studies have been carried out to investigate the potential relationship between hundreds of theoretical descriptors and cellular uptake values in PaCa2 cell lines in the following chapter.

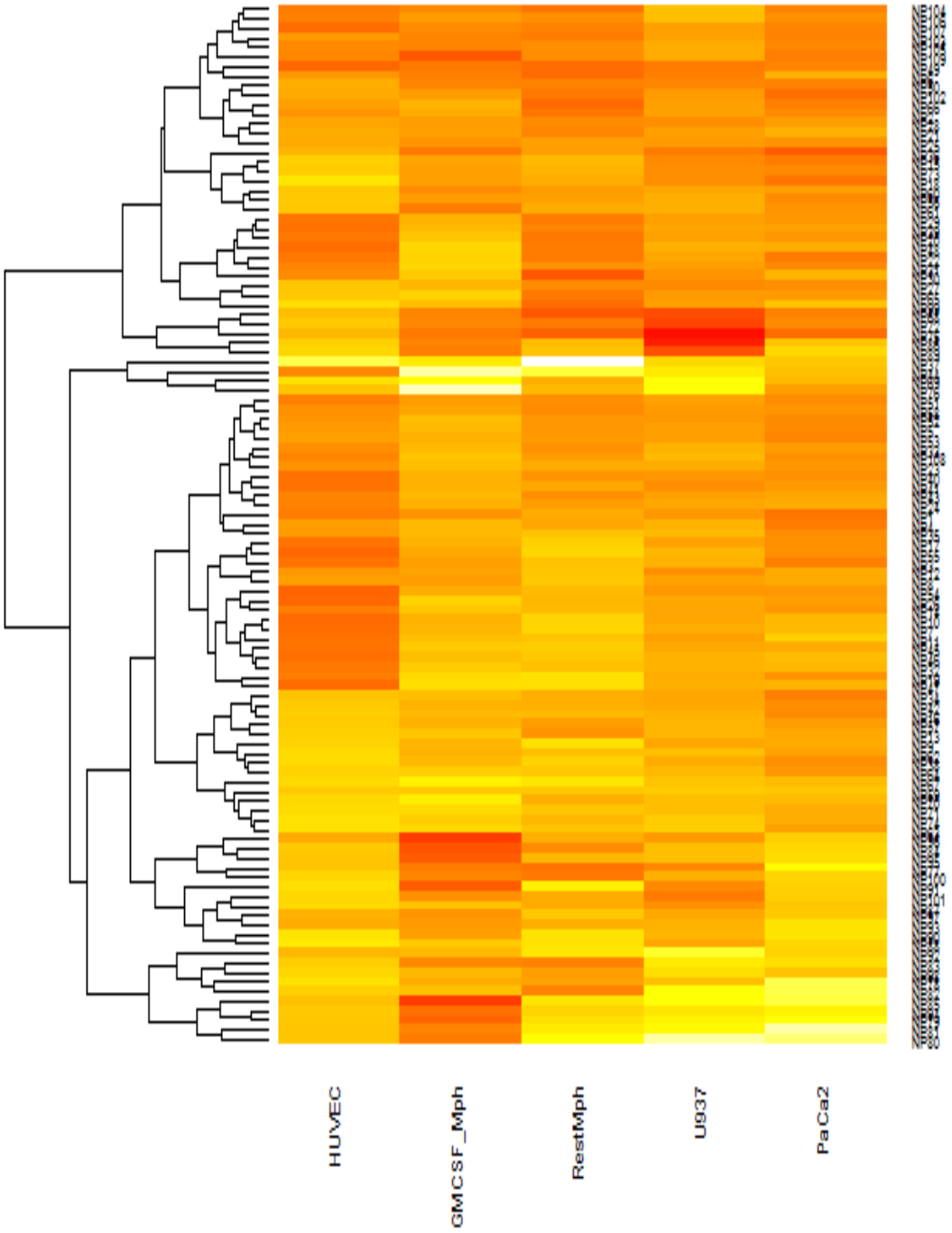

Figure 4.22 A cluster heat map displaying cellular uptake values of 109 NPs measured in five different cell lines including PaCa2, HUVEC, U937, GMCSF and RestMph

4.4 Concluding Remarks

Data visualisation is often carried out in order to identify patterns and extract useful information hidden within a given dataset before moving onto more complex statistical procedures. In particular, different visualisation techniques can be used for visual exploration of multi-dimensional data. They can visually display the relationships between multiple variables, handle limited datasets, and allow investigators to interactively make an analysis with the help of visual features such as colour. There are several techniques used in multi-dimensional data visualisation such as parallel co-ordinates, heat maps, projection and clustering methods.

In the context of nanotoxicology, the complexity is that no single parameter can describe the properties (e.g. physical, chemical and toxicological) of ENMs. In fact, there are various features including physical structure, chemical composition and surface characteristics that have been suggested to contribute to the effects and behaviour of ENMs in different environments. Moreover, toxicity investigations are usually carried out using various toxicity assays, exposure conditions and time points. A detailed characterisation and toxicity assessment often leads to the generation of large amounts of data that need to be analysed in detail and well understood. Therefore, there exists the need for a simple but yet effective method of converting multi-dimensional nanotoxicity and characterisation data (corresponding to multi-variables or features) into a more efficient format that can be visually explored and examined. Such visualisation techniques are necessary in order to get an overall picture of the properties describing individual toxicities and characteristics of ENMs when a large amount of information is involved. This also allows data to be efficiently visualised without any information loss.

In this chapter, two direct visualisation techniques, cluster heat maps and parallel co-ordinate plots, were used for data exploration purposes. The cluster heat map that displays the hierarchy of clusters in the form of a dendrogram was used to summarise multivariate toxicity outcomes and to display ENM cluster membership. The main intention here was to compare toxicity of ENMs measured using different assays under different experimental

conditions and to group them according to their toxicity level. Once the ENMs of high toxicity concerned were identified through clustered-heap map visuals, the potential parameters contributing to the toxicity of those particular materials were investigated by plotting them in parallel co-ordinates. The value of using parallel co-ordinates in the context of nanotoxicity modelling is that certain properties in high dimensions can be transformed into a lower dimensional space and hence, potential relationships between multiple variables can be visually identified in a two-dimensional space. The main purpose here was primarily to use parallel co-ordinate plots for rapid assessment of correlations between descriptors and toxicity endpoints.

Overall, it was shown through a number of case studies that direct data visualisation techniques can be successfully employed to convert multi-dimensional nanotoxicity and characterisation data into a more efficient format for the ease of visual exploration and examination. The exploratory data analysis results reported in this chapter gave a strong indication that a relationship exists between structural properties and toxicity and promoted the modelling work presented in Chapter 5 and 6.

Chapter 5
Nano-(Q)SAR Model Development: Decision Trees

Data-driven models that decode the relationships between the biological activities of ENMs and their physicochemical characteristics provide an attractive means of maximising the value of scarce and expensive experimental data. Although such structure-activity relationship (SAR) methods have become very useful tools for modelling nanotoxicity endpoints (nano-(Q)SAR), they have limited robustness and predictivity and interpretation of the models they generate can be problematic. New computational modelling tools or new ways of using existing tools are required to model the relatively sparse and sometimes lower quality data on the biological effects of ENMs. The most commonly used SAR modelling methods work best with large data sets, but are not particularly good at feature selection, and may not account for non-linearity in the structure-property relationships. To overcome these limitations, the application of a novel algorithm, a genetic programming-based decision tree construction tool (GPTree) to nano-(Q)SAR modelling, was described and demonstrated.

This chapter demonstrates the use of GPTree in the construction of accurate and interpretable nano-(Q)SAR models by applying it to four diverse literature datasets. It was shown that GPTree generates models with accuracies equivalent to, or superior to, those of prior modelling studies on the same datasets. GPTree is a robust, automatic method for the generation of accurate nano-(Q)SAR models with additional advantages that it works with small datasets, automatically selects descriptors, and provides improved interpretability of models.

5.1 Introduction

Established data-driven computational techniques such as quantitative structure-activity relationship ((Q)SAR) modelling and its qualitative variant ((Q)SAR), have proven to be useful in modelling biological response data for ENMs. Their use has increased significantly in recent years because they

provide rapid biological activity/toxicity predictions from structural properties where experimental data are incomplete, missing or difficult to obtain (Wang *et al.* 2014; Fourches *et al.* 2010; Puzyn *et al.* 2011a; Epa *et al.* 2012; Gajewicz *et al.* 2014; Kar *et al.* 2014; Chau and Yap 2012; Zhang *et al.* 2012; Pathakoti *et al.* 2014; Bigdeli, Hormozi-Nezhad and Parastar 2015; Burello and Worth 2011b; Le, Yan and Winkler 2015; Liu *et al.* 2014). Additionally, they are the only methods currently available that can generate quantitative predictions of biological effects of multifarious ENMs in very complex biological or ecological 'real world' environments. Published nano-(Q)SAR models have identified linear and non-linear relationships between nanomaterials properties and their biological effects, suggesting a potentially complex relationship between physical and compositional features of ENMs and toxicity. Given the current scarcity of hazard data in nanotoxicology (Oksel, Ma and Wang 2015) due to time, cost, and ethical factors, nano-(Q)SAR methods provide reasonably accurate results in a timely manner and make best use of these limited data. Maximising the usefulness of limited data will provide opportunities to design inherently safer ENMs by structural manipulations (e.g. safety by design research).

In the absence of suitable datasets for generating quantitative models of ENM toxicity using traditional methods, it has been decided to focus on tools that elucidate relationships between theoretically/experimentally derived descriptors and toxicity. In particular, the use of decision tree learning algorithms has been investigated to identify the optimum combination of physicochemical properties for effective predictions of biological activity of ENMs. Decision trees (DTs) have been recently suggested as a 'gold standard' SAR algorithm by Ma *et al.* (2015). Our method allows automatic construction of DTs from categorical toxicity data. DT models are transparent and can deal with small, large and noisy datasets, detect nonlinear relationships, allow automatic selection of input descriptors, provide a clear indication of which properties are most important for toxicity, and generate understandable rules.

This chapter describes the GPTree (genetic-programming based decision tree induction) approach, and demonstrates its potential in SAR modelling of ENM toxicity by a number of case studies. A large amount of nanotoxicity data

was compiled from the literature and modelled with the GPTree method. Since the details of the method have been reported in recent literature (Ma, Buontempo and Wang 2008; Wang *et al.* 2006; Buontempo *et al.* 2005), only a summary is provided in this chapter. Here, the successful application of a genetic programming-based decision tree construction algorithm to identify key physicochemical descriptors contributing to the toxicity of ENMs has been demonstrated.

5.2 Nano-(Q)SAR modelling methods and decision tree induction

In theory, any regression or classification method, such as multiple linear regression, partial least squares, decision trees, random forest, support vector machine, linear discriminant analysis and artificial neural networks, can be used to qualitatively or quantitatively relate physicochemical properties to a biological activity of the ENMs. However, one of the main issues for nano-(Q)SAR modellers currently is the lack of comprehensive hazard and exposure data for well-characterised ENMs. Therefore, it is reasonable to focus on methods/tools that can make the best possible use of limited existing data, rather than tools that work best with large data sets that are currently in short supply. Moreover, in the absence broad understanding of how ENMs damage cells, tools that can automatically identify the most relevant descriptors for predicting toxicological outcomes, can simplify model interpretation and may provide new mechanistic insights (Burden and Winkler 2009). One method that is well suited to achieving these aims is decision trees modelling. This selects a small set of relevant variables (e.g. descriptors) in a context-dependant way and associates the output value (e.g. toxicity) to each of these key variables. Automatic construction of DTs is a powerful data-mining tool used for classification and regression. It is tolerant of poor quality and missing data and can model linear and nonlinear structure-activity relationships. Like other sparse feature selection methods that exploit sparsity-inducing Bayesian priors (Burden and Winkler 2009), decision trees select a small subset of the most relevant descriptors and completely remove the less important ones. They identify linear and non-linear structure-activity relationships in a transparent, understandable, and intuitive way. To date, the

DT algorithm has been successfully used in a range of SAR modelling studies (Sussman *et al.* 2003; Arena *et al.* 2004; Andres and Hutter 2006; Han, Wang and Bryant 2008; Ma, Buontempo and Wang 2008) but its use in nano-(Q)SAR studies is surprisingly very limited, given its clear advantages (Bakhtyari *et al.* 2014).

5.3 Methodology

Decision tree models can be generated using a variety of algorithms. Most construction algorithms use a greedy search of the response surface that can lead to suboptimal solutions (local minima) and overfitting of training data. These limitations can be ameliorated by the use of genetic programming methods to construct DTs. Genetic programming is a member of the broad class of evolutionary algorithms that can efficiently search very large parameter spaces for locally optimal solutions to high dimensional materials spaces (Le and Winkler 2016). The application of evolutionary algorithms for discovery and optimisation of materials has been reviewed very recently (Le and Winkler 2016).

In 2004, DeLisle and Dixon (2004) developed a novel approach called EPTree that employs a genetic programming-style search to construct accurate DT models. A variant called the GPTree has been developed that uses a simpler fitness function and its successful application to modelling of ecotoxicity data has been demonstrated (Buontempo *et al.* 2005). As the details of the technique can be found in literature (Wang *et al.* 2006; Buontempo *et al.* 2005), only a basic overview of the method is provided here.

Briefly, GPTree begins with a random population of solutions and repeatedly attempts to find better solutions by applying genetic operators such as mutation and crossover (for descriptions of these operators see Le and Winkler (2016)). The first step is to construct a user-specified number of trees (usually a large number) starting from a random compound and randomly chosen descriptor. Once the initial population is generated, tournament selection is performed to identify the best tree to be used as a parent tree for genetic operators such as crossover. The best tree from the subset of trees is chosen by its fitness (e.g. accuracy). Genetic operators such as crossover and

mutation are used to form next generation of trees that added or replace the current generation. These steps are repeated until the user-specified number of generations has been created. The DT model with the highest accuracy of classification for the training set is selected as the optimal decision tree model. Figure 5.1 summarises the operations used to find the optimal DTs while key parameters used in GPTree are shown in Table 5.1.

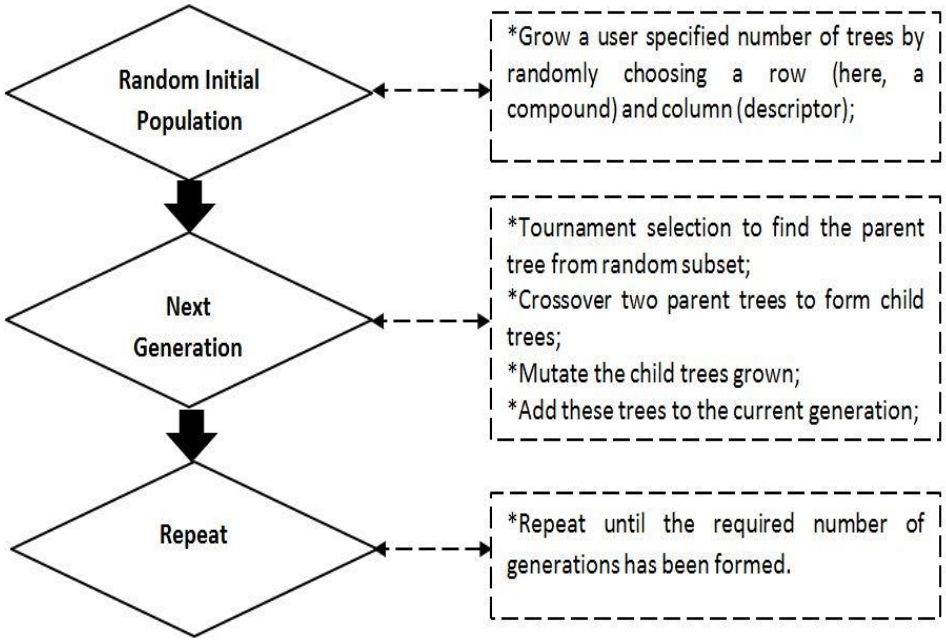

Figure 5.1 An overview of research methodology used in this chapter.

Table 5.1 GPTree parameters

yCOL	**Column number containing the class of the data set.**
nGen	Number of generations required.
nTrees	Number of trees in each generation required.
No. in tournament	Number of trees in the tournament to sort out the best for crossover operation
Winners included	The Elitism operator (The N best trees are placed directly into the next generation).
LIIAT	Low increase in accuracy tolerance (It forces a mutation for every tree if no improvement in the best accuracy has been seen for this many generations).
Mutation	% age of mutation
C in LN	Minimum number of cases in a leaf node

5.4 Results

The results of genetic-programming based DT models of four nanotoxicity datasets are presented in this section to illustrate the applicability of GPTree to SAR modelling studies of ENMs.

5.4.1 Case Study I – Zhang Dataset

5.4.1.1 Data pre-processing

A previously reported dataset containing the toxicological responses of 23 nanoparticles (NPs) together with a large pool of NP descriptors was used for GPTree analysis (Zhang *et al.* 2012). Liu *et al.* (2013b) used self-organising maps (SOM) to model toxicity data for 23 NPs (one of the metal oxides, Fe3O4, was excluded as it was impure) in order to group NPs with similar toxicological effects into the same clusters. Although their SOM-based clustering analysis revealed three distinct NP clusters, they suggested combining cluster 2 and 3 into a single cluster. Thus, Cluster 1 contained 16 NPs having no toxicological effects (i.e. negative response) while cluster 2 included 7 NPs of high toxicological concern (i.e. positive response). A set of 27 NP descriptors including element related descriptors, energy/enthalpy descriptors, size information and surface charge descriptors was also collected from Liu *et al.* (2013b) and used as input parameters in GPTree analysis. The initial dataset was then divided into training (18 NPs, 78% of dataset) and test set (5 NPs, 22% of dataset) as recommended by Sizochenko *et al.* (2015).

5.4.1.2 GPTree modelling results and statistical evaluation

The initial descriptor dataset and the categorical (toxic/nontoxic) biological data were used to generate 100 generations of decision trees, each generation consisting of 600 trees. The fittest 16 trees competed in each tournament and 0.015 of trees were mutated. These values were all chosen after a number of trial-and-error runs. The decision tree with best performance (Fig. 5.2) was selected based on its ability to predict the biological activities of the training and test sets, and its complexity (e.g. number of descriptors included). The statistical measures of the performance of the binary

classification tree generated by GPTree are presented in Table 5.2. Here, sensitivity represents the proportion of positives that are correctly predicted; specificity quantifies the proportion of negatives that are correctly identified while accuracy is the proportion of the true results including both true positives and true negatives among the total number of examined cases. The best performing tree model given in Figure 5.2 achieved the maximum value of accuracy, specificity and sensitivity (i.e. 100%) on both training and test datasets at the 24th generation. A Y-scrambling test involving repetitive randomisation of the response data was performed using the procedure of Wold, Eriksson and Clementi (1995). This demonstrates the statistical significance of the nano-(Q)SAR model by comparing its prediction accuracy to the average accuracy of random models (50% for a two class problem). The first step was to randomise the response data (toxicity class membership) of 18 compounds in the training set. For this purpose, a random number generator was used to allocate the integer between 1 (negative class) and 2 (positive class). GPTree analysis was then carried out on these scrambled response data with the same parameters used in the original model development. Simulations were run for 100 generations, each consisting of 600 trees, and the prediction accuracy of the best decision tree of the current generation was recorded. This process was repeated 3 times. The results of Y-scrambling (prediction accuracy of the best “random” trees in each of 100 generations, and number of leaf nodes) were averaged and compared to the results of the original model. In each case, scrambled data gave accuracies of 44, 41 and 47%, close to 50% expected by chance. This confirmed the high statistical significance of the nano-(Q)SAR model constructed from the experimental biological response data. As large and complex trees may overfit the data, resulting in the loss of ability of the model to generalise to untested compounds, tree complexity provides an additional model quality parameter (Ariew 1976).

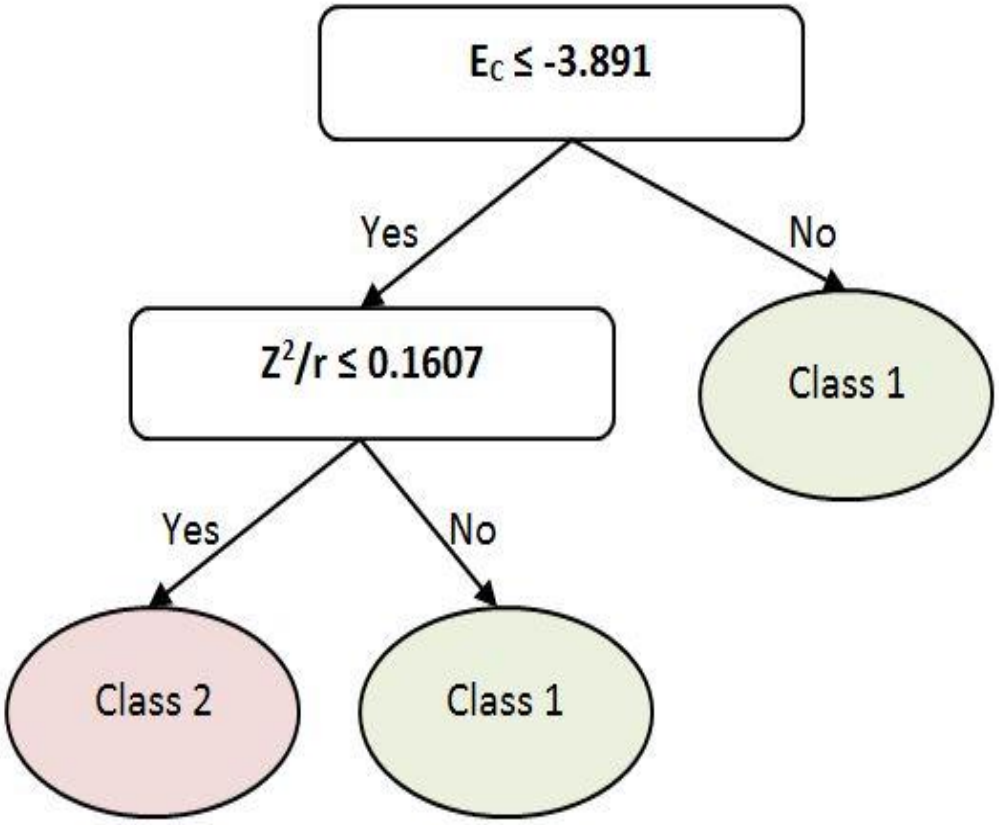

Figure 5.2 Decision tree produced by GPTree for general cellular toxicity dataset (Zhang *et al.* 2012). The statistical measures of the performance are given in Table 5.2.

Table 5.2 Classification performance of the decision tree induced by GPTree and shown in Figure 5.2.

Training Set			**Test Set**		
	Predicted Class			Predicted Class	
Actual Class	Nontoxic	Toxic	Actual Class	Nontoxic	Toxic
Nontoxic	13	0	Nontoxic	3	0
Toxic	0	5	Toxic	0	2
Sensitivity	100%		**Sensitivity**	100%	
Specificity	100%		**Specificity**	100%	
Accuracy	100%		**Accuracy**	100%	

5.4.1.3 Model interpretation

One of the strengths of the decision tree method, compared to other widely used nano-(Q)SAR modelling approaches, is the ability to *interpret the model*. The descriptors selected by the GPTree model include NP conduction band energy, E_C, and ionic index of metal cation, Z^2/r. This finding is very consistent with past studies that identified these two descriptors as being important for the toxicity of metal oxide NPs (Zhang *et al.* 2012; Liu *et al.* 2013b). The conduction band energy values of NPs screened ranged between -5.5 and -1.5 while the ionic index of metal cation of the studied NPs were in the range of 0.054 and 0.615. GPTree analysis showed that NPs with a conduction band energy of less than -3.9 and an ionic index of less than 0.16 tended to show toxic responses. Again, these findings are consistent with the conclusions of earlier studies (Liu *et al.* 2013b) that metal oxide NP toxic effects increased when its conduction band energy is close to the cellular redox potential (in the range of [-4.8, -4.12]) and when its ionic index is low.

5.4.2 Case Study II – Weissleder dataset

5.4.2.1 Data pre-processing

This dataset consisted of 105 iron-oxide based NPs investigated for cellular uptake by Weissleder *et al.* (2005). The NPs had the same metal core, super paramagnetic iron oxide, but different surface chemistries. The biological response values used in this case study were the cellular uptake of NPs in human pancreatic cancer cell line (PaCa2). The cellular uptake values of 105 NPs ranging between 170 and 27 542 NP/cell were obtained from Fourches *et al.* (2010). For binary classification, a criterion of Chau and Yap (2012) was considered: the NPs having cellular uptake of more than 5000 NPs per cell were considered to have good cellular uptake (class 2 - positive class) while NPs with cellular uptake of less than 5000 particles per cell were considered to have poor cellular uptake values (class 1 - negative class). According this criterion, 56 NPs belonged to class 2 and the remaining 49 NPs were in class 1 resulting in a balanced data set. The data set was split into a

training set (84 NPs) and test set (21NPs) that containing NPs distributed across the range of the cellular uptake values.

Although no experimental characterisation data was provided in the original paper (Weissleder *et al.* 2005), all NPs screened in this study contained the same magnetic iron oxide core decorated with different small molecules which enabled the computation of the theoretical descriptors based on the chemistry of the surface modifiers. Two different descriptor datasets were separately used as input data in modelling part. Firstly, a total of 690 1D and 2D descriptors were calculated using DRAGON 6 software (Mauri *et al.* 2006). After removing those descriptors with little variation across the nanoparticles, 389 chemical descriptors were retained. Secondly, a pool of 147 chemically interpretable descriptors was used (Winkler private communication) (Epa *et al.* 2012). These two descriptor datasets were modelled separately in GPTree analysis to investigate the relationship between descriptor values and the cellular uptake of NPs in PaCa2 cell line.

5.4.2.2 GPTree modelling results and statistical evaluation

For the descriptor dataset of 389 Dragon descriptors, 100 generations of trees were produced, each generation consisting of 600 trees (a larger number of trees provided no advantages and slowed the calculations down). Sixteen trees competed in each tournament and 10% of trees were mutated each time. These values were chosen after a number of trial-and-error runs in which the adjustable parameters, such as the number of generations, number of trees in each generation, number of trees in each tournament and the age of mutation were varied. The best performing decision tree (Figure 5.3), selected by model prediction accuracy for the training and test sets, had performance parameters given in Table 5.3. This tree model achieved a training accuracy of 98% and test accuracy of 86% at the 54th generation and no improvement was observed subsequently.

The risk of chance correlation was verified by the Y-scrambling test, which was repeated 3 times following the procedure explained in section 3.12. In comparison to the original dataset, lower test accuracy values (39, 44 and 55%) and also higher complexities (23, 23, 21 leaf nodes) of the randomised models confirmed that the developed nano-(Q)SAR model which achieved

higher test accuracy (86%) with less complexity (14 leaf nodes in total) was not due to chance factors.

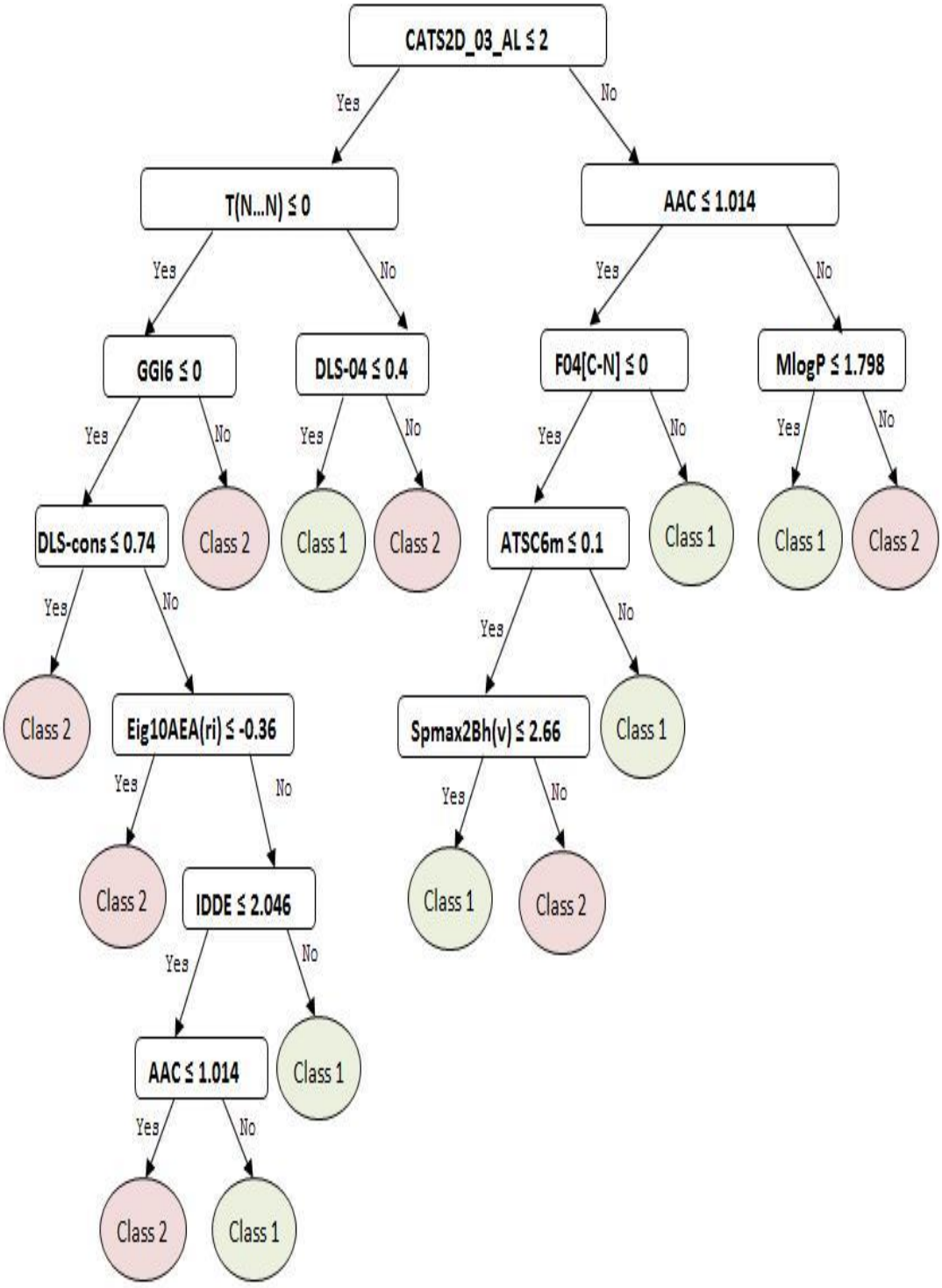

Figure 5.3 Decision tree produced by GPTree for nanoparticle cellular uptake dataset (Weissleder *et al.* 2005) using an initial pool of 389 DRAGON descriptors.

Table 5.3 Classification performance of the decision tree induced by GPTree and shown in Figure 5.3.

Training Set			**Test Set**		
	Predicted Class			Predicted Class	
Actual Class	Nontoxic	Toxic	Actual Class	Nontoxic	Toxic
Nontoxic	39	0	Nontoxic	9	1
Toxic	2	43	Toxic	2	9
Sensitivity	100%		**Sensitivity**	90%	
Specificity	95%		**Specificity**	82%	
Accuracy	98%		**Accuracy**	86%	

A similar modelling approach was followed for the second descriptor dataset. Overall, 1000 trees were grown in each generation while a maximum of 50 generations was used (no improvement was obtained with a higher number of generations required). 16 trees competed in each tournament and the mutation rate was set to be 10%. The best performing decision tree (shown in Fig. 5.4) was selected based on its ability to predict the class membership of NPs in the training and test sets. The performance parameters for the model are given in Table 5.4. At the 48th generation, the GPTree achieved a training accuracy of 99% and a test accuracy of 86%.

A Y-scrambling test was carried out to investigate the chance correlations and robustness of the best model selected. The results of Y-scrambling showed that the accuracy of the random response models (49, 58 and 39%) were not comparable to the original model (86%). Lower test accuracy values (39-58%) of the random response models despite their higher complexities (22, 24, 22 leaf nodes) were a good indicator of the absence of chance correlation in the developed nano-(Q)SAR model. Randomisation results confirmed that the developed nano-(Q)SAR model, which achieved higher test (86%) accuracy with less complexity (16 leaf nodes), was robust and not due to chance factors.

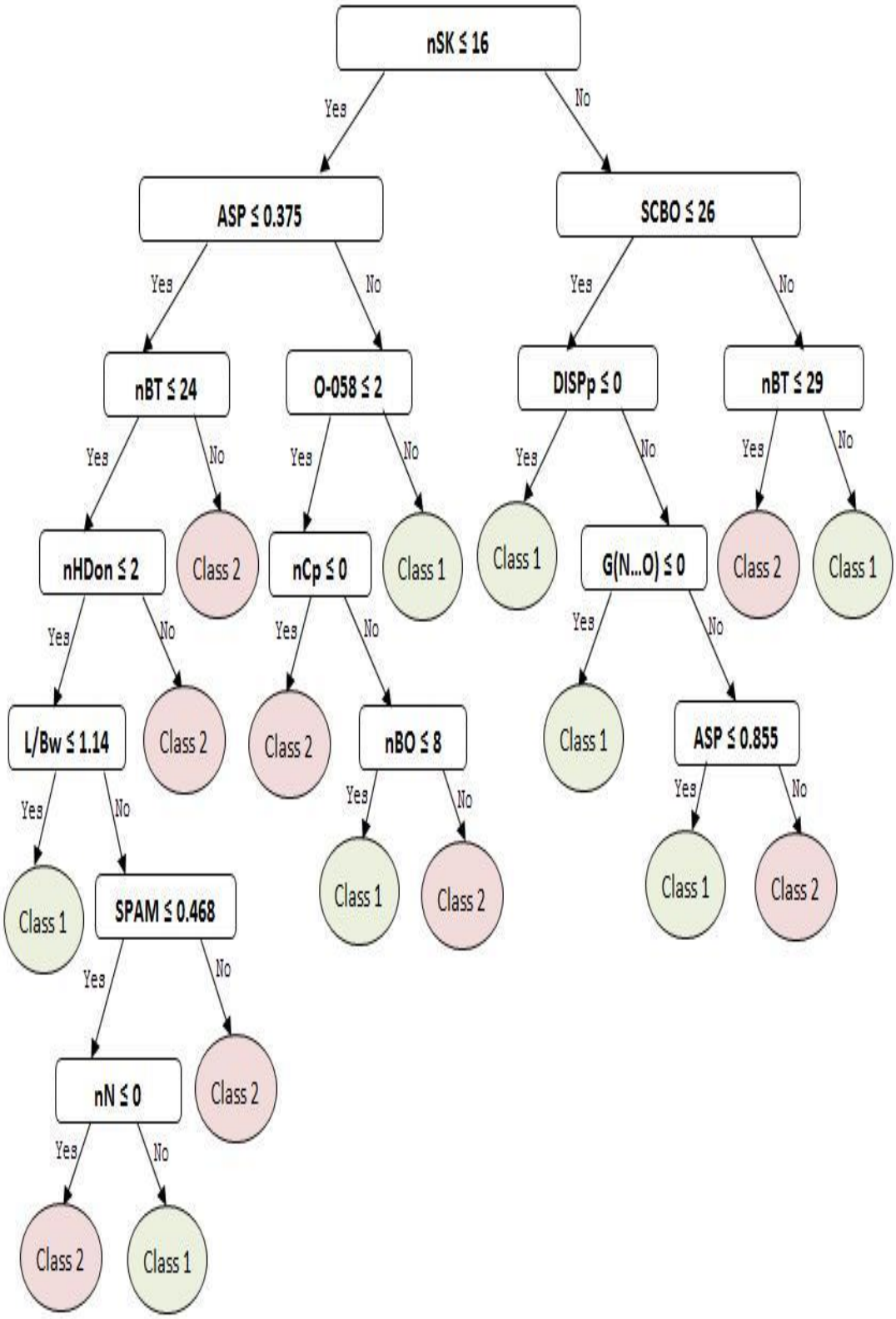

Figure 5.4 Decision tree produced by GPTree for nanoparticle cellular uptake dataset (Weissleder et al. 2005) using the descriptor dataset obtained from Epa et al. (2012).

Table 5.4 Classification performance of the decision tree induced by GPTree and shown in Figure 5.4.

Training Set			**Test Set**		
	Predicted Class			Predicted Class	
Actual Class	Nontoxic	Toxic	Actual Class	Nontoxic	Toxic
Nontoxic	39	1	Nontoxic	7	3
Toxic	0	47	Toxic	0	11
Sensitivity	98%		**Sensitivity**	79%	
Specificity	100%		**Specificity**	100%	
Accuracy	99%		**Accuracy**	86%	

5.4.2.3 Model interpretation

For the descriptor dataset of 389 Dragon descriptors, our GPTree model selected 12 descriptors related to lipophilicity (MlogP and CATS2D_03_AL), atomic masses (ATSC6m), symmetry associated with structure (AAC, IDDE), charge distribution (GGI6) and connectivity indices (Spmax2Bh) as the most important descriptors (see Table 5.5). Drug-like scores (DLS-cons and DLS-04) that are defined based on several parameters such as lipophilicity (MlogP), molecular weight and hydrogen bonding characteristics, were also found to be significant in explaining cellular uptake of different NPs in pancreatic cancer cells. In line with the earlier studies (Fourches *et al.* 2010), our analysis showed that lipophilicity, as measured by a MlogP lipophilicity descriptor, of NPs correlates well with their uptake. This lipophilicity descriptor successfully discriminated between two classes of NP uptake: 15 NPs with low values of MlogP, indicating the ability to penetrate lipid-rich zones from aqueous solutions (Turabekova and Rasulev 2004), were correctly located in Class 1 while 6 NPs with higher MlogP values were accurately located in Class 2.

Table 5.5 Descriptors selected from a pool of 389 descriptors for nanoparticle cellular uptake dataset

Descriptor Name	Descriptor Block	Interpretation
MlogP	Moriguchi octanol-water partition coeff.	Lipophilicity
CATS2D_03_AL	CATS2D Acceptor-Lipophilic at lag 03	Lipophilicity
DLS_04	Modified drug-like score	Lipophilicity, H-bonding and molecular weight
DLS_cons	DRAGON consensus drug-like score	Lipophilicity, H-bonding and molecular weight
AAC	Mean information index on atomic composition	Symmetry associated with structure
IDDE	Mean information content on the distance degree equality	Symmetry associated with structure
ATSC6m	Centred Broto-Moreau autocorrelation weighted by mass	Atomic masses
GGI6	Topological charge index of order 6	Charge distribution
Spmax2Bh(v)	Burden largest eigenvalue descriptor weighted by van der Waals volume	Connectivity Index
Eig10AEA(ri)	Eigenvalue n.10 from edge adjacency mat. weighted by resonance integral	Edge adjacency indices
T(N..N)	Sum of topological distances between N…N	Connectivity index
F04[C-N]	Frequency of C-N at topological distance 4	Connectivity index

For the second descriptor dataset, 13 parameters associated with hydrogen-bonding capacity (nN, O-058, nHDon), functional group counts (nCp), molecular shape (ASP, L/Bw), composition (nSK, nBT) and polarizability (DISPp) were identified by the GPTree model search as the best correlated with NP uptake (see Table 5.6). As reported elsewhere (Epa *et al.* 2012), strong correlation between hydrogen bonding capacity, molecular shape and cellular uptake was observed. Two of the selected descriptors, nBO and SCBO, can be viewed as a representation of the degree of unsaturation that specifies the amount of hydrogen that a compound can bind and hence can be related to the hydrogen bonding ability of a molecule. The findings of GPTree analysis regarding the large contribution of lipophilicity, hydrogen bonding and molecular shape descriptors in the cellular uptake behaviour of NPs is in great agreement with the results of previous nano-(Q)SAR studies.

Table 5.6 Descriptors selected from a pool of 147 chemically interpretable descriptors for nanoparticle cellular uptake dataset GPTree (the ones highlighted in yellow are in common with Epa *et al.* (2012))

Descriptor Name	Descriptor Block	Interpretation
nN	Number of N atoms	Hydrogen bonding capacity
O-058	(atom-centred fragments) =O	Hydrogen bonding capacity
SPAM	Average molecular span R	
NCp	Number of terminal primary C(sp3)	Functional group
DISPp	Displacement value / weighted by polarizability	Molecular shape and polarizability
nHDon	Number of donor atoms for H-bonds (N and O)	Hydrogen bonding capacity
ASP	Asphericity	Molecular shape
L/Bw	Length-to-breadth ratio by WHIM	Molecular shape
nSK	Number of non-H atoms	Chemical composition
nBT	Number of bonds	Chemical composition
nBO	Number of non-H bonds	Degree of unsaturation (hydrogen-bonding)
SCBO	Sum of conventional bond orders (H-depleted)	Degree of unsaturation (hydrogen-bonding)
G(N…O)	Sum of geometrical distances between N..O	Substructure descriptor

5.4.3 Case Study III – Gajewicz Dataset

5.4.3.1 Data pre-processing

The third dataset modelled with GPTree software consists of 29 descriptors (e.g. 16 quantum-mechanical descriptors, 11 image-based descriptors and 2 experimental measurements) representing the structural features of 18 metal oxide NPs (Gajewicz *et al.* 2014). The authors also measured the cytotoxicity of 18NPs to human keratinocyte (HaCaT) cell line using the CytoTox-Glo cytotoxicity assay and calculated LC_{50} values for all NPs.

Firstly, since GPTree can only work with categorical endpoints, 18 NPs were divided into two homogenous clusters, e.g. low toxicity (9 NPs) and high toxicity (9 NPs), based on a threshold value of 2.4. Activity threshold was chosen based on the natural grouping of NPs with balanced distribution between toxic and nontoxic ENMs. There was no object falling near the

decision boundary (between 2.32 and 2.48), hence, there was no need to exclude any compounds from the analysis. The selection of classification threshold value has a direct influence on the modelling results. However, choosing a different activity threshold, for example 2.0, results in an unbalanced split of 2 nontoxic and 16 toxic NPs for which no significant model could be constructed. To ensure the validity of the data split, k-means clustering method was applied using XLSTAT statistic package (Fahmy 1993). In k-means clustering analysis, the selected criterion was Determinant (W), as it allowed to remove the scale effects of the variables. The results of k-means clustering were identical to the results of data split based on a threshold value of 2.4. Accordingly, 9 NPs (Al_2O_3, Cr_2O, Fe_2O_3, Sb_2O_3, SiO_2, TiO_2, V_2O_3, Y_2O_3 and ZrO_2) were assigned to the low-toxicity cluster (class 1 - negative response) while the remaining 9 NPs (Bi_2O_3, CoO, In_2O_3, La_2O_3, Mn_2O_3, SnO_2, NiO, ZnO and WO_3) were assigned to the high-toxicity cluster (class 2 - positive response).

Secondly, for validation purpose, the dataset was split into training (10 NPs) and test (8 NPs) datasets in the same way as in Gajewicz *et al.* (2014).

5.4.3.2 GPTree modelling results and statistical evaluation

After data transformation and splitting, 100 generations of trees were produced by GPTree using the training and test datasets. Elitism between 2 and 16 trees surviving was tried but no elitism gave the best results in terms of accuracy, so the results are presented for no elitism. 16 trees were computed in each generation, and 0.5% of the trees were mutated since low values of mutation rate were found to be more suitable for this dataset. These values were all chosen after recording the accuracy of best trees and the average accuracy of each generation on the training data. The best performing tree was obtained at the 39th generation, which achieved an accuracy of 100% on both training and test data. This tree is shown in Figure 5.5 while performance parameters for the model are given in Table 5.7.

Following the same procedure described in case study I, a standard Y-scrambling test was applied to the shuffled data to show the robustness of the developed nano-(Q)SAR model. The predictivity of the selected model was confirmed by the lower values of the average test accuracies (39-54%) of the

randomised models, compared to the accuracy of the actual model as assessed by the prediction accuracy on test set.

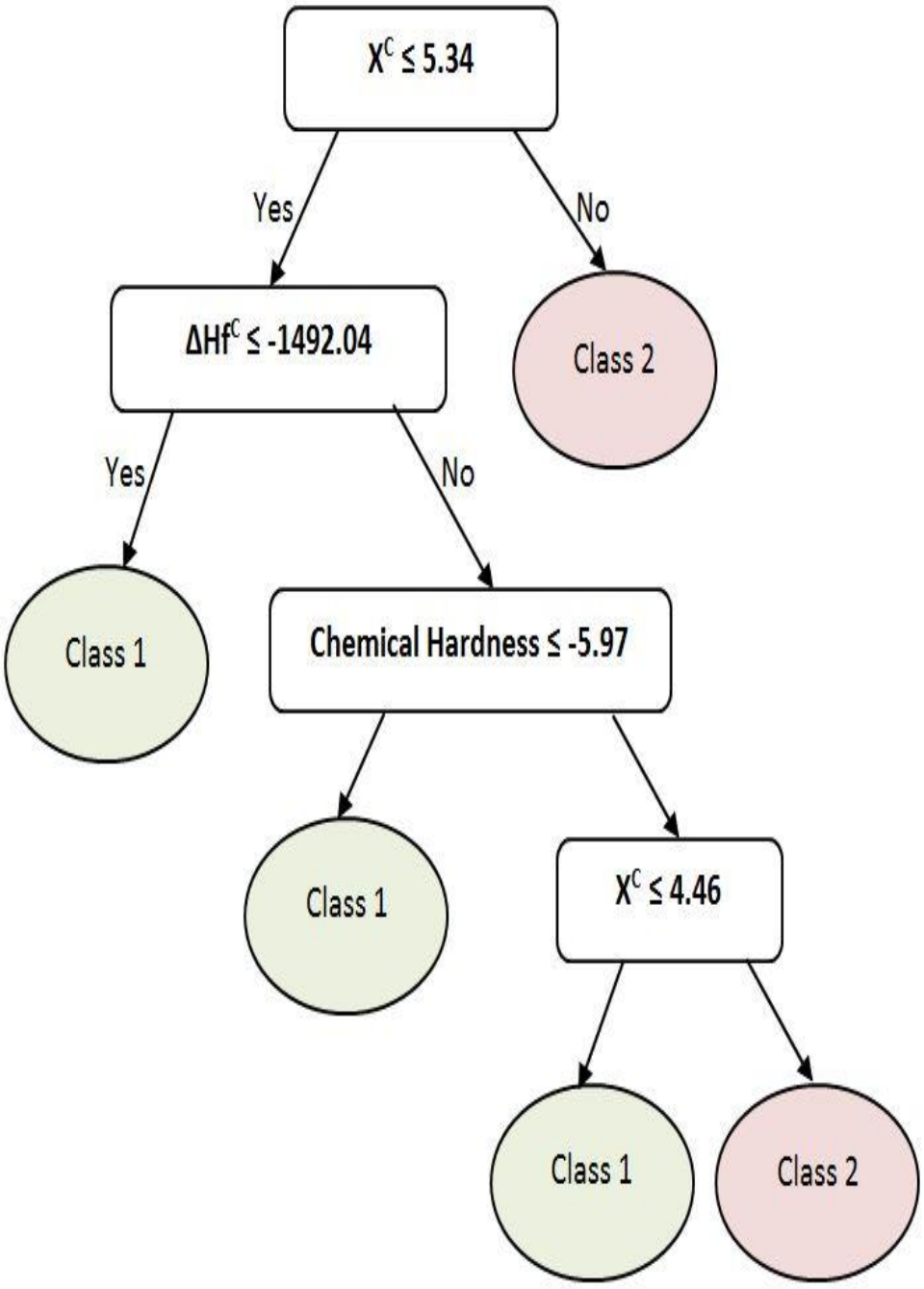

Figure 5.5 Decision tree produced by GPTree for cytotoxicity to human keratinocytes dataset (Gajewicz *et al.* 2014). The statistical measures of the performance are given in Table 5.7.

Table 5.7 Classification performance of the decision tree induced by GPTree and shown in Figure 5.5.

Training Set			**Test Set**		
	Predicted Class			Predicted Class	
Actual Class	Nontoxic	Toxic	Actual Class	Nontoxic	Toxic
Nontoxic	5	0	Nontoxic	4	0
Toxic	0	5	Toxic	0	4
Sensitivity	100%		**Sensitivity**	100%	
Specificity	100%		**Specificity**	100%	
Accuracy	100%		**Accuracy**	100%	

5.4.3.3 Model interpretation

As can be seen from Figure 5.5, the constructed decision tree model included following quantum-mechanical descriptors only: ΔHf^c (the enthalpy of formation of metal oxide nanocluster representing a fragment of the surface), X^c (Mulliken electronegativity of the cluster) and chemical hardness. Three descriptors were selected by GPTree with the most important one being the Mulliken electronegativity of the cluster (X^C). The results of GPTree are in very good agreement with the results of Gajewicz *et al.* (2014) who developed a nano-(Q)SAR model that utilised two molecular descriptors (e.g. ΔHf^c and X^c). As shown by the GPTree model given in Figure 5.5, metal oxide NPs with higher electronegativity were more toxic. Since the mechanistic interpretation of the constructed model based on these two descriptors is discussed elsewhere (Gajewicz *et al.* 2014), it will not be repeated here. The only extra descriptor selected by GPTree was chemical hardness, which corresponds to a half of the band gap of a chemical compound. Again, this finding is not surprising as the relevance of the band energy levels to adverse biological effects of metal oxide NPs has been previously reported by Zhang *et al.* (2012).

5.4.4 Case Study IV – Oh and Park

5.4.4.1 Data pre-processing

Oh and Park (2014) examined the role of surface properties in the exocytosis of gold NPs (GNPs) in macrophages. They reported the exocytosis rates of 12 GNPs expressed as the percentages of GNPs leaving the macrophage, and a set of 6 experimental descriptors including zeta potential, hydrodynamic diameter, and maximum wavelength both prior to and after protein coating (Oh and Park 2014). Bigdeli, Hormozi-Nezhad and Parastar (2015) extracted 12 nano-descriptors (e.g. size, surface area, aspect ratio, corner count, curvature, aggregation state, and shape) from TEM images of GNPs and calculated 10 descriptors such as charge densities, adjusted aspect ratio, charge accumulation values, spectral size, spectral surface area, spectral aspect ratio and spectral aggregation by combining TEM extracted image descriptors with experimental parameters. Our study used 28 descriptors, comprised of experimental parameters, TEM extracted image descriptors and nano-descriptors together with the observed exocytosis values of GNPs in the GPTree analysis.

The results of Oh and Park (2014) demonstrated that cationic GNPs exhibited the lowest rate of exocytosis while PEGylated ones showed the highest rate. They also noted that the remaining ones, anionic and zwitterionic GNPs, exhibited medium exocytosis rates. Based on these findings, the initial set of 12 GNPs was divided into three homogenous clusters, e.g. low (3 GNPs), medium (6 GNPs) and high exocytosis (3 GNPs). For validation purpose, 1 compound from each cluster was randomly selected resulting in the formation of a test set of 3 GNPs.

5.4.4.2 GPTree modelling results and statistical evaluation

Based on the initial pool of toxicity dataset and clustered toxicity data, 100 generations of trees were produced with each generation consisting of 600 trees. 16 trees competed in each tournament and 0.015 of trees were mutated. The best performing decision tree shown in Figure 5.6 was selected based on mode accuracy on classifying training and test datasets. The corresponding statistical performance measures are given in Table 5.8. This

tree model achieved both training and test accuracies of 100 at the 35th generation.

Y-scrambling was applied to randomised response data to demonstrate the robustness of the developed nano-(Q)SAR model. A random number generator was used to allocate the integer between 1 and 3. GPTree analysis was then carried out with the same parameters on the randomly shuffled response data. This process was repeated 3 times. The averaged test accuracies reached in Y-randomisation test runs (1-27%) were similar to those expected by chance (33%), much lower than achieved by the model (100%), indicating that the method has produced a robust model.

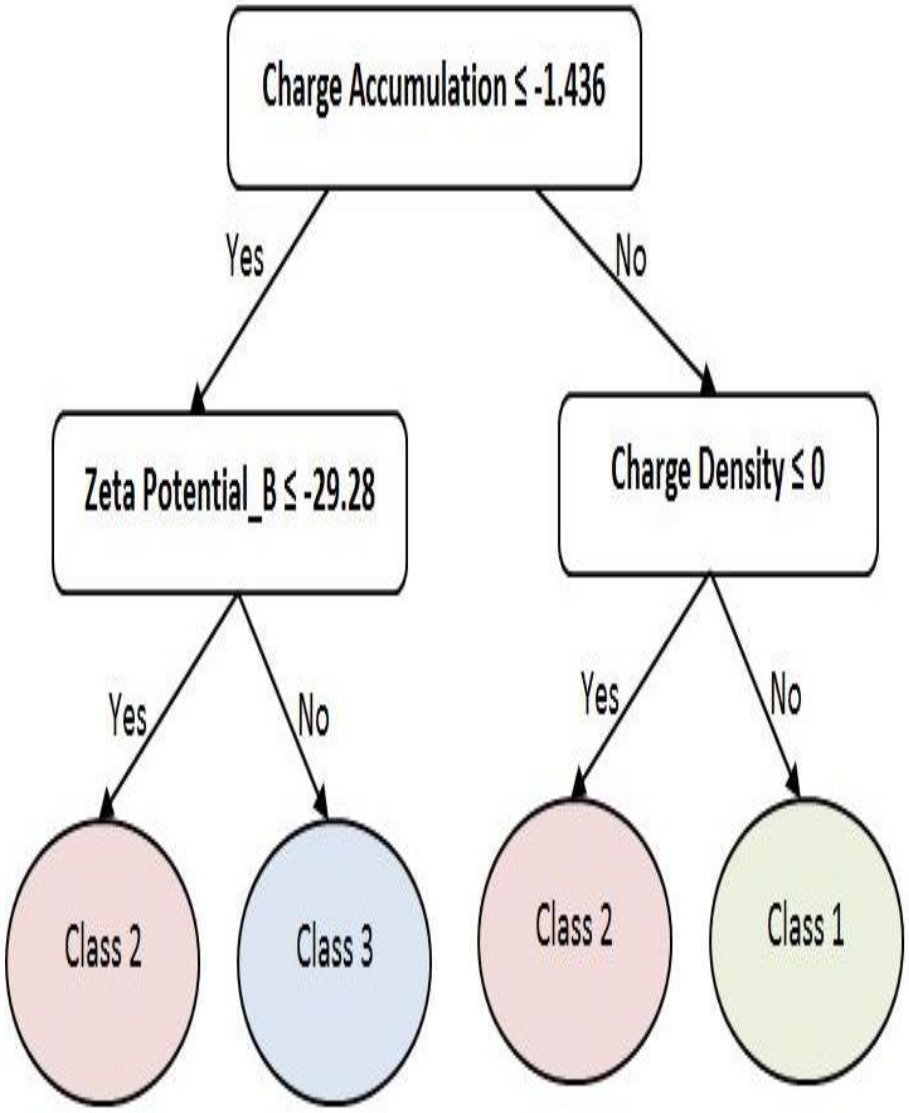

Figure 5.6 Decision tree produced by GPTree for exocytosis of gold nanoparticles dataset (Oh and Park 2014). The statistical measures of the performance are given in Table 5.8.

Table 5.8 Classification performance of the decision tree induced by GPTree and shown in Figure 5.6.

Training set				**Test set**			
	Predicted Class				Predicted Class		
Actual Class	Low	Mediu	Hig	Actual Class	Low	Mediu	Hig
Low	2	0	0	Low	1	0	0
Medium	0	5	0	Medium	0	1	0
High	0	0	2	High	0	0	1
Sensitivity	100%			**Sensitivity**	100%		
Specificity	100%			**Specificity**	100%		
Accuracy	100%			**Accuracy**	100%		

5.4.4.3 Model interpretation

The descriptors selected from a pool of 28 descriptors by the GPTree model include charge accumulation, zeta potential and charge density values before coating. This finding are completely consistent with the previous results of previous studies (Bigdeli, Hormozi-Nezhad and Parastar 2015) which showed that charge density, zeta potential, charge accumulation and circularity have the highest impact on the exocytosis of GNPs in macrophages. GPTree results showed that high (or positive) values of zeta potential prior to protein corona formation resulted in higher exocytosis of GNPs in macrophages. Also in line with the findings of previous studies (Bigdeli, Hormozi-Nezhad and Parastar 2015; Oh and Park 2014), our GPTree analysis results demonstrated that particle size had no effect on the exocytosis pattern of GNPs, while surface characteristics were the main factors influencing the exocytosis rate.

5.5 Discussion

Using four literature datasets, it has been demonstrated that GPTree was clearly capable of correctly classifying the biological response data from cells exposed to diverse NPs and of identifying the key NP descriptors associated with their toxicity. The accuracy of the model predictions was satisfactorily high and clearly highly statistically significant relative to the classification rate due to chance.

Interpretability of models was also an important reason for investigating the applicability of GPTree to modelling of NP biological effects. The data sets were chosen for the case studies because they have been modelled by others, allowing us to determine how the relatively sparse model parameters chosen by GPTree compared with these earlier studies and with the known mechanisms of toxicity where these have been identified or suggested. In the first general cellular toxicity case study, two parameters, the conduction band energy and ionic index of metal cation, were identified as suitable descriptors for metal oxide NPs. Previous studies (Zhang *et al.* 2012; Liu *et al.* 2013b) showed that cytotoxicity tended to increase with decreasing values of the ionic index, and for conduction band energies in the range of -5.5 and -3.9 eV, close to the estimated range of standard redox potential couples in biological medium (typically in the range of 4.84 - 4.12 eV) (Liu *et al.* 2013b; Zhang *et al.* 2012; Nel *et al.* 2006; Burello and Worth 2011c).

In the cellular uptake of NP case study, two different descriptor datasets were used to generate the nano-(Q)SAR model. For the descriptor dataset of 389 Dragon descriptors, 12 descriptors related to lipophilicity, atomic masses, symmetry associated with structure, charge distribution and connectivity indices were found to be predominantly affecting the cellular uptake behaviour of NPs. Additionally, the results showed that *drug-likeness* score can potentially be used to judge the NP's cellular uptake behaviour since it takes into account the most important parameters (lipophilicity and hydrogen bonding), which seem to have an influence on cellular uptake. For the descriptor dataset of 147 chemically interpretable descriptors, 13 descriptors representing the hydrogen-bonding characteristics, functional group counts, molecular shape, composition and polarizability were found to be significant

predictors of cancer cell uptake. The findings of GPTree analysis regarding the large contribution of lipophilicity, hydrogen bonding and molecular shape descriptors in the cellular uptake behaviour of NPs is consistent with earlier studies (Fourches, Pu and Tropsha 2011; Fourches *et al.* 2010; Chau and Yap 2012; Epa *et al.* 2012).

For the cytotoxicity to human keratinocytes dataset, the descriptors selected by GPTree were the enthalpy of formation of metal oxide nanocluster representing a fragment of the surface (ΔHf^c), the Mulliken's electronegativity of the cluster, X^c, and the chemical hardness. The former two descriptors are consistent with the properties reported to be important for cytotoxicity of metal oxide NPs (Gajewicz *et al.* 2014; Puzyn *et al.* 2011a). In addition, the chemical hardness corresponding to the reactivity was found to be an influential parameter on the cytotoxicity of NPs.

In the exocytosis of gold nanoparticles in macrophages case study, the optimal descriptors for predicting the exocytosis were the charge accumulation, zeta potential and charge density. These findings are in line with previous studies revealing an association between surface characteristics of GNPs, especially high positive surface charge, and their exocytosis patterns in macrophages (Oh and Park 2014; Bigdeli, Hormozi-Nezhad and Parastar 2015).

The two main issues hampering the development of computational models in nanotoxicology and limiting usefulness and reliability of data-driven models are the lack of nano-specific molecular descriptors and the scarcity of high-quality and systematically derived data on ENM characterisation and hazard. To build robust, predictive models, not only the amount of data but also about the diversity, quality, consistency, and accessibility of those data is critically important. Additionally, experimentally derived parameters used in models data can be highly dependent on experimental procedures (e.g. dispersion protocols, environmental conditions, concentrations, protein number and concentrations etc.). If the characterisation or biological data are not complete or representative of the material or the in vivo toxicity, then it is not possible to accurately model the relationships between NP physicochemical characteristics and biological activity, no matter how robust and accurate the

computational modelling approaches are. Ideally, a complete characterisation dataset should include not only intrinsic and primary properties of ENMs, but also their extrinsic properties influenced by the environments or changing over time. Computational models are well able to deal with such rich data and temporally dynamic data sets (Le *et al.* 2013).

It is now well recognised that the collection of a considerable amount of high quality data on both nano-characteristics and nano-toxicity is the key to successful application of SAR-like computational approaches like GPTree to ENMs. The acquisition of such data in a timely and cost effective manner can only be possible with the integration of more efficient data generation systems such as high-throughput toxicity screening (HTS) analysis and faster, more systematic and complete characterisation systems into nanotoxicity research. Once a significant amount of systematically obtained biological data for properly-characterised ENMs become available as a consequence of HTS testing efforts and standard ENM characterisation protocols/methods, the (Q)SAR-like computational methods will be much more valuable and effective in predicting ENM toxicity. Another important issue is the construction of an appropriate ontology for the nanosafety domain to support data integration from different sources and facilitate computational studies (Robinson *et al.* 2015). Such an ontology encompassing ENMs is currently under development in EU projects such as eNanomapper (eNanoMapper) .

The quantitative or qualitative nano-(Q)SAR approach is also very promising for other applications that link physicochemical characteristics of ENMs to endpoints such as the exposure, toxico-kinetics and environmental behaviour. Nano-(Q)SAR-like approaches can potentially identify links between different toxicity endpoints (e.g. cellular cytotoxicity and genotoxicity) or the same toxicity endpoints measured in different assays (e.g. cellular ATP assay and LDH release assay) or under different conditions (e.g. different cell lines such as A549 or CaCo2). As with toxicities of industrial chemicals, it is likely that SAR-type approaches that use in vitro assays as descriptors will be capable of predicting in vivo activity when sufficient data are available.

Finally, in order to increase confidence of the outcome of nano-(Q)SAR approach, computational modellers should manage the expectations of

experimentalists and regulators on the predictive capability of models based on small data sets with limited domains of applicability. More effort should be put into model interpretation using computational methods like GPTree to help understand the complex interplay between many physiochemical properties of NPs and their environments. Providing sensible interpretation and explanatory information regarding the observed system behaviour can be as important as developing statistically significant nano-(Q)SAR models itself.

5.6 Concluding Remarks

The focus of this study was to show how decision tree construction tool can accurately predict the toxicity and transport properties of NPs in cells, and elucidate the key physicochemical properties that lead to high toxicity of ENMs. It has been demonstrated using case studies that DT analysis is a powerful tool for categorical predictions of biological activity in nano-(Q)SAR investigations. The DT models were usually very sparse, ≤13 predictors selected from a large pool of descriptors, with an accuracy ranging between 98 - 100% and 86 - 100% on training and test data, respectively.

Overall, the genetic programming based decision tree construction algorithm shows considerable promise in its ability to identify the relationship between molecular descriptors and biological effects of ENMs. The selected decision tree models yielded (external) prediction accuracy of 86-100%. Other statistical test (e.g. Y-randomisation) was also performed to demonstrate the robustness of the selected models. In each case, the scrambled data gave much lower test accuracy data than the original data clearly proving the relevance of the selected nano-(Q)SAR models. This work is a first step in the implementation of genetic-programming based DT construction algorithm to nano-(Q)SAR studies. There are a number of opportunities to expand this work and fully evaluate the capabilities of GPTree in the context of nano-(Q)SAR toxicity modelling.

Chapter 6
Nano-(Q)SAR Model Development: Partial Least Squares

Regression methods are essential components of (Q)SAR applications. There are two methods that are commonly used to develop regression-based (Q)SAR models: Multiple Linear Regression (MLR) and Partial Least Squares (PLS). Although both methods have proved their applicability in (Q)SAR modelling, the latter provides several advantages that are particularly attractive in nano-(Q)SAR research. For example, unlike MLR, PLS can handle collinear input data and underdetermined dataset (e.g. fewer data objects than variables). PLS also has the advantage in that it can simultaneously derive accurate and easily interpretable models for more than one response variable. These advantages make PLS especially useful for regression applications in nano-(Q)SAR modelling.

This chapter shows the use of an empirical regression method, PLS, as a tool in nano-(Q)SAR model development by applying it to five different nanotoxicity dataset. Prior to applying PLS regression, the datasets are independently centred and scaled to unit variance in order to bring all of the variables into proportion with one another. PLS is then carried out on the pre-processed data. The results suggested that the PLS approach is well suited to estimate the parameters influencing the toxicological response and to model the relationship between descriptors describing the physicochemical properties of a number of ENMs and toxicity endpoints measured on the same set of materials.

6.1 Introduction

PLS is a linear regression technique which can be considered as an effective tool in handling large datasets associated with nanotoxicology research. It can be used as a visual aid to identify the key features that are potential sources of the observed toxicity and to formulate the relationship between physicochemical properties of ENMs and their biological activity. Although PLS is sometimes regarded as the extension of the MLR method (Keri and Toth 2003; Scior *et al.* 2009), it employs different strategies to

establish a linear relationship between independent and dependent variables. Unlike MLR, PLS works well when there are several noisy and inter-correlated descriptors in the dataset and it allows multiple responses to be modelled simultaneously (Eriksson and Johansson 1996). PLS avoids collinearity problem by constructing new latent variables governing the process which are then used for modelling and predicting the response variable. Additionally, PLS has the advantage of easy implementation and interpretation while the main disadvantage of this method is its inability to capture non-linear correlations.

This chapter focuses on the application of regression analysis to model the association of physicochemical properties with biological activity. PLS was selected and used as a regression method to correlate descriptors of ENMs with their toxicity.

6.2 Methodology

In this chapter, regression-based nano-(Q)SAR models were developed for toxicity potential of diverse ENMs using five different datasets.

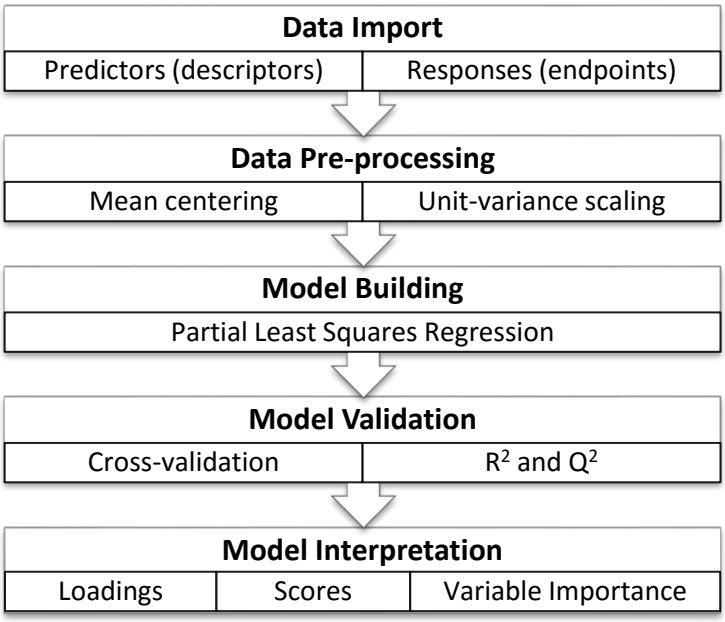

Figure 6.1 Modelling steps followed in the development of mathematical nano-(Q)SAR equations

Fig. 6.1 illustrates the modelling approach followed in developing PLS models. The pre-processing of data prior to PLS consisted of mean centring and unit variance scaling: the descriptors and toxicity values were standardised by subtracting the mean and dividing by the standard deviation. Pre-processing of the data (e.g. data standardisation) and PLS analysis were both carried out using the SIMCA-P 10 software.

6.3 Results

The results of the regression based nano-(Q)SAR models of six nanotoxicity datasets are presented in this section to illustrate the applicability of PLS to (Q)SAR modelling studies of ENMs.

6.3.1 Case Study I - Wang Dataset

The original dataset consisted of a large number of toxicity endpoints and experimental descriptors measured for 18 ENMs (e.g. carbon-based and metal oxide ENMs). From the set of 18 ENMs, a homogeneous group of compounds including 10 metal-based ENMs was selected and used in the development of PLS models. The complete dataset used in this study is given in Table 6.1.

Table 6.1 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.		

Initially, the relationship between 33 physicochemical descriptors (x variables) and 18 toxicological responses (y variables) was modelled simultaneously using the PLS method. The resulting graphs given in Fig. 6.2, PLS score and weight plots, provide an overview of the relationship between descriptors and toxicity endpoints.

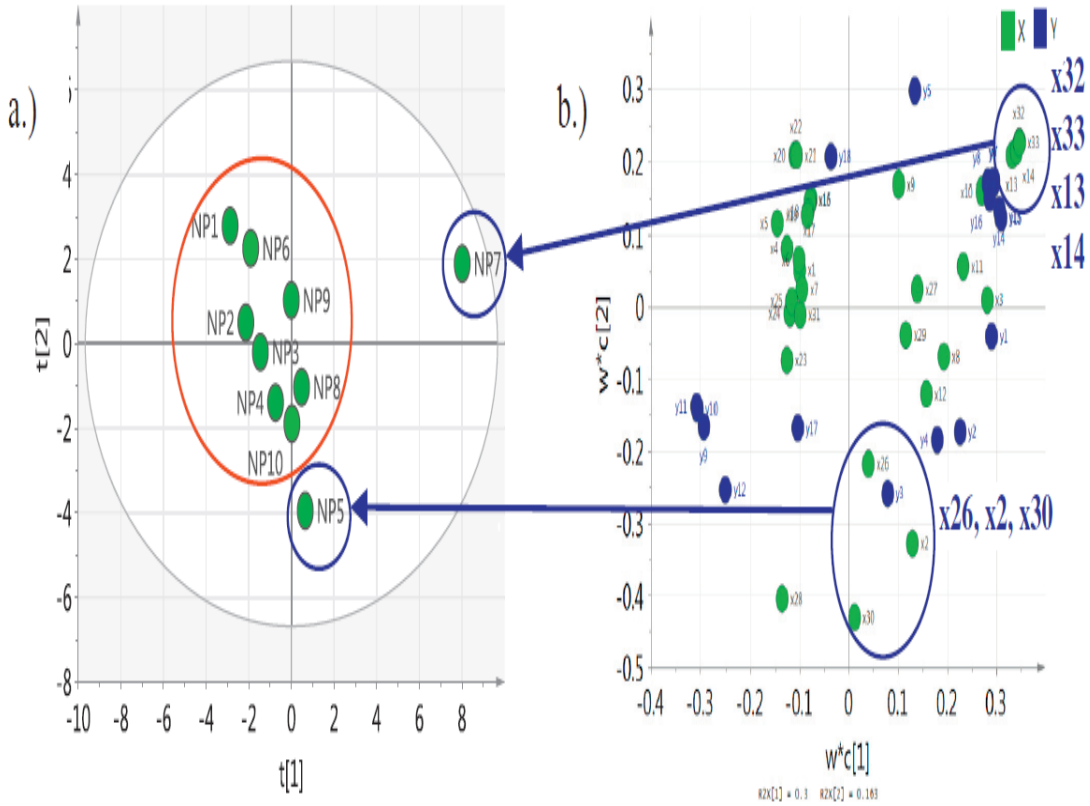

Figure 6.2 (a) PLS t1/t2 score plot which reveals the relationship between observations (i.e. nanomaterials); (b) PLS weight plot (loading plot) corresponding to Fig. 6.2a

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 t1/t2 score plot given in Fig.6.2a 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.

By looking at the PLS weight plot (Fig.6.2b) showing how the x-variables are combined to form PLS X-scores (t1/t2), the descriptors contributing to the positioning and separation of NPs can be identified. By comparing these two plots given in Fig. 6.2, 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.

The PLS weight plot given in Fig. 6.2b can be further employed to identify the activity-activity relationships between different toxicity endpoints. It can be seen from the loading plot 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, one 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 can be observed. Viability (y9-12) and MTT assay (y17) responses lie on the other side of the origin since they show the percentage of viable cells and their higher values are associated with the low level of toxic effects.

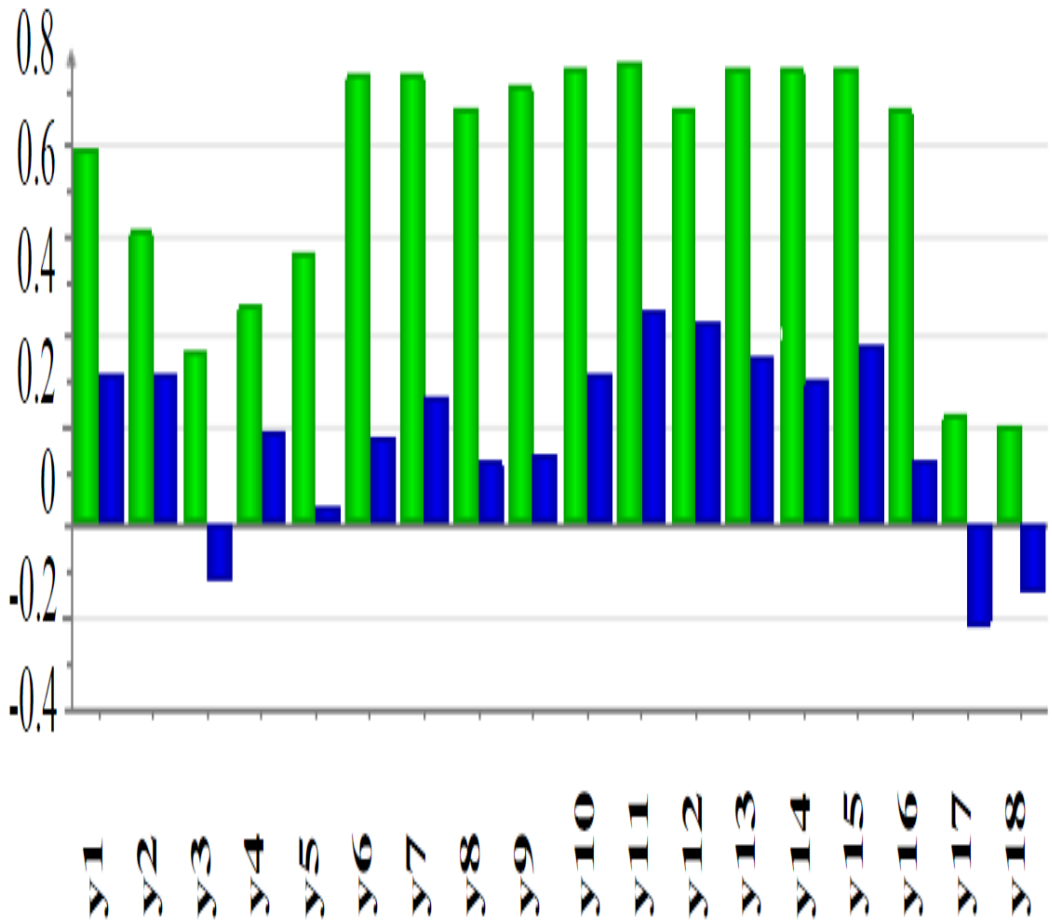

Figure 6.3 R2 (green) and Q2 (blue) values showing the goodness of fit and the goodness of prediction, respectively.

The cumulative R^2 and Q^2 values of the each variable are given in Fig. 6.3. 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.

As a second step, PLS was 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) were replaced with a new single variable (y1), principal component that accounts for 95% of the total variation. PLS score plots given in Fig. 6.4a and b, t[1]/t[2] and u[1]/[u2], show the relationships among observations in the X space and Y space, respectively.

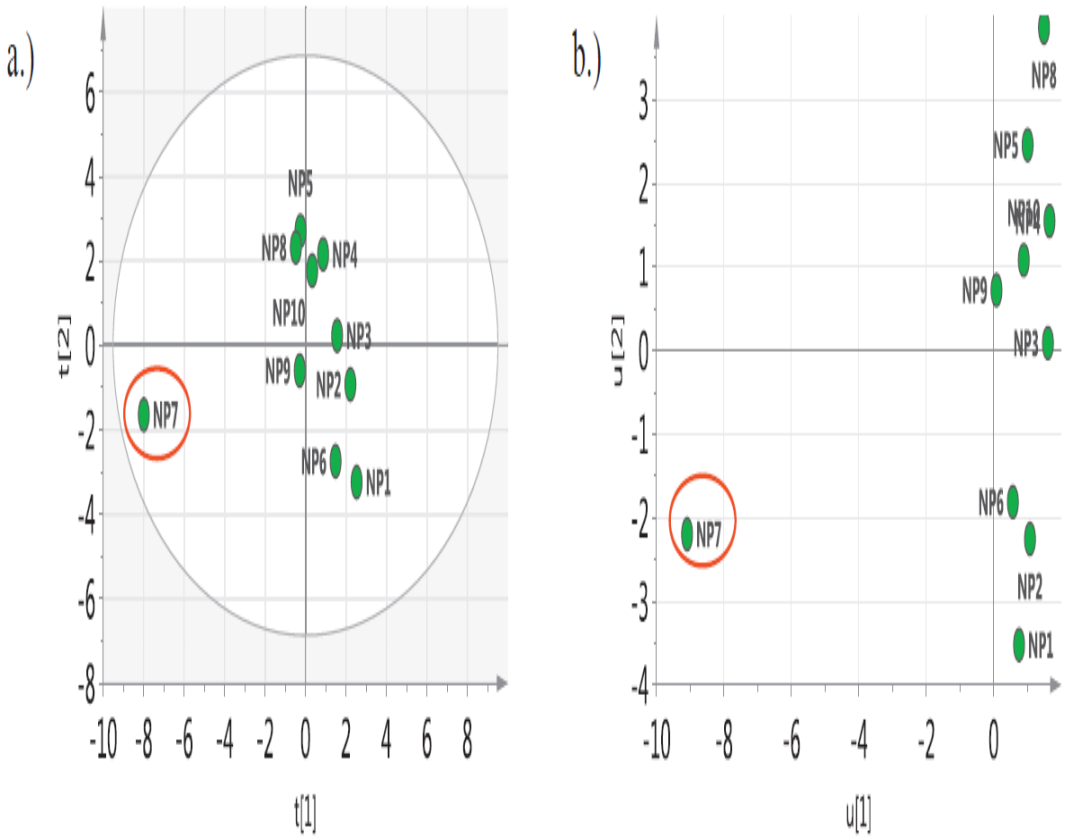

Figure 6.4 (a) PLS t1/t2 score plot which reveals the relationships between observations (i.e. nanomaterials) in the X space; (b) PLS u1/u2 score plot which reveals the relationships between observations in the Y space

It is clear from the score plots of the model that zinc oxide (N7) is separated from the main cluster, in both X and Y space. The weight plot given in Fig. 6.5 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 away from the origin have large influence.

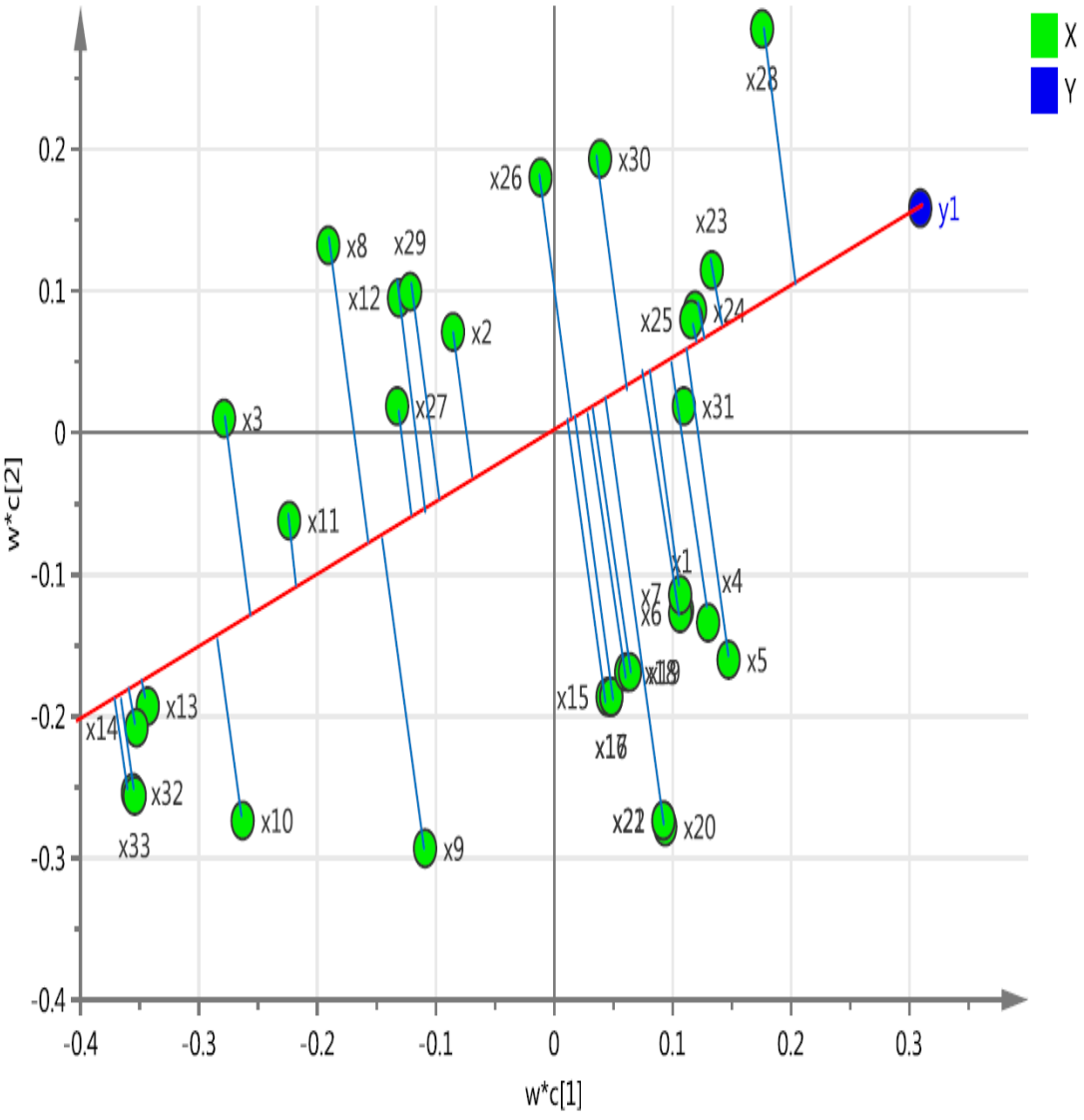

Figure 6.5 PLS weight plot with an illustration of how to interpret a weight plot (w*c [1]/ w*c [2])

Therefore, the impact of variables on the viability can be summarised as follows:

- Variables that have a 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 make a 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.

6.3.2 Case Study II - Gajewicz Dataset

PLS was performed to find the quantitative relationship between the cytotoxicity of 18 metal oxide NPs and quantum-mechanical descriptors which were identified as relevant features by DT analysis. Three descriptors which were previously identified as relevant features were used to model the cytotoxicity: ΔHf^c (the enthalpy of the formation of metal oxide nanocluster representing a fragment of the surface), X^c (Mulliken electronegativity of the cluster) and η (chemical hardness).

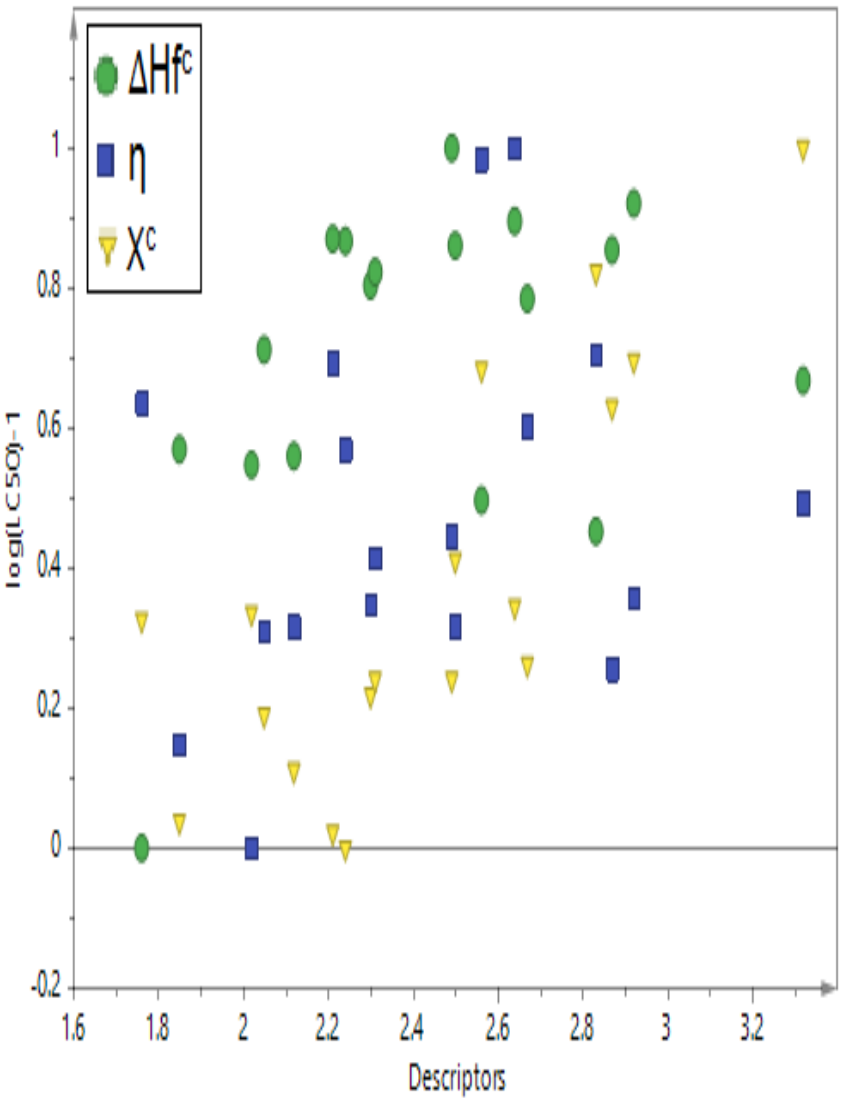

Figure 6.6 Scatter plot displaying cytotoxicity (log(LC50)-1) versus 0-1 scaled values of quantum mechanical descriptors

Figure 6.6 displays the correlation between log values of $(LC_{50})^{-1}$ and the three quantum-mechanical descriptors. A statistically significant model was built using cytotoxicity data and the three descriptors. The data used was converted to Z-scores by centring and scaling to unit variance prior to model construction. Two latent variable model (Fig. 6.7), explaining 71% of the variance of the independent variables and 92% of variation the dependent variable, was found to be the optimal model since it achieved the highest prediction accuracy (70%) with the lowest error metric (0.12).

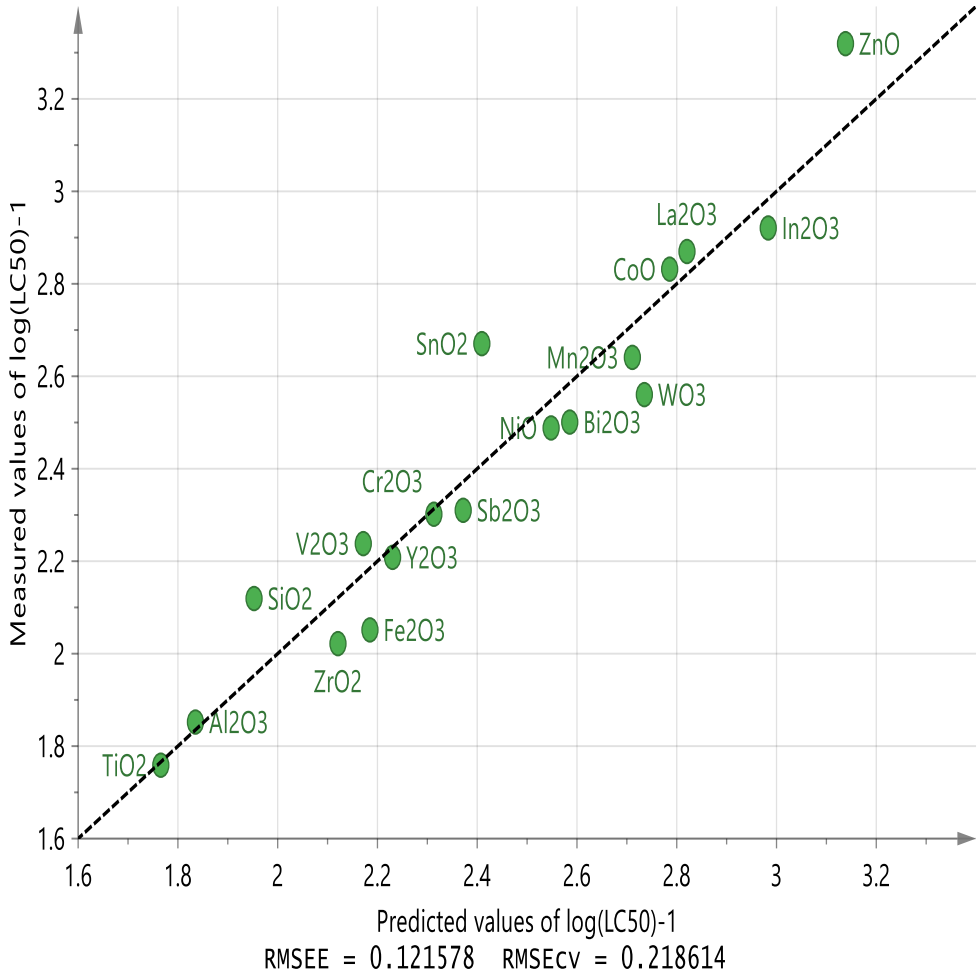

Figure 6.7 Plot of experimentally measured versus predicted values of log (LC50)-1

The correlation coefficients showing how well the PLS model fits the data (R^2Y) and predicts new data according to cross validation (Q^2Y) were calculated to be 0.92 and 0.7, respectively. Large values of R2 (>0.8) and Q2 (>0.5) indicated that the developed model was statistically significant and had good predictivity. The room mean square error of estimation (RMSEE) and root mean square error of cross-validation (RMSEEcv), which indicate how close the fitted line to data points, were 0.12 and 0.22, respectively. Outliers in the PLS model can be found based on widely scattered data points that are far away from the regression line (e.g. SnO_2).

The variable importance (VI) plot given in Fig. 6.8 summarises the significance of the descriptors for predicting the response variable. A VI score

larger than 1 indicates “very important” descriptors while values lower than 0.5 shows “unimportant” independent variables. In line with the previous findings reported in Chapters 4 and 5, electronegativity of the cluster (Xc) was observed to be the most important descriptor (VI score=1.45), followed by formation enthalpy of metal oxide nanocluster (VI score=0.77) and chemical hardness (VI score=0.55). The error bars shown in the variable importance plot represent 95% confidence intervals estimated using the jack-knife (e.g. the coefficient can be considered significant when the confidence interval does not include zero). Clearly, toxicity is most sensitive to changes in electronegativity, as the confidence interval does not cross zero.

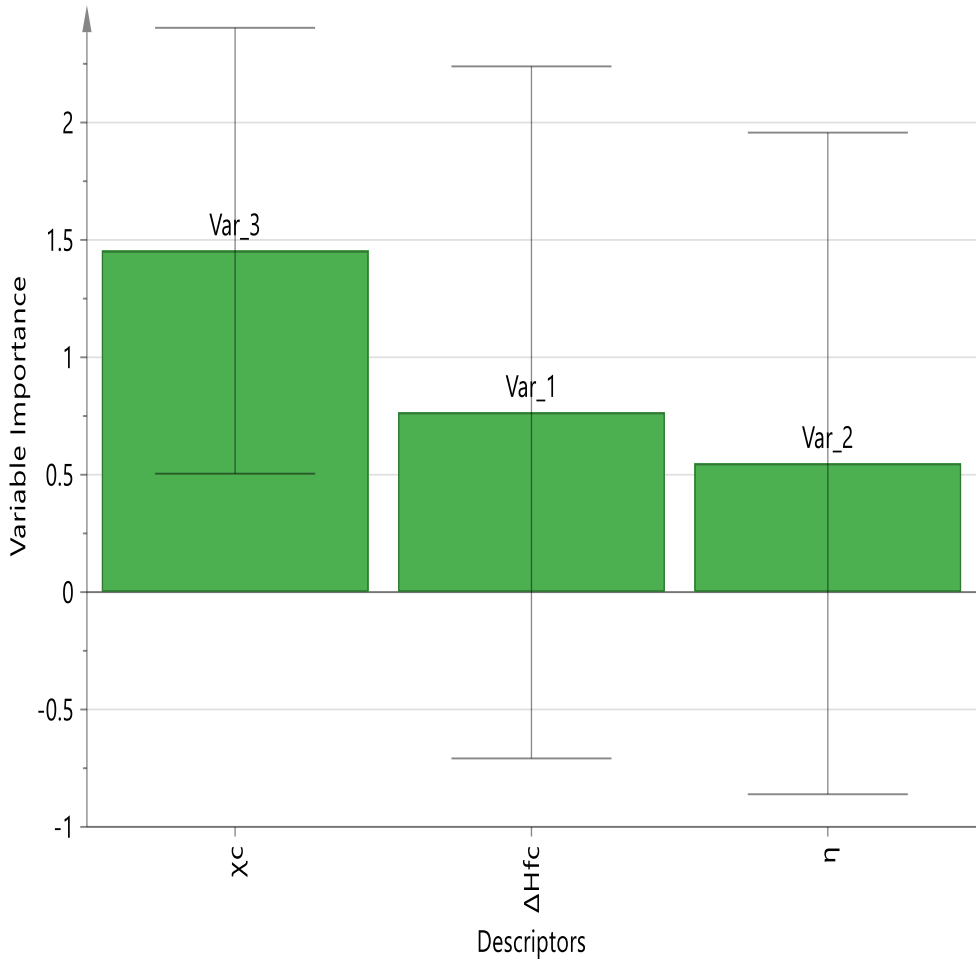

Figure 6.8 Variable importance plot showing the contribution of each descriptor to the PLS model

6.3.3 Case Study III - Oh and Park Dataset

The dataset including the exocytosis rate of 12 GNPs and a pool of nano-descriptors was imported into the software SIMCAP and PLS regression analysis was performed on auto-scaled data. Since all the descriptors are not calculated or measured on the same unit, unit variance scaling was employed prior to model building. PLS for the centred and scaled data gave five latent variable model describing 99% variability of the dependent variable and 92% of the variance of the independent variables. The correlation coefficient R^2Y and cross-validation correlation coefficient Q^2Y were 0.99 and 0.84 respectively, indicating very good model performance. Figure 6.9 shows the measured versus predicted values of exocytosis rates of 12 GNPs.

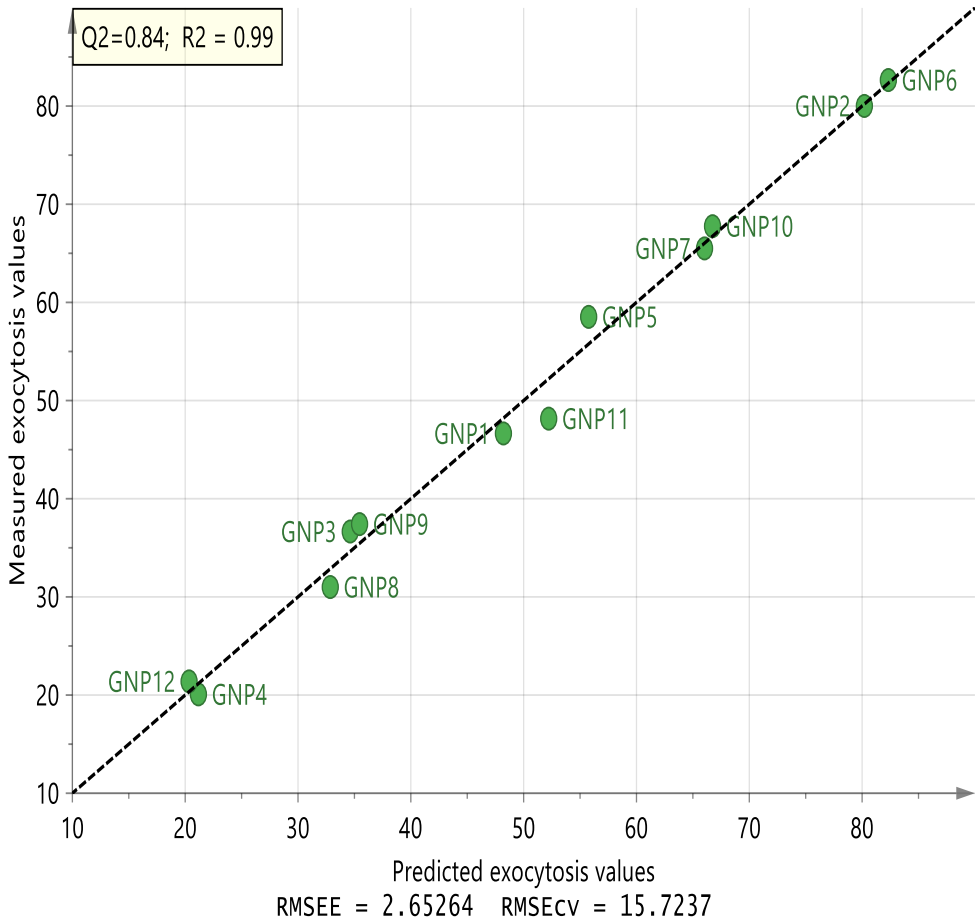

Figure 6.9 Plot of experimentally measured versus predicted values of exocytosis

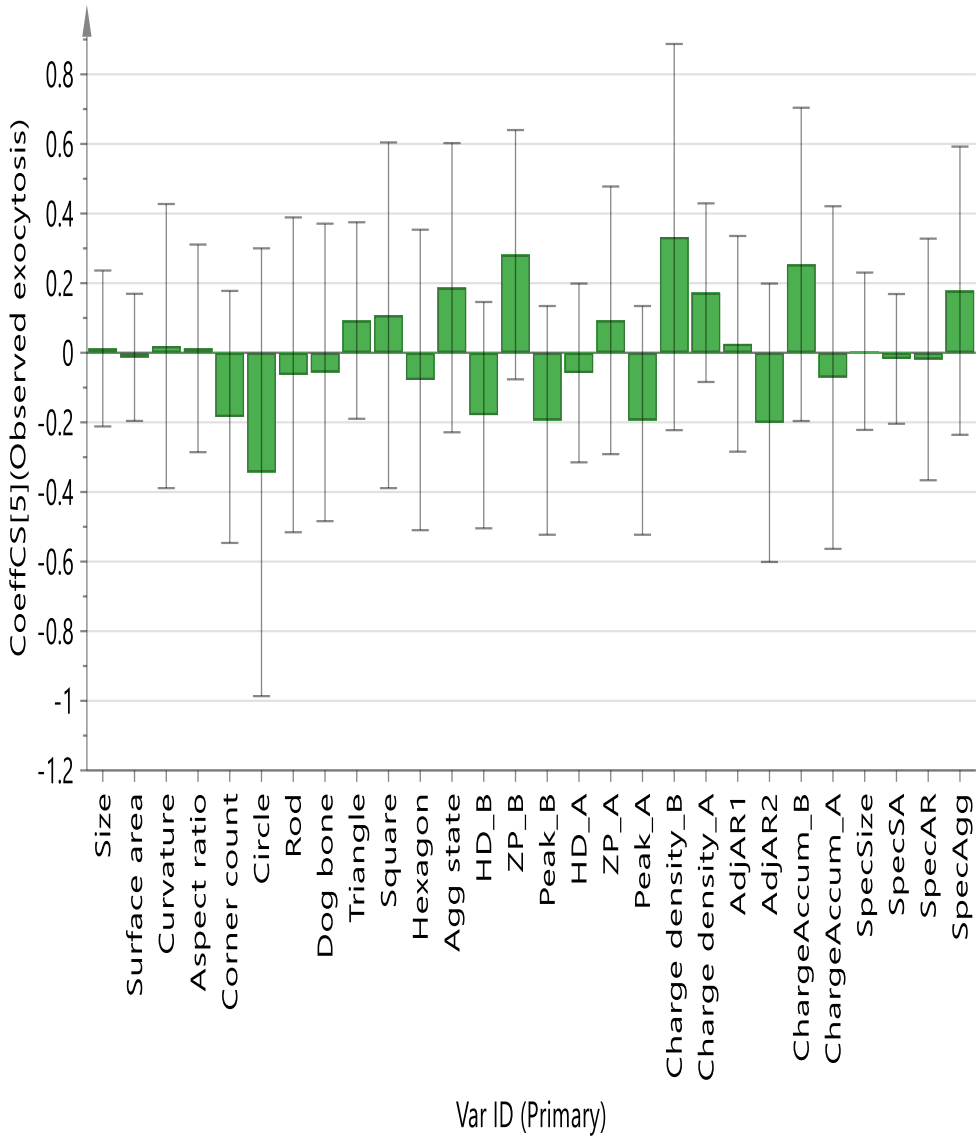

Figure 6.10 PLS coefficients related to mean-centred and scaled X variables for 5 latent variable model.

The coefficient plot given in Fig. 6.10 illustrated that most of the descriptors were redundant and should be excluded. The magnitude of the coefficient describes the variation in the response variable when the descriptor varies between 0 and 1 and shows how strongly the response-variable is dependent on the X-variables. Coefficient values below 0 express negative correlation: as the value of X variable increases, the value of Y variable decreases. Another way of visualizing the relative importance of the descriptors is the variable importance plot given in Fig. 6.11.

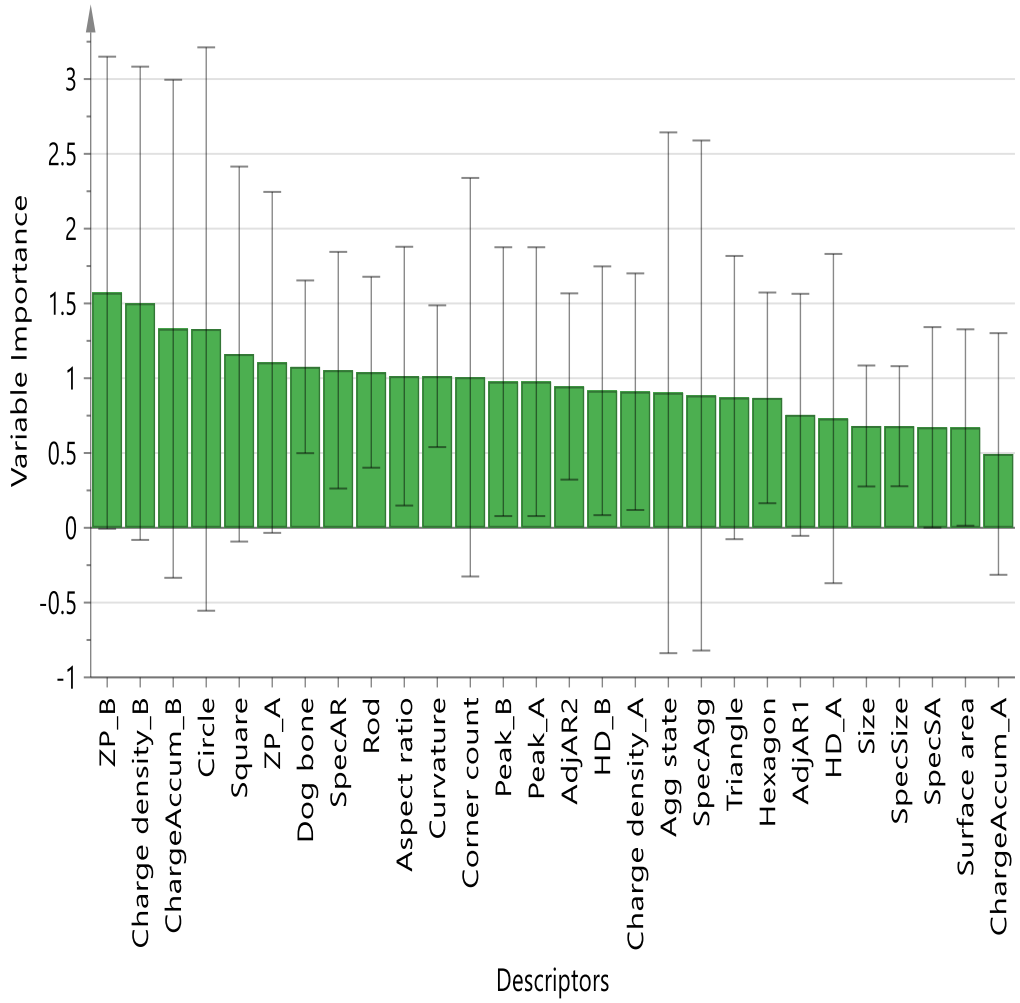

Figure 6.11 Variable importance plot showing the contribution of each descriptor to the PLS model (jack-knifed confidence intervals are shown on the plot)

The variable importance (VI) plot given in Fig. 6.11 summarises the contribution of the each descriptors to the model. A predictor can be considered significant when the VI score is greater than 1. Evaluation of the variable importance plot together with coefficients, five descriptors (shape descriptors (circle and square), zeta potential B, charge density B and charge accumulation B) were identified to be the most influential parameters. A new PLS model based on these five descriptors was built. Figure 6.12 shows the observed versus predicted values of exocytosis. 71% variance of all the independent variables and 76% variance of the dependent variable was explained by two extracted PLS components.

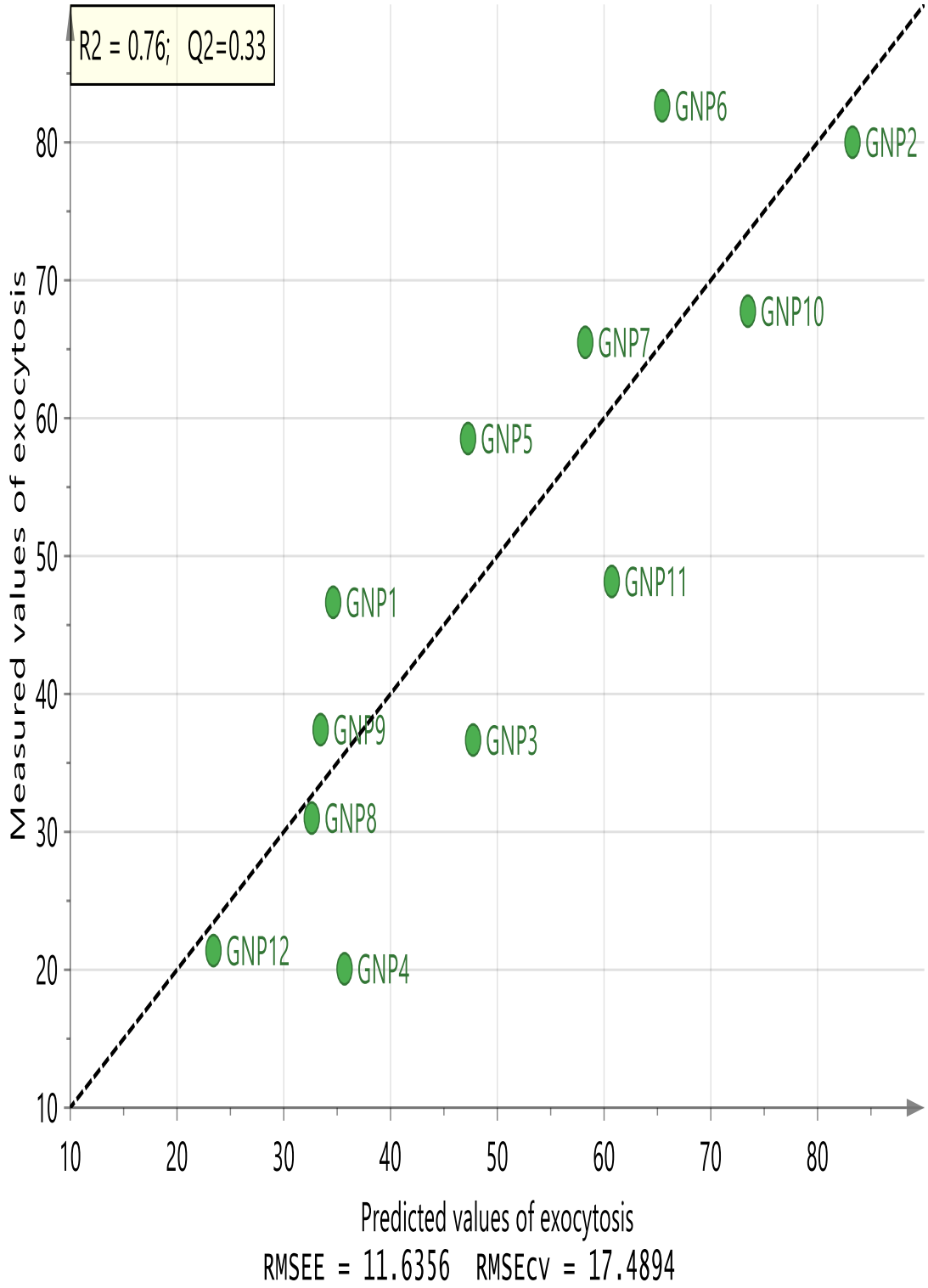

Figure 6.12 Plot of experimentally measured versus predicted values of exocytosis

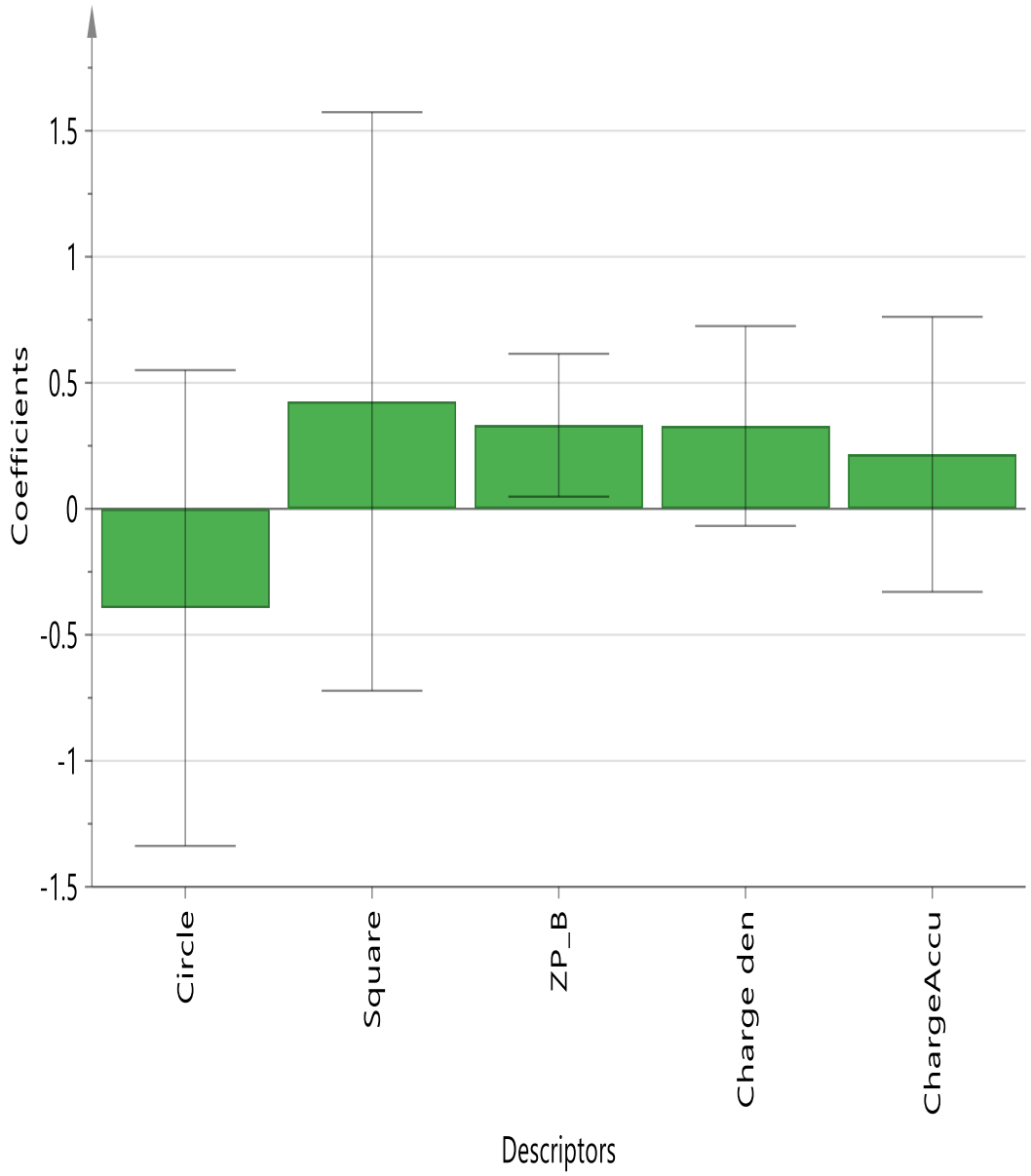

Figure 6.13 The coefficients plot

Concerning the validation metric Q^2, a critical value of 0.4 is generally admitted for biological models. Therefore, the Q^2 value of 0.33 is not an acceptable value in light of the (Q)SAR models and indicates poor predictive ability. The coefficients plot given in Fig. 6.13 summarises the relationship between exocytosis rate and the five descriptors. This plot also illustrates a very poor model since most of the coefficients are insignificant as indicated by the confidence intervals crossing zero.

6.3.4 Case Study IV - Shaw Dataset

PLS analysis of the 44 various ENMs, with biological activity profiles (i.e. ATP content, reducing equivalents, caspase-mediated Apoptosis, and mitochondrial membrane potential) as dependent variables and the four experimentally measured descriptors as the independent variables resulted in a three-component PLS model. The PLS model explained 87% of the variance of X variables and the percentage variation in Y that was explained was about 16%. The coefficient overview plot given in Fig. 6.14 shows the coefficients for all response variables while the variable importance plot (Fig. 6.15) displays the relative significance of each descriptor for response variables being modelled.

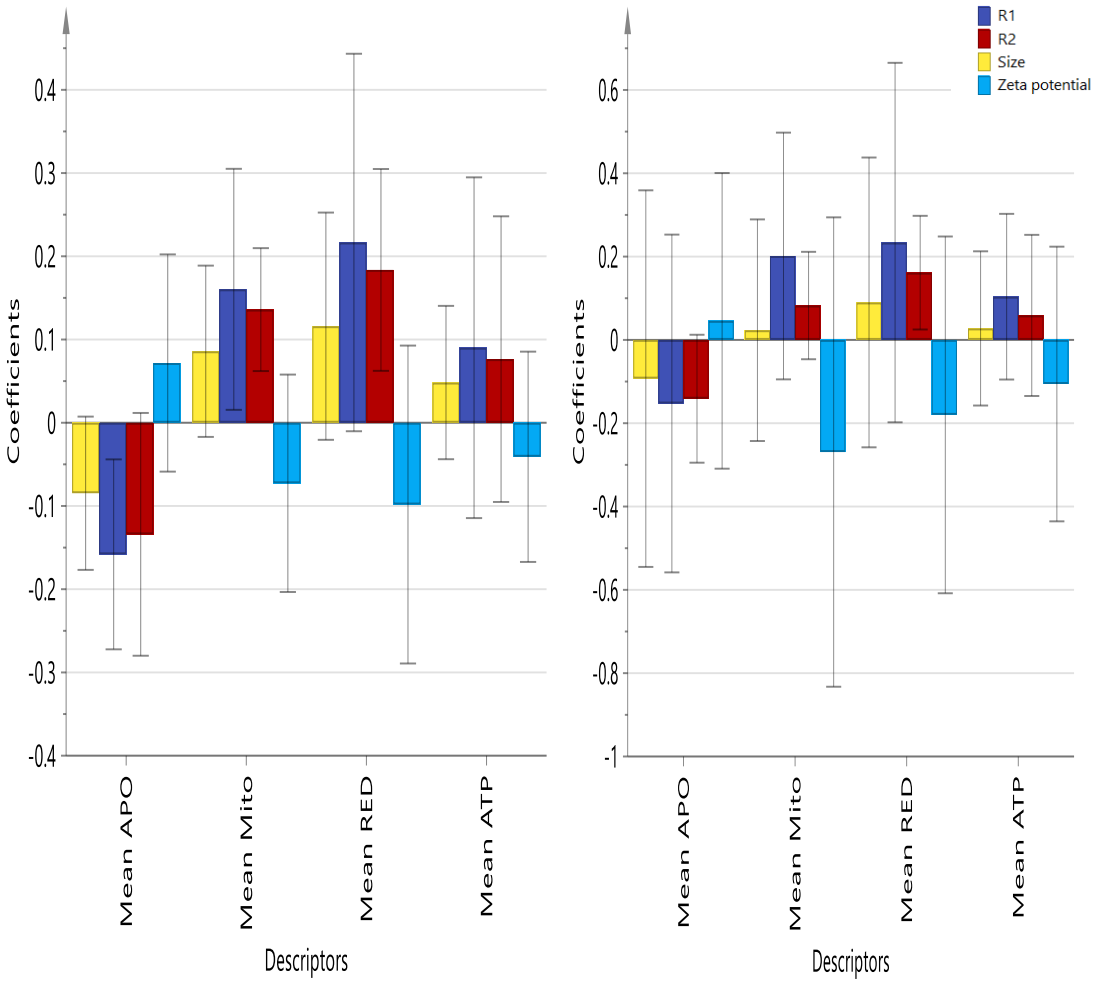

Figure 6.14 Coefficient overview plots for the first (left) and second (right) PLS components

Both the coefficients overview plot and variable importance plot show that relaxivity values, R1 and R2, were the dominant factors governing the toxicity of 44 various NMs while size and zeta potential were less significant than others. Confidence interval bars also confirm the significance of R1 and R2 since they do not include the value of 0.

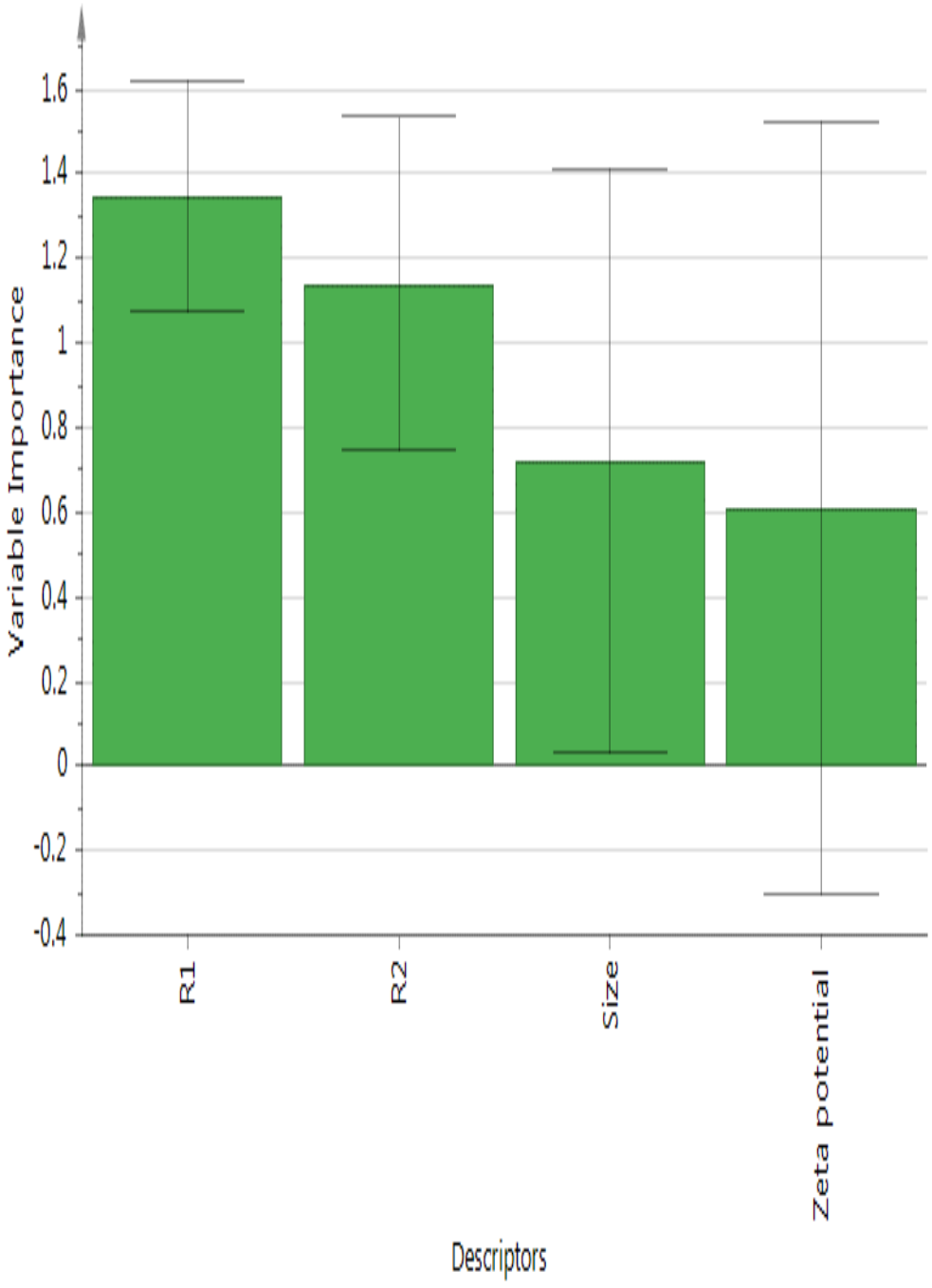

Figure 6.15 Variable importance plot

The cross-validation coefficient, Q2, was less than 0.1, indicating very poor internal predictivity. Although PLS can handle multiple toxicity endpoints, simultaneous modelling of multiple endpoints usually cause a significant decrease in the predictive capability of the developed model. Therefore, a new PLS model using reducing equivalents assay results was developed. However, only a modest increase (10%) was obtained in the predictivity which suggested that, although the relative importance of relaxivity values for the toxicity was clearly shown in the PLS analysis, the toxicity values and four experimental descriptors gave poor regression models with low predictivity. The main obstacle was the limited number of descriptors representing the characteristics of 44 NMs.

6.3.5 Case Study V - Nanommune Dataset

When applying PLS to the Nanommune data, consisting of normalised Apoptosis results measured at four different doses, and three experimentally measured parameters (particle size, hydrodynamic size and zeta potential) and three indicator variables derived from core-type, shape and surface charge range, a four component model with the following (cumulative) performance statistics resulted: R^2X= 0.91, R^2Y=0.60, Q^2cum=0.24.

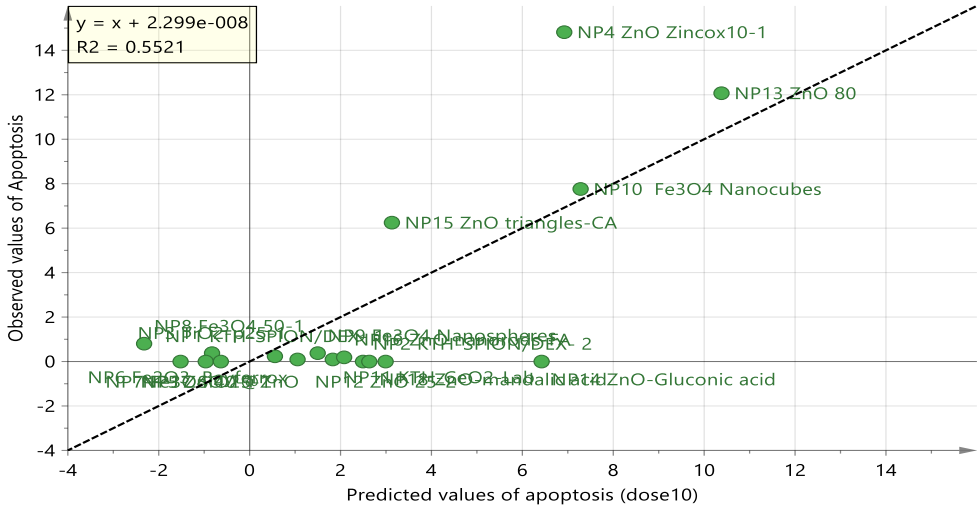

Figure 6.16 Plot of experimentally measured versus predicted values of Apoptosis at dose = 10 µg/ml

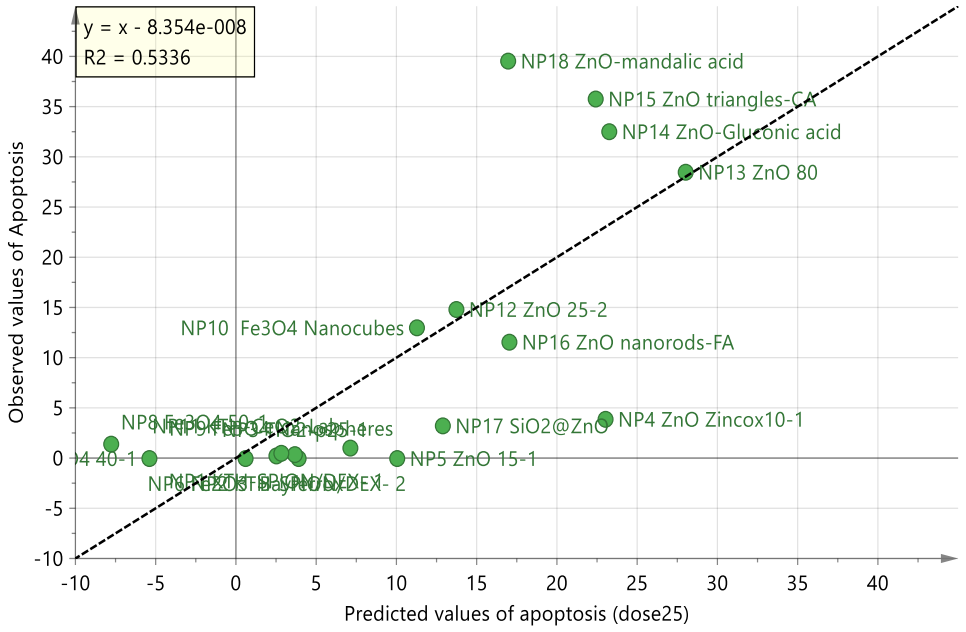

Figure 6.17 Plot of experimentally measured versus predicted values of Apoptosis at dose = 25 µg/ml

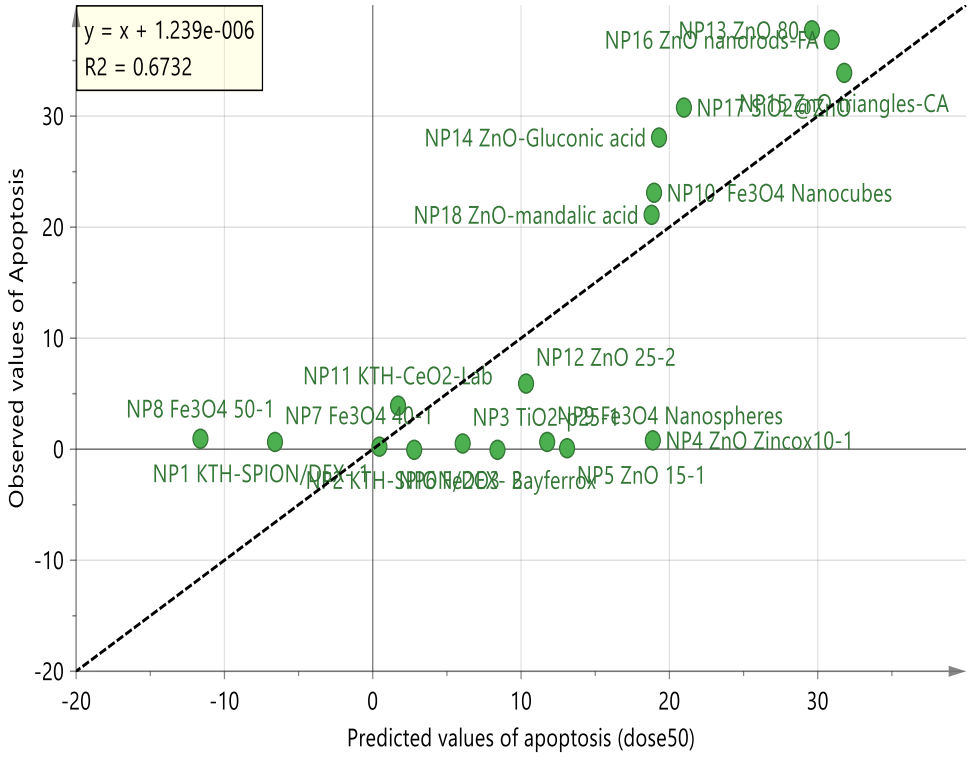

Figure 6.18 Plot of experimentally measured versus predicted values of Apoptosis at dose = 50 µg/ml

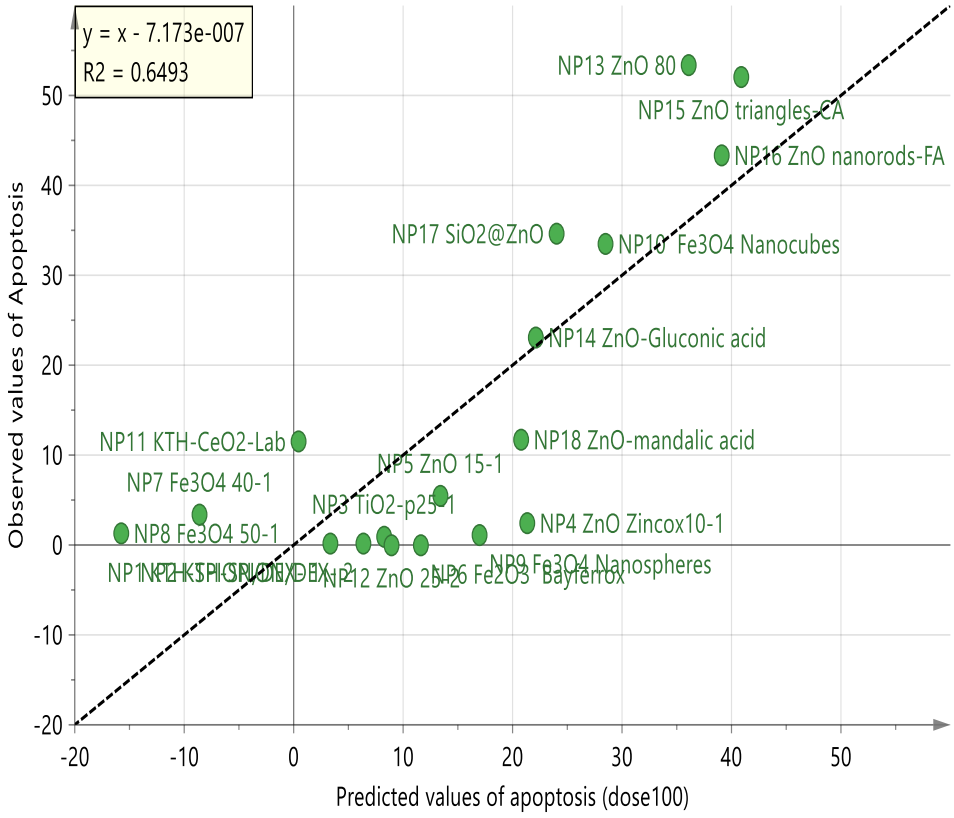

Figure 6.19 Plot of experimentally measured versus predicted values of Apoptosis at dose = 100 µg/ml

Figures 6.16 – 6.19 show the observed versus predicted values of Apoptosis at four different doses. As can be seen from these plots, the goodness-of-fit values (R^2) ranged between 0.54 - 0.67. One clue for understanding this deficiency in model fit was obtained by evaluating the distance of each NPs from the regression line. It was observed that one of the zinc oxide NPs, commercial Zincox 10 (NP4) was the main outlier that have a substantial effect on model fit. Regression models developed by excluding this NPs gave significantly higher model statistics (>0.7).

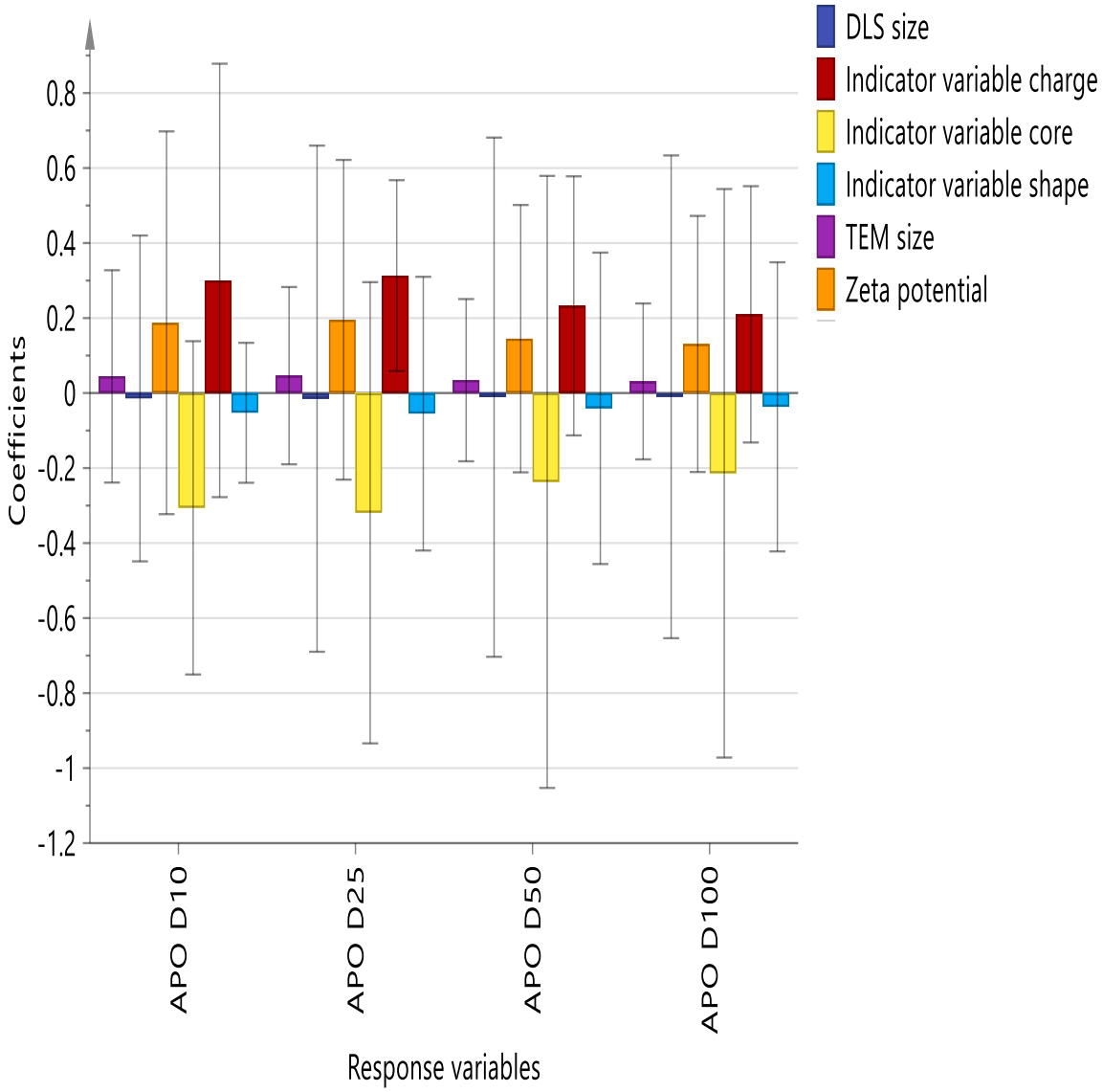

Figure 6.20 Coefficients overview plot

The coefficient overview plot given in Fig. 6.20 shows the coefficients for all response variables while the variable importance plot (Fig. 6.21) displays the relative significance of each predictor for the response variables being modelled. It is clear from these plots that the type of material core is the main factor controlling toxicity, while the surface characteristics (e.g. surface charge) showed relatively lower, but still substantial correlation with toxicological outcomes.

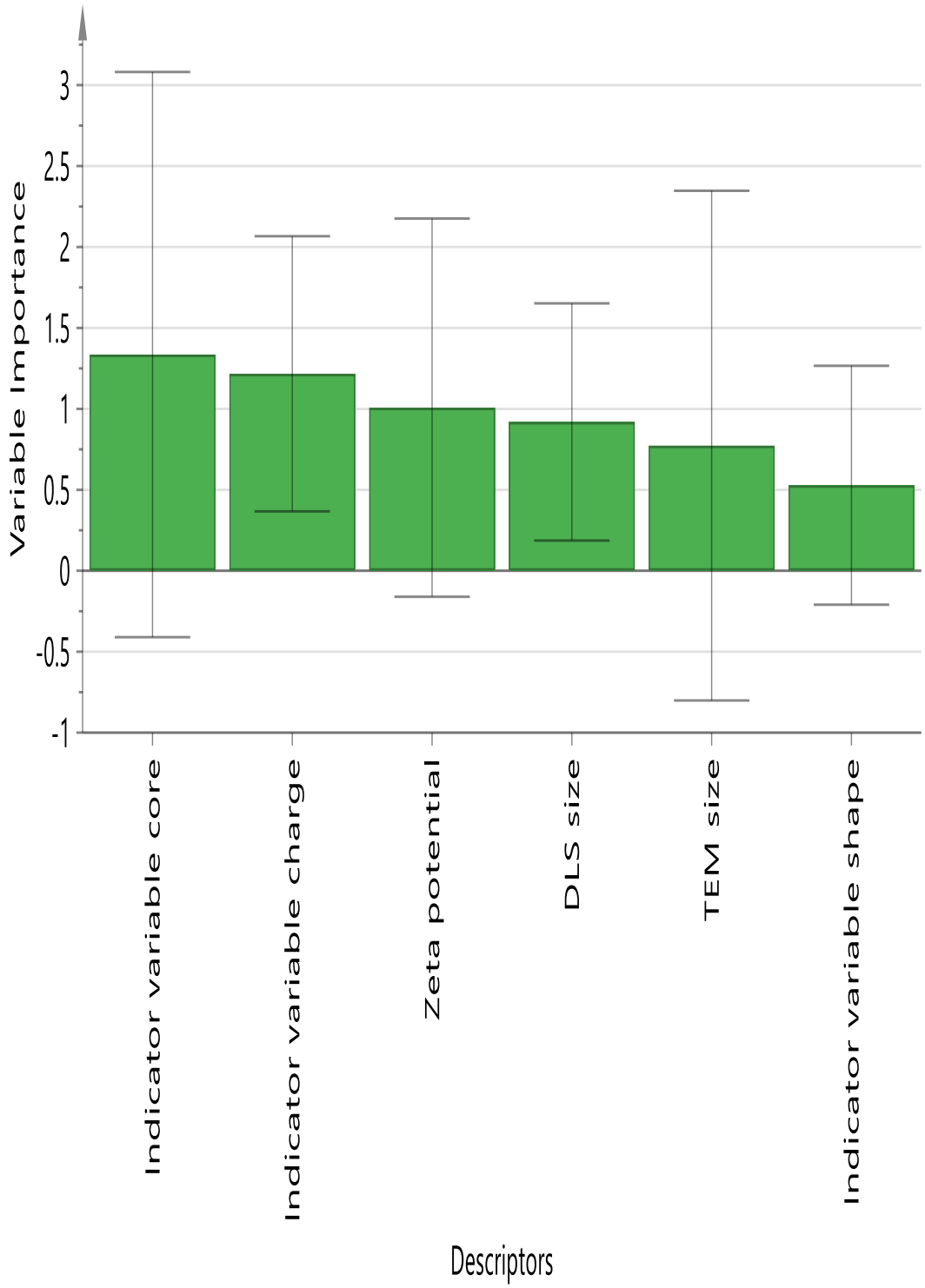

Figure 6.21 Variable importance plot

6.4 Discussion and Concluding Remarks

In this chapter, the use of a regression-based PLS approach to uncover and model the potential relationship between the toxicity and a number of structural and compositional features was introduced using five different case studies. Cross-validation was used as a diagnostic tool to assess the predictivity of developed models. PLS models were interpreted and assessed in terms of validity using several performance statistics such as a model's fit (R^2), prediction ability (Q^2), and plots displaying the measured versus predicted values of the response variables and coefficients. The coefficients and variable importance plots were used to identify the overall contribution of each predictor to the model.

In the first case study, the correlation between the 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. Although some case-specific correlations between the properties of ENMs and their biological activity were observed, it was not possible to generalise these findings for external ENMs.

In the second case study, a statistically significant PLS regression model based on three descriptors was developed. The PLS model achieved an R^2 value of 0.92 and a Q^2 value of 0.70 indicating that the correlation between the measured and predicted response values was significant. As the cumulative value of Q2 is satisfactorily larger than the 0.5 threshold, the PLS model can be considered as statistically significant and capable of being predictive. In the third case study, a good PLS model was built using the exocytosis data and the descriptors. Five descriptors, including two shape descriptors, zeta potential, charge density and charge accumulation after protein coating, were found to be the most significant predictors for exocytosis, since they had variable importance values higher than 1. A new PLS model based on these five descriptors only was developed. However, a significant decrease in the predictivity over the previous model was observed. PLS analysis of Apoptosis data belonging to 44 various ENMs and the four

experimental descriptors resulted in poor regression models, which might be a consequence of the very limited number of experimental descriptors available. In the last case study, analysis was performed on the dataset including four different response variables (e.g. Apoptosis measured at different doses) and the size descriptors. The results suggested that material core is the most influential factor governing the toxicity of metal oxide NPs, followed by an indicator variable encoded based on the magnitude of the zeta potential (e.g. +1 for values >+10, 0 for values between -10 and +10, -1 for values <-10). The analysis revealed that zinc oxide-core NPs (e.g. indicator variable -1) had higher toxicity Jurkat cell lines, compared to those that had an iron oxide-core. Positive coefficient values of zeta potential indicator variable suggested that NPs with high positive surface charge (for >+10) seemed to be more toxic than negatively charged NPs (<-10).

It is shown in this chapter that PLS analysis can successfully be used to assess the relative importance of descriptors for toxicity endpoints and to link ENM properties to toxicological outcomes. In addition to structure-activity correlations, PLS was also used to explore activity-activity relationships (e.g. case study 1), when multiple toxicity endpoints associated with the same set of ENMs were available. However, it was observed that the influences of nano-characteristics on different types of toxicity endpoints were significantly different. It was concluded that in order to develop predictive models, a more local approach should be taken, focusing on a single toxicological endpoint at a time since the parameters contributing to the particular types of side effects are different.

Chapter 7
Risk Reduction Stretegies for Nanomaterials

Predictive models such as (Q)SAR have great potential to fill in data gaps on nanotoxicity and to be used as a priority-setting method for risk assessment of ENMs. Once all the potential risks are identified by means of toxicity screening methods including in silico models (e.g. (Q)SAR), the next step is the implementation of risk reduction measures for those risks that are outside the range of tolerable limits.

This chapter a) reviews the need for risk management and reduction of ENMs; b) presents the list of risk mitigation measures that are applicable to ENMs; and c) provides an overview of the concepts of efficiency and cost of these risk reduction measures. The key task is to collect knowledge on available risk reduction measures applicable for ENMs with the ultimate aim of supporting a selection of the most suitable risk control measures in terms of efficiency and cost. To that end, an extensive literature review has been carried out and a questionnaire survey seeking information from organisations that are involved in nano-related activities has been conducted.

7.1 Introduction

Nanotechnology is an emerging field of science and engineering that has already been applied to a variety of industrial fields. Given the ever increasing use of ENMs in industry, it is essential to properly assess all possible risks that may occur as a result of exposure to ENMs (Kuempel, Geraci and Schulte 2012). Recent studies have shown that the distinctive characteristics of ENMs that have made them superior to bulk materials for some uses presumably, might also have a substantial impact on the level of risk they pose (Sharifi *et al.* 2012; Arora, Rajwade and Paknikar 2012). However, the potentially complex nature of ENMs presents a challenge for the existing general and product-specific regulation (Falkner and Jaspers 2012). In order to facilitate sustainable manufacturing of ENMs, it is desirable to develop transparent and comprehensive tools for risk assessment and management (Linkov *et al.* 2007).

The risk assessment process involves identification and evaluation of occupational, consumer and environmental exposure to hazardous substances, while risk management primarily focuses on the selection and implementation of effective measures to control and minimise risks. Over recent years, the need for coherent risk management strategies for ENMs has become apparent, leading to the publication of numerous technical reports and nano-specific guidelines (NIOSH 2012; NIOSH 2013; HSE 2013; FNV 2011). Numerous control-banding tools have been proposed (Zalk, Paik and Swuste 2009; Riediker *et al.* 2012; ANSES 2010; Jensen *et al.* 2013), that associate pre-defined hazard and exposure levels with risk management measures and link hazard with physical characteristics in a qualitative or semi-quantitative way. However, there are a number of critical issues and research needs in this field, the addressing of which is essential to ensure that risk management practices of nanomaterials are fully effective in real world contexts:

- Emerging strategies for the risk management of nano-enabled products through the lifecycle need to be considered to make risk management decisions;
- Pragmatic criteria affecting real-world implementation of risk management should be included in decision making;
- Risk assessment and risk management should be linked quantitatively (Gilbert, Adams and Buckingham 2011).

This chapter focuses on the development of a Risk Management Measures (RMM) inventory for ENMs based on the review of data available from the literature and nanosafety projects and a web-based questionnaire seeking information from companies that are involved in nanotechnology-related activities. RMM in this context can be defined as the collection of individual measures in the control strategy to reduce the hazard, emission and exposure to a nano-substance. It contains a list of existing risk reduction strategies (e.g. vacuum cleaner, dust suppression systems, glove boxes and exhaust ventilation) that are considered to be relevant for ENMs through their lifecycle. The RMM inventory contains information on two main criteria for comparing different risk reduction measures, efficiency and cost. The aim

here is to support the choice of an effective and economical risk control option when dealing with ENMs.

The efficiency of RMM can be measured based on the percentage reduction of exposure when using risk prevention measures compared to the uncontrolled case or below (exposure) limit which should not be exceeded when RMM is introduced. Apart from the efficiency of the control measures, their costs (e.g. associated with the installation, operation and maintenance of the risk reduction measures) are considered when deciding on the optimum risk control method since the achievement of risk reduction at the lowest possible cost is the common goal of several risk management approaches. A quantitative estimate of the cost of implementing a risk control measure is required to ensure that health, environmental and economic benefits are kept in balance.

The key goal of this chapter is to review risk management measures and tools for ENMs and to collect information on the cost and efficiency of these measures. The focus is on supporting the risk assessment and management of ENMs by ensuring that adequate risk control measures are in place.

7.2 Risk management of ENMs

Risk management decisions and actions are taken in response to risks identified in the risk assessment process. It is generally agreed that traditional risk management frameworks and tools do not cover all the issues associated with manufacturing, handling and using nanomaterials and hence need to evolve to become more sensitive to nano-specific issues (Marchant, Sylvester and Abbott 2008). Although a revised risk management methodology for nano-scale objects has not been approved yet, there are a number of technical reports and guidelines published by standard setting bodies (EPA 2012; ISO 2014; ISO 2012; ISO 2008) that provide guidance on risk management issues and control measures relating to ENMs. Additionally, there are a large number of guidance documents on working safely with ENMs that are published by international organisations, European projects and individual laboratories(OECD 2010a). For more detailed information, the reader is referred to the OECD‘s technical report(OECD 2010a) which

presents an excellent summary of existing guidelines for safe use and handling of ENMs in laboratories.

As in traditional risk management approach, once all potential hazards are identified, assessed and thoroughly evaluated, risk reduction strategies should be considered in a systematic approach (e.g. hazard control hierarchy). Essentially, there are two ways of mitigating or reducing the risk: hazard control through modification of ENM properties while maintaining their original features and functionality and exposure control reducing the release of ENM from industrial processes or consumer products or limiting the exposure of workers and consumers to ENM by means of administrative measures and behavioural guidelines. The main aim of hazard control is to remove the hazard from workplace through improved materials, equipment or process design. Although mitigating the health and environmental risks of nanoscale materials by integrating the safety into the design plan is considered to be one of the most powerful risk reduction strategies, its application to ENMs is challenging mainly because of the knowledge gaps on how to make ENMs safe and the difficulty of retaining the desired properties while changing the product design(Schulte *et al.* 2013) . Ideally, the hazard potential and exposure to hazardous material should be eliminated while maintaining the desired functionality. If the physical removal or replacement of a material that produces hazard is not practical, additional exposure control measures such as engineering controls, administrative controls and personal protective equipment (PPE) can be introduced and implemented to minimise exposure to the substance and hence reduce the health and environmental risks of ENMs. Additionally, understanding the behaviour of the ENM in different environments and identifying information gaps are additional issues that would be helpful when framing the problem of risk.

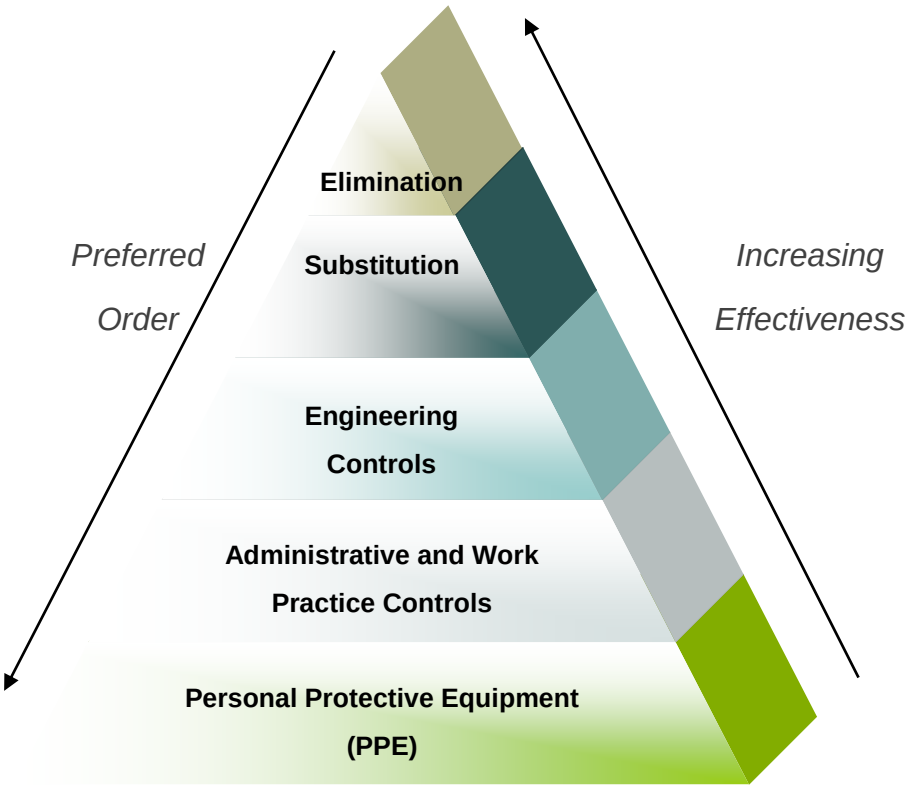

Figure 7.1: The traditional hierarchy of risk reduction measures

It has been mentioned in many of the published guidelines that risk management measures should follow the standard hierarchy of control strategies in order to eliminate hazard or reduce exposure (NIOSH 2012; NIOSH 2013; EPA 2012; Eija-Riitta Hyytinen 2015). The traditional hierarchy of controls given in Figure 7.1 describes the order that should be followed when choosing between viable control options for controlling risks in a reliable and cost effective manner. According to the traditional hierarchy of control, the most effective hazard control is the elimination of all hazards within a process (e.g. by replacing the process or use of a non-hazardous substance). If the complete elimination of hazard and risk at source is not practical, risk should be minimised by substituting the process or compound with a less hazardous (i.e. safer) alternative. The third most effective risk management strategy is the use of engineering controls, which require physical change to the workplace. The remaining control measures, namely administrative controls that are designed to enforce operational procedures to minimise release to a working area and PPE aiming to protect an individual person from risks to

health and safety, are least effective when used on their own because they rely on human behaviour and supervision. Ideally, these measures should be used in conjunction with more effective control measures if control at source of risk is very impractical. However, given the uncertain risks around ENM, the administrative controls affecting worker behaviour often play a greater role in risk management of ENM.

7.3 Methodology

Published literature from 2008 to 2015 was searched for studies on risk management of nanomaterials using Web of Science database. The following keywords have been used to identify the relevant studies: nano*, risk assessment, risk management, risk reduction, risk prevention, risk management measures, risk reduction measures, risk control measures, risk management strategies. The bibliographies of the identified articles were searched for further relevant studies. The project search on CORDIS with the same keywords revealed five relevant EU-funded projects, namely Scaffold, NanoMicex, NanoSafePACK, GUIDEnano, SANOWORK. The scientific findings from these projects were also inspected to find out whether they obtained information that may be relevant for the development of RMM inventory. Additionally, a further literature review was conducted to identify papers containing quantitative data on the efficiency and costs of each measure. These selection criteria have evolved from their importance in decision-making on risk management and allow the systematic selection of risk reduction measures rather than solely relying on expert judgement. Efficiency of risk reduction is the most important characteristic as it determines if exposure can be reduced by the RMM to a value (at least) lower than prescribed regulatory thresholds such as DNEL (Derived No-Effect Level). This helps the decision maker decide which, if any, RMM to select in a specific scenario. Cost is another important criterion to consider. For example, if implementing a RMM is 30% effective in reducing the exposure and an alternative RMM is 35% effective but costs five times more, then the decision maker may decide to go for the first option despite its lower efficiency, if both measures lead to a situation in which the exposure level is below DNEL. Cost of risk reduction needs to be considered for the selected RMM to ensure that

risk reduction to below these thresholds is as inexpensive as possible.

Finally, in addition to the review of projects and scientific literature, a questionnaire was developed to survey organisations involved in the manufacture, distribution, supply, handling, use and disposal of ENMs and to understand the efficiency and cost of the control measures that are currently available. The potential participants and their contact information were identified from nano-safety projects, nano-related websites, European NanoSafety Cluster Compendium 2015 and personal communications with relevant individuals. The RMM questionnaire was organised around several categories: general information about respondent and his/her organisation, engineering controls, organisational measures personal protective equipment (PPE) and future research directions. The draft questionnaire was tested internally by the world's leading chemical companies and revised according to their feedback. The participants were initially restricted to European nanotechnology companies. In the second stage, the questionnaire was distributed to a wide audience (e.g. Universities, Laboratories, Institutes, Technological Centres, SMEs, Industries etc.) in order to obtain a more holistic overview of the whole nanotechnology field. The following results cover the 36 participants (14 large-, 6 medium-, 8 small- and 8 micro-sized institutions/companies) who completed the survey.

7.4 Results and discussion

There are a number of ongoing studies and projects dedicated to improving the knowledge and understanding of risk management and reduction of ENMs. A short description of relevant EU-funded projects, together with their relevance to RMM inventory, is given in Table 7.1. It should also be noted that most of these projects are recent or ongoing and results are, in most cases, not yet published. Although the review of relevant projects allowed identification of the main sources of information relevant to RMM inventory, only a small amount of data is currently available from these projects. As the projects reach their conclusion, much more data will be available with time.

Table 7.1 EU-funded research projects for risk assessment and mitigation of ENMs

Project	Duration	ENMs	Aim	Relevance for RMM
SUN	2013-2017	Ag, TiO_2, WC-Co, CuO, SiO_2, MWCNTs and organic pigment	Development of a Decision Support System (DSS) to facilitate safe and sustainable manufacturing and risk management of NMs	Data on in-use efficiencies and protection factors for engineered ventilation control and PPE
Scaffold	2012-2015	TiO_2, SiO_2, Cellulose Nanofiber(NF), CNF, Nanoclays	Development of risk management models and tools for NMs in the construction industry	Data on the efficiencies of collective protections (e.g. LEV, glove-box) and PPEs
NanoMicex	2012-2015	ZnO, Fe_2O_3, TiO_2, Al_2O_3, $CoAl_2O_3$	Development of methods and strategies to reduce the potential risks of workers' exposure to NMs in the pigment/ink industry	Data on the efficiencies of common RMMs (PPE and engineering controls) against ENMs
NanoSafePACK	2011-2014	Nanoclays, Ag, SiO_2, ZnO, $CaCO_3$	Development of a best practices guide for safe handling and use of ENMs in packaging industry	Data on the efficiencies of PPE and Engineering Controls (LEV systems and filtration) against common nanofillers
GUIDEnano	2013-2017	Pristine synthesised NMs	Assessment and mitigation of nano-enabled product risks on human and environmental health	Data on the efficiencies of. safer-by-design approaches and exposure control measures (e.g. fumehoods, closed systems and ventilation) tested on ENMs
SANOWORK	2012-2015	ZrO_2, Polyamide andTiO_2 NF, TiO_2 and Ag nanosols, CNTs,	Development and implementation of design option-based risk remediation strategies for NMs	Data on the efficiency of safety-by-design approach in decreasing nanoaereosolisation and control hazard determinant properties (ROS production, surface ions dissolution)

The results of the literature review on risk management measures of ENMs with a focus on their risk mitigation efficiencies and costs are summarised in this section. Overall, the literature search retrieved more than a hundred of articles, of which 15 peer-reviewed papers were on risk management methods of ENMs and 7 tools were identified for further analysis. Additionally, the questionnaire survey resulted 36 responses by: micro- (8), small- (8), medium- (6) and large-sized (14) nanomaterial producers and integrators, which were analysed and discussed in this paper.

By making use of the information and data collected through project review, literature review and the questionnaire, this section is structured as follows: in Section 7.4.1, a general review of existing risk management models/tools for ENMs is given, Section 7.4.2 introduces RMM relevant for ENMs and Section 7.4.3 presents preliminary findings on two criteria (e.g. efficiency and cost) for characterizing risk control measures introduced in Section 7.4.2.

7.4.1 Existing Risk Management Approaches for ENMs

The safe and healthy workplace for employees exposed to ENMs is essential but challenging, which can be achieved by identifying and managing risks, such as recognition of hazards, assessing exposures, characterising actual risk, and implementing measures to control the identified risks. In this section, the existing tools, scoring systems and strategic approaches for minimizing risks of exposure to ENMs are briefly described based on the literature information. The basic nano-tools for risk management and prioritisation are given in Table 7.2.

Table 7.2 Risk prioritisation and management tools for ENMs

Tool	Description
CB Nanotool (Zalk, Paik and Swuste 2009)	A control banding tool for assessing risks associated with ENM operations and selecting effective engineering controls
Stoffenmanager Nano (Van Duuren-Stuurman *et al.* 2012)	A generic online tool for ranking potential human health risks as well as risk management measures applicable to ENMs
ANSES Nano (Riediker *et al.* 2012; ANSES 2010)	A control banding tool for managing the potential risks of ENMs
Swiss precautionary matrix (Höck *et al.* 2010; de Ipiña *et al.* 2015)	A risk prioritisation tool for safe handling of synthetic NMs
NanoSafer (Jensen *et al.* 2013)	A semi-quantitative risk prioritization tool for managing ENMs in the workplace
NanoRiskCat (Hansen, Jensen and Baun 2014)	A conceptual decision support tool for risk categorisation and ranking of ENMs
A low-cost/evidence-based tool (Genaidy *et al.* 2009)	A low-cost/evidence-based for assessing and managing the risks associated with exposure to Carbon Nanofiber

Risk management tools used to mitigate risk and manage exposure can be divided into three main categories: qualitative, semi-quantitative and quantitative. Qualitative or semi-quantitative tools are currently favourable for the control of potential risks associated with ENMs since there is still lack of knowledge or understanding in relation to the safety assessment of nano-scale materials (Boldrin *et al.* 2014). A control banding approach is a potential solution to assess and manage workplace risks where there is limited information, particularly relating to safety procedures and workplace exposure limits. It combines risk assessment and management to simplify risk complexity in the scarcity of input data (NIOSH 2009). To date, a number of control banding tools such as CB Nanotool (Zalk, Paik and Swuste 2009), ANSES Nano (Riediker *et al.* 2012; ANSES 2010), NanoSafe r(Jensen *et al.* 2013) and Swiss precautionary matrix(Höck *et al.* 2010) have been developed to protect the health of workers handling ENMs. A low-cost/evidence based tool (Genaidy *et al.* 2009) was one of the earliest control banding tool developed for assessing and managing the potential risks resulting from workers' exposure to Carbon Nanofibers. Similarly, Hansen, Jensen and Baun (2014) developed a systematic tool, NanoRiskCat, to support companies and regulators in their first-tier assessment and communication on the hazard and exposure potentials of consumer products containing ENMs. The outcome is related to five coloured dots representing the qualitative exposure potential for professional end-users, consumers and the environment, and the hazard potential for humans and the environment. Each dot is assigned one of four different colours (red, yellow, green, and grey) indicating high, medium, low or unknown level of exposure/hazard potential, respectively. With the obtained results, users can identify the top priority to apply proper risk measures for the reduction of the exposure and hazard risks. Most of these nano-tools seem to use reasonable approaches and provide promising results, while their main limitations are the extensive input data requirements and solely theoretical, rather than observational, considerations being made. More detailed information about the existing tools for risk management and prioritisation of ENMs can be found elsewhere (Work 2013; Brouwer 2012).

Table 7.3 Existing risk management strategies for ENMs

Ref.	Description
(Kuempel, Geraci and Schulte 2012)	— It provided a detailed overview on making use of current hazard data and risk assessment techniques for the development of efficient risk management guidelines for nanomaterials (NMs). — The authors proposed an integrated approach for risk management of ENMs including research and tools, risk characterisation, risk management and workplace actions.
(Schulte *et al.* 2013)	— This paper provided an overview on the application of risk management approaches for NMs. — The authors concluded that risk management process for NMs should be an internal part of an enterprise-wide risk management system, including both risk control and a medical surveillance program that assesses the frequency of potential side effects among groups of employees (potentially) exposed to NMs. They also suggested that the medical surveillance can be used to estimate the effectiveness of risk management program.
(Yokel and MacPhail 2011)	— This extensive review drawn together finding from a broad range of research on risk assessment and management of ENMs and outlines some good workplace practices. — The authors investigated the elements of occupational health protection and hierarchy of exposure control, including primary prevention (e.g. elimination, substitution, engineering controls, environmental monitoring, administrative controls and PPE), secondary prevention (e.g. medical examination of workers) and tertiary prevention (e.g. diagnosis, therapy and rehabilitation), for NMs.
(Goudarzi *et al.* 2013)	— The researchers proposed A 10-step qualitative risk management model for nanotechnology projects: the basic knowledge of the work; a thorough risk assessment; identifying nanoparticles; identifying hazardous nanoparticles; obtaining latest information; evaluating exposure routes; identifying risks; performing actions; documenting the whole process; and reviewing the risk management.

(Ling *et al.* 2012; Luther 2004)	— The investigators constructed a risk management strategy to protect employees working with NMs based on the precautionary risk management and reported the results of case studies with NMs. — Overall, they developed four risk management approaches: technology control (removing potential hazards from raw materials, manufacturing processes, mechanical equipment and factory facilities and other operating environments, changing operating pattern, confining production process systems), engineering control (adopting additional protective methods such as preventing and limiting sources of risk, using local ventilation and high efficiency particulate filters), personal protective equipment (breathing apparatuses, gloves or protective clothing), and working environment monitoring (exposure monitoring and special health examinations).
(Eddy *et al.* 2014; Fadel *et al.* 2015)	— These papers outlined latest efforts and outcomes in regard to risk assessment and management of NMs. — The authors highlighted the importance of integrating risk and life cycle analyses to guide engineering design using multi criteria decision analysis.
(Groso *et al.* 2010)	— The researchers introduced a methodology for nano-safety and health management. — The procedure they developed employs a schematic decision tree to classify risks into three hazard classes with each class being provided with a list of required risk mitigation measures (technical, organisational and personal).
(Chen *et al.* 2011)	— This paper provided an overview of eco-toxicological effects and risk management of NMs. — The authors noted that a NM risk assessment framework should include three main steps: (1) Emission and exposure pathway, nanoparticle characteristics and exposure metric, (2) Effects and impacts on both ecosystem and human health, (3) Risk assessment (risk characterisation and risk levels).
(GRIDELET *et al.* 2015)	— The authors proposed a new risk assessment approach based on the “control banding” approach comprising five occupational hazard bands (1-5). — The methodology they proposed considers exposure based on seven parameters including the main properties of the NMs, their emission potential, the condition of use and exposure characterisation parameters such as duration and frequency.

A number of risk management strategies proposed for use with ENMs are summarised in Table 7.3, including risk management approaches, methods and models. Kuempel, Geraci and Schulte (2012) suggested an integrated procedure for risk management of ENMs including research and tools (toxicology & epidemiology, exposure and risk analysis), risk characterisation (weight of evidence, severity & likelihood, variability & uncertainty), risk management (occupational safety & health guidance, exposure limits, communication) and workplace actions (engineering controls & PPE, exposure monitoring, worker training, medical monitoring). Schulte *et al.* (2013) proposed that risk management process for NMs should be a part of an enterprise-wide risk management system, including both risk control and a medical surveillance program assessing the frequency of adverse effects among groups of workers exposed to NMs. Goudarzi *et al.* (2013) proposed a 10-step qualitative risk management model for detecting significant risks in a systematic approach and providing decisions and suitable actions to reduce the exposure and hazard to an acceptable level. Ling *et al.* (2012) developed a risk management strategy based on the precautionary risk management, which is a modified version of Luther's method (Luther 2004). The risk management strategies were constructed according to the different levels of precautionary risk management, which includes the measures relating to technology control, engineering control, personal protective equipment, and monitoring of the working environment for each level.

Fadel *et al.* (2015) highlighted that the use of multi-criteria decision analysis (MCDA) for risk management purposes and the integration of risk and life cycle analysis using MCDA can be helpful to support the next generation of sustainable nano-enabled product designs and effective management of ENM risks. In the European project SCAFFOLD, the structure, content and operation modes of the Risk Management Toolkit (de Ipiña *et al.* 2015) were developed to facilitate the implementation of "nano-management" in construction companies with the consideration of 5 types of nanomaterials (TiO_2, SiO_2, carbon nanofibres, cellulose nanofibers and nanoclays), 6 construction applications (Depollutant mortars, self-compacting concretes, coatings, self-cleaning coatings, fire resistant panels and insulation materials) and 26 exposure scenarios, including lab, pilot and industrial

scales. The proposed risk management model included the following main tools: Risk management to open checklist for diagnostic, implementation or audit; Risk assessment to evaluate the identified risks; Planning to schedule the implementation of control measures specified in the evaluation tool; Key performance indicators to define, customise, calculate and visualise the indicators; Documents and templates to provide a list of templates with procedures, instructions, registers and manuals. Groso *et al.* (2010) developed a practical, user-friendly hazard-classification system for the safety and health management of nanomaterials. The process starts using a schematic decision tree that allows classifying the nano laboratory into three hazard classes similar to a control banding approach (from Nano 3 - highest hazard to Nano 1 - lowest hazard). For each hazard level they provide a list of required risk mitigation measures (technical, organisational and personal) such as protective measures, technical measures, organisational measures, personal measures and cleaning management. Yokel and MacPhail (2011) reviewed the exposures, hazards and risk prevention measures of ENMs, in particular the occupational exposure assessment and the approaches to minimise exposure and health hazards including engineering controls such as fume hoods and personal protective equipment, and the efficiencies of the control measures. The recommendations to minimise exposure and hazards were largely based on common sense, knowledge by analogy to ultrafine material toxicity, and general safety and health regulations, due to the lack of available information and/or un-verified research findings. Chen *et al.* (2011) reviewed the eco-toxicological effects of ENM and the existing regulations that can be related to ENMs. They concluded that the variety of ENMs and their properties make the identification and characterisation of ENMs a challenging task, and hence, an improvement in sensitivity and selectivity of analytical methods to detect and quantify ENMs in the environment is essential. They proposed a risk assessment framework as a practical alternative for the environmental assessment and effective management of ENMs. Based on the occupational hazard band (OHB) method, a new approach to assess the risks inherent in the implementation of powders was developed (GRIDELET *et al.* 2015), which considers exposure based on seven parameters which take into account the characteristics of the materials used, their emission potential, the

conditions of use, as well as classic parameters of exposure characterisation like duration and frequency. The result of the reflection is then positioned on a hazard versus exposure matrix from which 4 levels of priority of action are defined, as in the classical OHB method used to manage pure chemical risk.

In summary, most researchers appear to agree on the conclusion that although there is no need to develop an entirely new risk management paradigm to manage ENM risks, there is a clear need to expand existing practices to better address nano-related issues and ensure safe production, handling and use of ENMs. Although the existing risk management approaches applies well for ENM, their ability to transform from one form to another which leads on to changes in exposure and hazard (and hence risk) makes the process much more complex. At present, the main limitation in the field of ENM risk management is the insufficiency of the hazard/exposure research data that will be used to adopt existing risk management approaches and translated into modified practices. This problem is originated by not only the lack of data available, but also lack of systematic approaches for collecting and managing the information needed. One strategy to overcome this limitation in a timely manner is to collate available data from various sources (e.g. literature, ongoing/completed projects and nanotechnology companies) through the RMM inventory.

7.4.2 Risk control measures relevant for ENMs

Most of the technical exposure control methods (e.g. glove boxes, dust suppression systems, fume cupboard, safety cabinet, good hygiene practices and personal protective equipment) can be applied to ENMs since these measures rely on the bulk properties of nanoscale materials, not on their nano-specific properties. However, their performance in controlling ENM exposure should be evaluated since control measures that are proven to be effective for controlling exposure to traditional particles might give unsatisfactory results in the case of nano-scale particles (Jahnel, Fleischer and Seitz 2013). Table 7.4 gives a list of traditional RMM that are considered to be relevant for ENMs.

Table 7.4 The proposed classification system for technological alternatives and risk management measures of ENMs

Product/Substance Controls	
Substitution of hazardous material	Surface modification
Limiting concentration of hazardous ingredient	Embedding in matrix
Change of physical form and solubility	Packaging
Change in physicochemical properties	Granulation, controlled aggregation, purification
Process and Waste Controls	
Change of env. conditions (e.g. humidity)	Reduction/cleaning of air emissions
Automation	Reduction/cleaning of general waste
Suppression systems- wetting at point of release	Disposal of general waste
Suppression systems- Knockdown suppression	Reduction/cleaning of nano-specific waste
Use of mechanical transportation	Disposal of nano-specific waste
Containment of operator (e.g. cabin with filtered air for operator)	
Engineering (enclosure, isolation and ventilation) Controls	
Physical containment (e.g. covers, sealing heads)	Glove bags and glove boxes
Chemical fume hoods	Enclosed (isolated) operations
Biosafety cabinets	Sealed operations
Local exhaust ventilation systems (e.g. with enclosing, capturing or receiving hoods)	
Mechanical room ventilation	Dilution (general exhaust) ventilation
Natural ventilation	Laminar flow booths & benches
Good Work Practices and Administrative Controls	
Cleaning and maintenance of process equipment	Management systems
Vacuum cleaner with an air filter (e.g. HEPA)	Operating practice
Spill containment measures	Supervision
Workplace housekeeping	Monitoring
Personal hygiene facilities	Health surveillance
Restricted or prohibited process areas	Worker training
Personal Protective Equipment Controls	
Body protection	Face / Eye protection
Hand protection	Feet protection
Respiratory protection	

Between 2006 and 2011, NIOSH conducted site visits to 46 U.S. companies that produce and/or use ENMs and collected information on the most frequently used engineering controls, housekeeping methods and PPE types (Schubauer-Berigan *et al.* 2015). Their assessment showed that the most frequently employed engineering controls for reducing occupational exposures to ENMs were local exhaust ventilation (59%) and chemical fume hoods (54%) followed by ventilated enclosures (50%), enclosed production (48%) and glove boxes (22%). Additionally, 37% and 30% of the visited companies were observed to be employing wet wiping and HEPA vacuum as housekeeping methods, respectively. Moreover, the most frequently used PPE type was observed to be gloves (89%) followed by lab coats/Tyvek suits (83%) and respirators (76%) (Schubauer-Berigan *et al.* 2015). Similarly, it was noted in NIOSH's guidance document (NIOSH 2013) that the most common control measures used for ENMs are fume hoods, local exhaust ventilation systems, filtered vacuum cleaners, walk-in ventilated enclosures and isolation techniques such as negative pressure rooms or boxes.

In 2007, Conti et al. carried out an international survey among 83 nanotechnology companies and research laboratories to find out (nano-specific) health and safety programs and risk control measures implemented by these organisations to ensure safe working practices and environmental protection (Conti *et al.* 2008). The results demonstrated that the most common type of engineering control measure was fume hoods (66%) followed by some kind of exhaust filtration (49%). 82% of the interviewed companies said they had nano-specific PPE recommendations for their employees. Schmid, Danuser and Riediker (2010) conducted a survey between 1626 Swiss Companies investigating the quantity of nanoparticles and current protection measures that are in place. Closed process was identified to be the most common protection method in liquid applications while PPE was observed to be the most prominent safety measure followed by local exhaust ventilation in case of powder applications. Similarly, in 2010, NEPHH project conducted a survey on occupational health and safety procedures that are in place in nano-manufacturing sector with the aim of collecting information on engineering controls, PPE and waste management (NEPHH 2010). They reported that the majority of their respondents (66%) use fume hoods, followed by laminar flow

clean blench (34%), glove boxes (29.8%), biological safe cabinet (27.7%), cleanrooms (23.4%), glove bag (21.3%), closed piping system (21.3%), pressure differentials (19.1%), separate HVAC (8.5%) and chemical box (2.1%) to reduce worker exposure to ENMs (NEPHH 2010). Moreover, 95% of survey respondents indicated that they employ PPE and/or clothing recommendations for their employees while only 78% kept the use of PPE compulsory when handling ENMs. In terms of waste management, only 31% were observed to use nano-specific spill control methods and the most common equipment cleaning technique was identified as “wet wipe”. Moreover, the majority of the respondents were observed to treat nano-waste as any other chemical waste(NEPHH 2010).

The review of literature on efficiency of different control measures for ENMs showed that the most widely used RMM according to these surveys (e.g. local exhaust ventilation and chemical fume hoods) have indeed high efficiencies in reducing ENM emissions and particle concentrations (Methner 2008; Sahu and Biswas 2010; Methner 2010).

In the RMM questionnaire, respondents were asked to score four risk management categories (engineering controls-elimination and substitution, engineering controls-technical measures, organisational measures and personal protective equipment) in terms of their relevance to their firms’ activities in risk reduction process on a scale of 1 to 4, with 4 being most relevant and 1 being least relevant for reducing potential risks that are associated with ENMs. The answers given to this question by survey respondents are summarised in Fig 7.2.

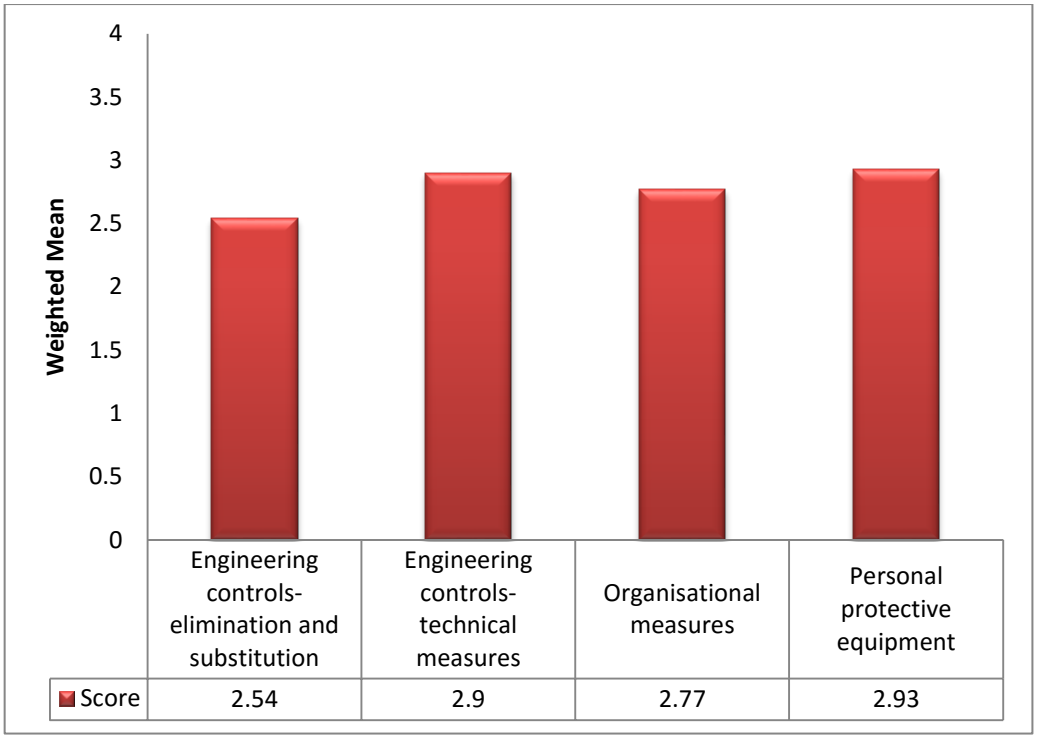

Figure 7.2 (Weighted mean) Relevance of risk management measures for survey-respondent institutions on a scale of 1-4

In this context, relevance can be considered as a subjective parameter, which is estimated from a questionnaire survey of 36 nanotechnology organisations. Overall, the respondents selected the personal protective equipment (e.g. body, hand respiratory and face protection) and the technical measures (e.g. design of manufacturing processes that reduce workers' contact with raw nanomaterials, such as containment, isolation and ventilation) to be the most relevant control strategies for ENMs followed by organisational measures (e.g. monitoring, health surveillance and good hygiene practices). Despite their high efficiency, survey respondents ranked substitution and elimination (e.g. physical manipulation of raw materials into forms that reduce hazard or exposure such as change of physical state and coating) as the least relevant control methods. This finding is consistent with previous core surveys in that the most common risk reduction strategies were observed to be based on isolating people from hazard through engineered measures or PPE, rather than eliminating hazard at source. It needs to be noted here that the use of PPE for risk reduction purpose should be the last option as it relies on human competence.

7.4.3 Efficiency of risk control measures for ENMs

There are two important criteria that need to be considered when deciding on the optimum risk control method: efficiency and cost. These two criteria are important because they signify the technical, economical and contextual feasibility of risk control options.

Although it is widely agreed that traditional methods used to control exposure to particles can be implemented to ENMs, there is a need to re-test their level of control against ENMs (Tyshenko 2015). Currently, there is a lack of knowledge on the efficiencies and practicality of particular risk management measures for control of worker exposure to ENMs. A number of studies (quantitatively) examining the efficiency of different control measures for ENMs are summarised in Table 7.5, while the data collected from reviewed projects are given in Table 7.6 and 7.7.

Table 7.5 Studies evaluating the efficiency of control measures for ENMs

Measure	NM Type	Efficiency	Ref
Process change (harvest wait time)	CNTs and/or graphene	99.6 and 100% reduction in conc.	(Heitbrink, Lo and Dunn 2015)
Process change (isolation valves)	CNTs and/or graphene	99.9% reduction in con.	(Heitbrink, Lo and Dunn 2015)
Process ventilation (exhaust fan)	CNTs and/or graphene	82.6% reduction in WBZ	(Heitbrink, Lo and Dunn 2015)
Exhaust ventilation system-with enclosure	CNTs	93-96% filtration efficiency on average	(Lo *et al.* 2012a)

Biological safety cabinet	CNTs	36% reduction in con. in WBZ and 40% reduction outside the hood	(Lo *et al.* 2012b)
Canopy hood	CNTs	15-20% increase in conc.	(Lo *et al.* 2012b)
Custom fume hoods and biological safety cabinet	Epoxy/CNT nanocomposites	Process/Background conc. in BZ Ratios; None: 5.9, Custom hood: 24.4, BSC:0.66	(Cena and Peters 2011)
Fume hood (fan ON and OFF)	Titanium tetraisopropoxide	Particle number con. reduced from 150 000 to ~6 300 particles/cm3	(Sahu and Biswas 2010)
Cabin air filter-high fan speed	Diesel engine exhaust	55% and 48.9% reduction in exposure based on particle number and surface area con.	(Wang and Pui 2011)
Cabin air filter-medium fan speed	Diesel engine exhaust	65.6% and 60.6% reduction in exposure based on particle number and surface area con.	(Wang and Pui 2011)
Personal protective clothing (cotton, polyester and Tyvek)	Nanoalumina	Mass of NP deposit (C:3364, P:2463, T:2121 µg/swatch) Mass of NP release (C:1674, P:1312, T:877 µg/swatch)	(Tsai 2015)
Ventilated feeder enclosure	Nanoalumina	Particle number con. reduced from 6060 to 360 part./cm3	(Tsai *et al.* 2012)
Ventilated full enclosure	Nanoalumina	Particle number con. reduced from 360 to -520 part./cm3	(Tsai *et al.* 2012)
Ventilated feeder enclosure	Nanoclay	Particle number con. reduced from 97 380 to -20 part./cm3	(Tsai *et al.* 2012)

Ventilated full enclosure	Nanoclay	Particle number con. reduced from -20 to340 particle/cm3	(Tsai *et al.* 2012)
Unventilation full enclosure	Nanoclay	Particle number con. reduced from -20 to 0 particles/cm3	(Tsai *et al.* 2012)
Sealed and unsealed respiratory protection device	Nanoscale NaCl aerosol	When RPD is sealed, the protection factor is 100-1000 000 greater than the protection factor in an unsealed fit.	(Brochot *et al.* 2012)
Local exhaust ventilation with a custom-filtered flange	Nanometal oxides	92% reduction in emission and 100% reduction in particle conc.	(Methner 2010)
Local exhaust ventilation	Nanometal oxides	88-96% reduction in conc.	(Methner 2008)
Thermo-denuder	CNT-containing polystyrene	99.9% reduction in the number of released NP	(Ogura *et al.* 2013)

Many researchers have employed different approaches (e.g. percent reductions based on mass or particle number concentrations, process to background ratios etc.) to quantify the efficiency of control measures being tested. Most of these studies have concluded with a set of recommendations for controlling worker exposure to ENMs. Overall it has been recommended that, after substitution of hazardous material and process changes, isolation of emission sources is the top priority to control and prevent worker exposure to ENMs while, ventilation system used for removing or diluting air containment is the next priority to consider (Tsai *et al.* 2012). It has been also demonstrated by many researchers that combination of isolation with ventilation remarkably increases the performance of exposure control systems (Tsai *et al.* 2012; Lo *et al.* 2012b; Heitbrink, Lo and Dunn 2015; Mazzuckelli *et al.* 2007).

Table 7.6 The experimental penetration factor (e.g. the ratio between the number conc. of particles inside and outside the protective device) of PPE (Fito 2015)

ENPs	PPE	PF_{Av} %	ENPs	PPE	PF_{Av} %
ZnO	Aut. Mask	7.40	Fe_2O_3	Latex Gloves	0.040±06
ZnO	Half Mask 1	8.50	Fe_2O_3	Nitrile Gloves	0.03±0.07
ZnO	Half Mask 2	12.00	Fe_2O_3	Lab coat	2.0±0.5
Fe_2O_3	Aut. Mask	5.52	ZnO	Latex Gloves	0.00±0.09
Fe_2O_3	Half Mask 1	6.58	ZnO	Nitrile Gloves	0.00±0.1
Fe_2O_3	Half Mask 2	8.55	ZnO	Lab coat	0.8±0.2
TiO_2	Aut. Mask	6.24	Al_2O_3	Latex Gloves	0.35±0.19
TiO_2	Half Mask 1	5.88	Al_2O_3	Nitrile Gloves	1.2±0.8
TiO_2	Half Mask 2	6.51	Al_2O_3	Lab coat	5.0±1.4
Al_2O_3	Aut. Mask	6.50	TiO2	Latex Gloves	0.04±0.03
Al_2O_3	Half Mask 1	9.99	TiO2	Nitrile Gloves	0.0±0.4
Al_2O_3	Half Mask 2	6.26	TiO2	Lab coat	8.5±1.9
$CoAl_2O_3$	Aut. Mask	7.80	$CoAl_2O_3$	Latex Gloves	0.0±0.4
$CoAl_2O_3$	Half Mask 1	7.16	$CoAl_2O_3$	Nitrile Gloves	0.0±0.4
$CoAl_2O_3$	Half Mask 2	7.87	$CoAl_2O_3$	Lab coat	12±4

Table 7.7 Scores for modifying respiratory and dermal exposure through protective measures (Scaffold 2015)

RMM	Score	RMM	Score
General Ventilation		**Localised Controls**	
No general ventilation, room size<100m3	10	No control measure	1
Mechanical and/or natural ventilation, room size<100m3	3	Limiting emission (e.g. wetting a powder, spraying of water)	0.3
Spraying booth, room size<100m3	0.1	Local exhaust ventilation (LEV)	0.3
No general ventilation, room size=100-1000m3	3	Containment of the source without LEV	0.3
Mechanical and/or natural ventilation, room size100-1000m3	1	Containment of the source with LEV (e.g. fume cupboard)	0.03
Spraying booth, room size100-1000m3	0.3	Glove boxes/bags	0.001
No general ventilation, room size>1000m3	1	**Gloves**	
Mechanical and/or natural ventilation, room size>1000m3	1	No gloves	1
Spraying booth, room size>1000m3	1	Woven clothing	0.3
Respiratory PPE		Gloves-Non-woven permeable, not connected well to clothing or arms	0.3
No PPE	1	Gloves-Non-woven permeable connected well to clothing or arms	0.1
FFP2 filtering half masks	0.4	Gloves-Non-woven impermeable, not connected well to clothing or arms	0.03
FFP3 filtering half masks	0.2	Gloves-Non-woven impermeable connected well to clothing or arms	0.09
P2 replaceable filter Half Mask	0.4	**Clothing**	
P3 replaceable filter Half Mask	0.2	No clothing	1

A1P2 combined half mask	0.2	Woven clothing	0.09
A1P3 combined half mask	0.1	Non-woven permeable	0.03
Full-Face masks with P3 filters	0.1	Non-woven impermeable	0.009
A powered filtered device incorporating a TH1 hood	0.2	**Personal Enclosure**	
A powered filtered device incorporating a TH2 hood	0.1	No cabin for workers	1
A powered filtered device incorporating a TH3 hood	0.05	Cabin without specific ventilation system	0.1
		Separated room with independent clean air supply	0.03

The survey results on the efficiency of RMM are not presented here since insufficient (quantitative) data on RMM's efficiencies have been collected from survey participants at the time of writing this thesis. Undoubtedly, knowledge of the efficiency of RMM is crucial if the approach is to be applicable to REACH. As mentioned earlier, the main difficulty here is defining which nano-form the efficiency applies to. When there is no information on the efficiency of control measures specific to ENMs, the default efficiencies can probably be used for initial assessment purposes although it should not be considered exhaustive. Specialised databases including scenario-specific efficiency values of risk management measures, such as TNO's exposure control efficiency library (Fransman *et al.* 2008), can be a good starting point for this purpose.

7.4.4 Cost of risk control measures for ENMs

The achievement of environmental protection at low cost is an integral feature of several risk management principles (e.g. European Commission's Precautionary Principle (Communities 2000), UK Health and Safety Executive's As Low as Reasonably Practicable (ALARP) principle (Report 2001)) and regulations (e.g. REACH Authorisation's Analysis of Alternatives (ECHA 2011) and Socioeconomic Analysis (ECHA 2012)).

Given the significant uncertainties around ENM risk and ambiguous risk perception of stakeholders, evaluation of costs is even more critical to support a rational risk management approach. Helland et al. (2008) report that small firms identified cost as the biggest barrier to occupational risk management(Helland, Kastenholz and Siegrist 2008). Fleury et al (2011) pinpoint difficulties in implementing risk management for nanocomposites based on acceptable risk thresholds, and propose risk management and cost evaluation based on the ALARP principle (Fleury *et al.* 2011).

To illustrate how efficiency and cost criteria can be integrated, emerging findings from the RMM questionnaire (Fig. 7.3) on respondents ranking on cost (on a scale from 1 to 4 with 1 meaning low cost and 4 meaning high cost) are compared with the occupational risk control hierarchy for efficiency (Fig. 7.1). Survey respondents rank PPE for face/eyes, hand, feet and body, together with natural ventilation (e.g. open windows) and user-friendly packaging as the least expensive RMMs. In most cases, the respondents did not specify whether their responses were related to one piece of PPE for single or repeated use. It should also be noted that PPE is the least effective category in the occupational risk control hierarchy and would not be useful in situations where significant risk reduction is required. However, the effectiveness of PPE in real-life conditions might be higher if they are used adequately. Process control and change (e.g. Automation and closed loop process control) are rated as the more expensive RMMs. On the other hand, most of the engineering controls (e.g. glove bags and boxes, LEV with enclosure such as safety cabinets and fume cupboards) are little higher than PPEs in cost, but more preferred according to the hierarchy of occupational risk hierarchy, suggesting that engineering controls could have the optimum trade-off between efficiency and cost for medium to high risk scenarios. Elimination and substitution (e.g. change of physical state, change in physicochemical properties, surface modification) have high efficiency in the occupational risk control hierarchy but also ranked among the most expensive RMMs by respondents, suggesting that they will be used in high risk scenarios.

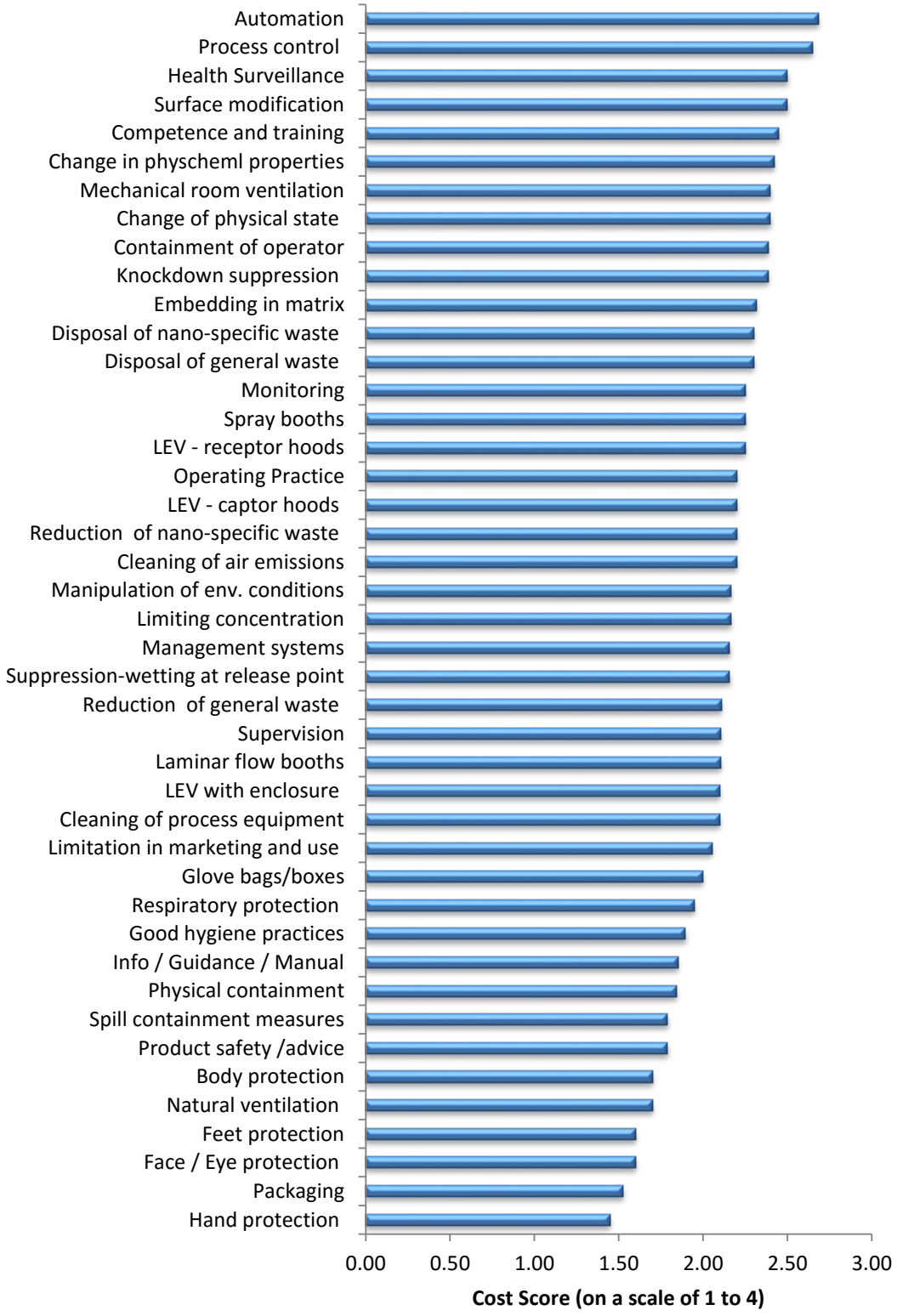

Figure 7.3 (Weighted mean) Cost of risk management measures on a scale of 1 (lowest) to 4 (highest)

7.5 Concluding remarks and direction for future research

In this chapter, relevant scientific literature and projects were reviewed in terms of available risk management practices for ENMs. A questionnaire was also designed to learn about current practices in ENMs handling in the workplace and the results are presented.

Nanotech companies participating in the survey were asked to score the importance of following research directions on a scale of 1-4 in order to understand their perspective on future research needs:

1. identification and categorisation of ENMs (e.g. classification of nano-enabled materials based on key parameters or biological interactions),
2. data collection (e.g. scientific data pertinent to hazard and exposure), standardisation (e.g. definitions, control limits, measurement methods and metrics, etc.),
3. safety-by-design research (e.g. integrating safety into design),
4. development of new measurements (e.g. developing a combination of different analytical methods for determining nanomaterial mass concentration, particle concentration, morphological information, etc.),
5. risk prediction/management tools (e.g. tools for the predictive risk assessment and management including databases and ontologies).

The answers given to this question are shown in Figure 7.4.

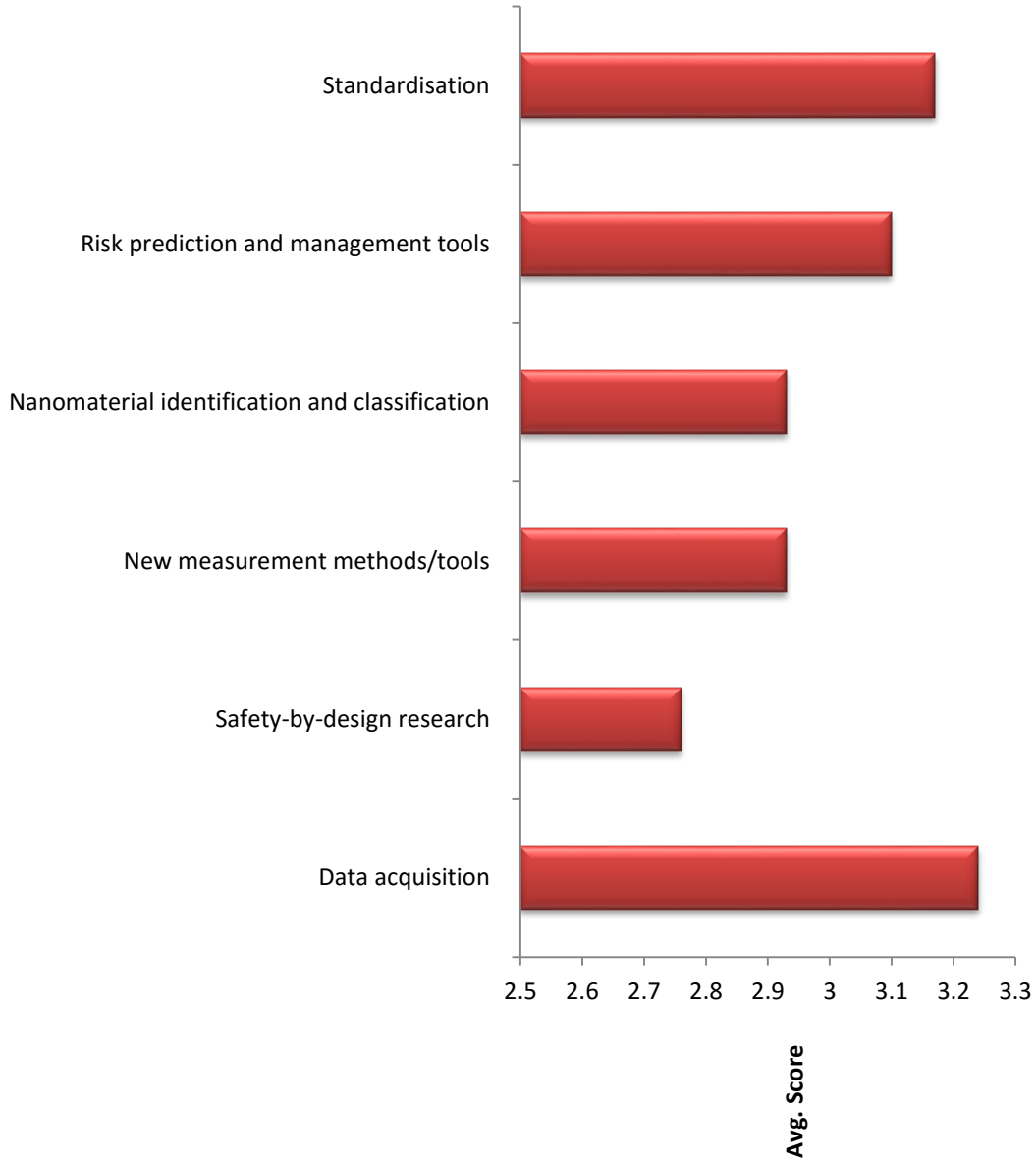

Figure 7.4 The importance of future research direction on a scale of 1 (lowest) to 4 (highest)

Data acquisition (e.g. scientific data pertinent to hazard and exposure) was ranked to be the most important research area followed by standardisation (e.g. definitions, control limits, measurement methods and metrics, etc.), risk prediction and management tools, nanomaterial identification and classification (e.g. classification of nano-enabled materials based on key parameters or biological interactions) and new measurement methods/tools (e.g. developing a combination of different analytical methods for determining nanomaterial mass concentration, particle concentration, morphological information etc.). The apparently less importance attributed by

companies to safety-by-design research may be caused by the high degree of uncertainty regarding the potential impact of manipulating nano-characteristics on the performance of final product. However, the ability to remove the source of risk through safety-by-design approaches (e.g. use of a nanoform encapsulated in micro/macro form that reduce human and environmental exposure while preserving nanoscale reactivity) is one of the most effective risk management strategies and deserves further investigation.

The existing challenges in risk management of ENMs are not only scientific but also related to insufficient communication and integration between different scientific disciplines, which might lead to unnecessary overlapping of studies. More focused research, integrated processes, and more dialogue is required. In part, this is currently being addressed by a growing number of European projects and international efforts.

Chapter 8
Conclusion and Future Research

8.1 Conclusion

Despite the clear benefits that nanotechnology can bring to various sectors of industry, there are serious concerns about the potential health risks associated with ENMs, intensified by the limited understanding of what makes ENMs toxic and how to make them safe. As the use of ENMs for commercial purposes and the number of workers/end-users being exposed to these materials on a daily basis increases, the need for assessing the potential adverse effects of multifarious ENMs in a time- and cost-effective manner becomes more apparent. One strategy to alleviate the problem of testing a large number and variety of ENMs in terms of their toxicological properties is through the development of computational models that decode the relationships between the physicochemical features of ENMs and their toxicity. Such data-driven models can be used for hazard screening, early identification of potentially harmful ENMs and the toxicity-governing physicochemical properties, and accelerating the decision-making process by maximising the use of existing data. Moreover, these models can also support industrial, regulatory and public needs for designing inherently safer ENMs. Therefore, the idea of using time- and cost-saving computational approaches such as (Q)SAR in nanotoxicology has gained popularity in recent years and attracted the interest of regulators and researchers aiming at moving from animal-based individual toxicity assessments toward a more integrated hazard screening approach.

The work described in this thesis has been mainly concerned with the investigation of the applicability of computational (Q)SAR methods to modelling of ENMs' biological effects. It is the main purpose of the study to determine the potential of the (Q)SAR technique to support risk assessment of ENMs, as well as the current limitations of this approach. Particular attention is paid to the capability of the computational approaches to identify physicochemical features contributing to the toxicity of ENMs by making use of existing experimental data. The use of exploratory data analysis methods

has also been considered to rank and prioritise ENMs based on their toxicities for monitoring and regulation purposes. Additionally, it has attempted to take the issue of risk assessment of ENMs a step further by investigating the existing risk reduction measures that are applicable to ENMs.

More specifically, this study is motivated by two research questions: (1) Can the (Q)SAR modelling approach be applied to ENMs; (2) How can computational approaches help identify hazardous category of ENMs and the physicochemical characteristics contributing to their toxicity. To examine these questions, a large amount of experimental data has been accumulated on various aspects of ENMs toxicity and in-depth case studies have been conducted using multiple data exploration and modelling methods including a novel decision tree construction tool. By addressing these areas, this study advances our understanding of the usefulness of computational models for predictive nanotoxicology and risk assessment of ENMs. It also contributes to revealing physicochemical properties that are likely to affect the toxicity of ENMs.

The study is started with a critical review of literature on the potential and challenges of (Q)SAR model development for ENMs (Chapter 2), which has led to the conclusion that the main issue that complicates the implementation of data-driven computational approaches in nanotoxicology is the lack of comprehensive experimental data and lack of information about where to find existing data that are particularly suitable for modelling investigations. To address this need, the primary sources of nano-(Q)SAR data have been summarised (Chapter 3). The compiled list of nano-(Q)SAR data sources can serve as a starting point for future modellers. Moreover, the existing nanotoxicity datasets have been analysed in the context of their ability to be used for developing nano-(Q)SARs. It has been concluded that the quality and quantity of the available nanotoxicity datasets is far from ideal from the (Q)SAR modelling point of view, but still useful for testing the hypothesis that ENM toxicity is a function of one or more physicochemical properties as long as any data analysis acknowledges its limitations. Moreover, in the absence of large volume and variety of high quality experimental data that causes large knowledge gaps in safety assessment of ENMs, the issue of

making the best possible use of existing data through computational approaches becomes more important in order to make better decisions.

The findings from visual exploratory data analysis have led to some interesting conclusions. Firstly, among several metal oxide NPs, zinc oxide is repeatedly found to exhibit the highest in vitro toxicity. Although core composition has a role in determining biological activity, surface characteristics are found to be the primary driver of Zinc oxide NPs toxicity, since surface-modified zinc oxide NPs has exhibited a significantly different level of toxicity. Secondly, nanotubes have shown toxic potential, largely associated with their size: the longer the nanotubes, the higher the toxicity. More importantly, the research indicates that the impact of physicochemical properties on toxicity is usually case-specific and more complex than previously assumed. Surprisingly, particle size has been shown to make a very small contribution to toxicity whereas two key factors, material core and surface properties, have directly influenced the toxicity at the nano-scale and the extent of their influence differs among ENMs. This finding suggests that the typical approach of toxicity assessments that is primarily based on the core composition of materials should be modified for ENMs as surface properties greatly affect the toxicological responses. Lastly, it has been observed that the influence of particular characteristics on different toxicity endpoints differs considerably, suggesting that more local predictive models focusing on one toxicity endpoint at a time should be constructed.

As the available nanotoxicity data is far from ideal for modelling purposes, the choice of nano-(Q)SAR tools used in this study has been made by considering the nature of the existing data (e.g. limited datasets, collinear input data) and desired outcomes (e.g. easily-interpretable models). Previous research on in silico analysis of ENMs toxicity has shown that although computerised (Q)SAR models are useful for modelling nanotoxicity endpoints, they have limited robustness and predictivity, and interpretation of the models they generate can be problematic. The main problem is caused due to the most commonly used (Q)SAR modelling methods working best with large data sets, but are not particularly good at feature selection, and cannot handle collinear input data. Ideally, new computational modelling tools or new ways of using existing tools are required to model the relatively sparse and

sometimes lower quality data on the biological effects of ENMs. To overcome these limitations, the application of a novel algorithm, a genetic programming-based decision tree construction tool to nano-(Q)SAR modelling has been described. Using four literature datasets, it has been demonstrated that this approach is clearly capable of identifying the key physicochemical descriptors associated with the toxicity of ENMs. It is shown that this approach generates models with accuracies equivalent to, or superior to, those of prior modelling studies on the same datasets. In the general cellular toxicity case study, two parameters, the conduction band energy and ionic index of metal cation, have been identified as suitable predictors for metal oxide NPs.

The second case study revealed that theoretical descriptors related to lipophilicity, hydrogen-bonding capacity, atomic masses, charge distribution and connectivity indices are predominantly affecting the cellular uptake behaviour of NPs. For the cytotoxicity to human keratinocytes dataset, the descriptors shown to be good predictors of cytotoxicity of metal oxide NPs are the enthalpy of the formation of metal oxide nanocluster representing a fragment of the surface, electronegativity and hardness. In the exocytosis of gold nanoparticles in the macrophages case study, the optimal descriptors for predicting the exocytosis were found to be the charge accumulation, zeta potential and charge density. It has been shown that the positive values of zeta potential prior to protein corona formation result in higher exocytosis of GNPs in macrophages. Overall, the genetic programming-based decision tree construction algorithm shows considerable promise in its ability to identify the relationship between molecular descriptors and biological effects of ENMs. The selected decision tree models have yielded a (external) prediction accuracy of 86 - 100%. This work is a first step in the implementation of genetic-programming based DT construction algorithm to nano-(Q)SAR studies. There are a number of opportunities to expand this work and fully evaluate its capabilities in the context of nano-(Q)SAR toxicity modelling.

Regression methods are essential components of (Q)SAR applications. There are two methods that are commonly used to develop regression-based (Q)SAR models: Multiple Linear Regression (MLR) and Partial Least Squares (PLS). Although both methods have proved their applicability in (Q)SAR modelling, the latter provides several advantages that

are particularly attractive in nano-(Q)SAR research. For example, unlike MLR, PLS can handle collinear input data and underdetermined datasets (e.g. fewer data objects than variables). PLS also has the advantage in that it can simultaneously derive accurate and easily interpretable models for more than one response variable. These advantages make PLS especially useful for regression applications in nano-(Q)SAR modelling. The use of an empirical regression method (PLS) as a tool in nano-(Q)SAR model development has been successfully demonstrated by applying it to five different nanotoxicity datasets. The results have suggested that the PLS approach is well suited to assess the relative importance of descriptors representing physicochemical properties of ENMs for toxicity endpoints and to link the key descriptors with toxicological outcomes in a quantitative manner.

Predictive models such as (Q)SAR have great potential to fill in data gaps on nanotoxicity and to be used as a priority-setting method for risk assessment of ENMs. Once all the potential risks are identified by means of toxicity screening methods including in silico models (e.g. (Q)SAR), the next step is the implementation of risk reduction measures for those risks that are outside the range of tolerable limits. While the risk management of ENMs receives significant attention, there is still a research gap in the scientific literature on how to select and implement appropriate risk reduction measures in order to protect nanotechnology workers' health. To take the issue of risk assessment of ENMs a step further and to address this research gap, the suitability of the existing risk management measures for ENMs has been investigated. Evaluative evidence on their cost and efficiency has been collected through literature review and a specialised questionnaire survey conducted among 36 organisations that are involved in nano-related activities. The aim here is to support the selection of the most suitable measures (e.g. based on their efficiency and cost) in order to control and reduce the risks resulting from exposure to potentially hazardous ENMs. Research has revealed that the most frequently employed engineering control measures for reducing exposure to ENMs are local exhaust ventilation and chemical fume hoods (e.g. high efficiency, relatively high cost). It has also been observed that safety-by-design approaches (e.g. change of physical state, change in physicochemical properties, surface modification) have high efficiency in the

occupational risk control hierarchy but also very costly, suggesting that they will be used in high risk scenarios.

8.2 Future Directions

While this thesis has provided strong evidence that data-driven computational methods can provide useful information for hazard screening and risk assessment of ENMs, much research remains to be done on order to be able to develop optimal and regulatory acceptable nano-(Q)SAR models. Clearly, more comprehensive and high-quality datasets are necessary before obtaining optimal nano-(Q)SAR models. To improve the accuracy of computational models, quality issues associated with experimental data used to develop the model in the first place must be tackled. Moreover, the development of novel descriptors that are able to express the specificity of nano-characteristics would also be of interest. Another problem that complicates the development of predictive models is the heterogeneity of the ENM family. There is a need to generate homogeneous datasets that include specific types or individual classes of ENMs since different types of ENMs are likely to have different mechanisms of toxicity. Lastly, the application of a genetic-programming based construction algorithm to nano-(Q)SAR modelling has resulted in accurate and easily interpretable models. To further prove the usefulness of this approach and illustrate its versatility, there is a need for more case studies on large toxicity datasets associated with a set of ENMs with similar core composition but varying physicochemical properties (e.g. size, shape, surface charge etc.) to be examined under realistic and identical experimental conditions.

The limited knowledge of nano-EHS issues points to important gaps in research on the environmental and health risks associated with nanotechnology. Clearly, much research remains to be done on the risk management of ENMs, including identification and categorisation of ENMs (e.g. classification of nano-enabled materials based on key parameters or biological interactions), data collection (e.g. scientific data pertinent to hazard and exposure), standardisation (e.g. definitions, control limits, measurement

methods and metrics, etc.), safety-by-design research (e.g. integrating safety into design), development of new measurements (e.g. developing a combination of different analytical methods for determining nanomaterial mass concentration, particle concentration, morphological information, etc.), and risk prediction/management tools (e.g. tools for the predictive risk assessment and management including databases and ontologies).

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Publications

The publications that have resulted from this research, including both published and submitted work, are given below, followed by conference presentations and invited talks:

Journals:

- **Accurate and Interpretable nanosat Models from Genetic Programming-based Decision Tree Construction Approaches**

  C. Oksel, D. Winkler, C. Y. Ma, T. A. Wilkins, and X. Z. Wang.
  Nanotoxicology (2016) [Epub ahead of print]
  doi: 10.3109/17435390.2016.1161857

- **Review of Risk Management Along the Lifecycle of Nano-enabled Products**
  C. Oksel, V. Subramanian, E. Semenzin, C. Y. Ma, D. Hristozov, X. Z. Wang, N. Hunt, A. Costa, W. Fransman, A. Marcomini, and T. A. Wilkins.
  Environmental Science: Nano (Under Review)

- **Current Situation on the Availability of Nanostructure–biological Activity Data**
  C. Oksel, C. Y. Ma, and X. Z. Wang.
  SAR and (Q)SAR in Environmental Research 26 (2) (2015).
  doi: 10.1080/1062936X.2014.993702

- **(Q)SAR Modelling of Nanomaterial Toxicity: A Critical Review**
  C. Oksel, C. Y. Ma, J. J. Liu, T. A. Wilkins, and X. Z. Wang
  Particuology 21 (2015) 1-19.
  doi: 10.1016/j.partic.2014.12.001

- **Structure-activity Relationship Models for Hazard Assessment and Risk Management of Engineered Nanomaterials**
  C. Oksel, C. Y. Ma and X. Z. Wang.
  Procedia Engineering 102 (2015) 1500-1510.
  doi: 10.1016/j.proeng.2015.01.284

- **Nano (Q)SAR: Challenges, Pitfalls and Perspectives**
  R. Tantra, C. Oksel, T. Puzyn, J. Wang, K. N. Robinson, X. Z. Wang, and T. A. Wilkins.
  Nanotoxicology 9 (5) (2015) 636-642.
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- **A Method for Assessing Nanomaterial Dispersion Quality Based on Principal Component Analysis of Particle Size Distribution Data**
  R. Tantra, C. Oksel, K. N. Robinson, A. Sikora, X. Z. Wang, and T. A. Wilkins.
  Particuology 22 (2015) 30-38.
  doi: 10.1016/j.partic.2014.10.004

Book chapters:

- **Chapter 8: Literature Review of (Q)SAR Modelling of Nanomaterial Toxicity**
  C. Oksel, C. Y. Ma, J. J. Liu, T. Wilkins, and X. Z. Wang
  Modelling Toxicity of Nanoparticles,, Publisher: Springer
  (in press)

  Chapter 14: Visualizsation of Multi-dimensional Data For Nanomaterial Characterisation
  J. J. Liu, J. Li, C. Oksel, C. Y. Ma, and X. Z. Wang
  Nanomaterial Characterisation: An Introduction, Publisher: Wiley (In Press)

Conference presentations:

- **Investigation of Genetic Programming-based Decision Tree Construction Approach for Nano-(Q)SAR Modelling**
  C. Oksel, C. Y. Ma, and X. Z. Wang
  CompNanoTox2015, Malaga, Spain (NOV 2015)

  Linking the Structure of Engineered Nanomaterials with their Toxicity
  C. Oksel, C. Y. Ma, and X. Z. Wang.
  NanoTech 2015, Washington, US (JUN 2015)

  Structure Activity Model for the Toxicity of Nanoparticles
  C. Oksel, C. Y. Ma, and X. Z. Wang.
  SUN-SNO-GUIDENANO Sustainable Nanotechnology Conference, Venice, Italy (MAR 2015)

  (Q)SAR Modelling of Nano-toxicity: Future Directions and Availability of Nanostructure Activity Data
  C. Oksel, C. Y. Ma, and X. Z. Wang.
  (Q)SAR2014, Milan, Italy (JUN 2014)

  Quantitative Structure-Activity Relationship Models for Hazard Assessment and Risk Management of Engineered Nanomaterials
  C. Oksel, C. Y. Ma, and X. Z. Wang.
  WCPT7 Conference, Beijing, China (MAY 2014)

  Data-driven Modelling of Engineered Nanomaterial Toxicity
  C. Oksel, C. Y. Ma, and X. Z. Wang.
  7th International Nanotoxicology Congress, Antalya, Turkey (APR 2014)

Invited talks:

- **In Silico Modelling of Nanomaterial Toxicity: Current Status and Future Directions**
  C. Oksel, C. Y. Ma, and X. Z. Wang.
  2nd Sustainable Nanotechnology School, Venice, Italy (JAN 2016)

- **Structure-activity Relationship Models for the Prediction of Nanomaterial Toxicity**
  C. Oksel, C. Y. Ma, and X. Z. Wang.
  MODENA Workshop, Plovdiv, Bulgaria (MAR 2015)

- **Predictive Toxicity Modelling and Risk Reduction Strategies for Nanomaterials**
  C. Oksel, C. Y. Ma, and X. Z. Wang.
  John Moores University, Liverpool, UK (NOV 2014)

- **(Q)SAR Modelling of the Toxicity of Engineered Nanomaterials**
  C. Oksel, C. Y. Ma, and X. Z. Wang.
  EU Joint Research Centre, Ispra, Italy (SEP 2014)

- **Current status of (Q)SAR Modelling in Risk Assessment of Nanomaterials**
  C. Oksel, C. Y. Ma, and X. Z. Wang.
  1st Sustainable Nanotechnology School, Venice, Italy (SEP 2014)

- **Hazard Prediction and Risk Reduction in the Production Process of Nanomaterials**
  C. Oksel, C. Y. Ma, and X. Z. Wang.
  Process Safety and Loss Prevention Workshop, Leeds, UK (SEP 2014)

- **In Silico Modelling of Nanomaterial Toxicity**
  C. Oksel, C. Y. Ma, and X. Z. Wang.
  ES1205 Workshop, Pula, Croatia (JUN 2014)

- **Assessing the Quality of Nanomaterial Dispersions using Principal Component Analysis**
  C. Oksel, C. Y. Ma, and X. Z. Wang.
  OECD Workshop, Vienna, Austria (FEB 2014)

- **Quantitative Structure-activity Relationship ((Q)SAR) Models for Nanomaterials**
  C. Oksel, C. Y. Ma, and X. Z. Wang.
  MODENA Workshop, Rome, Italy (AUG 2013)

- **Quantitative Structure-activity Relationship ((Q)SAR) Models**
  C. Oksel, C. Y. Ma, and X. Z. Wang.
  Quality-Nano Toxicity Modelling, Edinburgh, UK (MAR 2013)