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Identifying nanodescriptors to predict the toxicity of nanomaterials: a case study on titanium dioxide†

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Since the evaluation of nanomaterial (NM) hazards by animal testing is expensive, time-consuming and critical from an ethical point of view, much interest is being given to the development of alternative testing strategies such as computational (predictive) models based on *in vitro* testing. However, the variations in *in vitro* experimental conditions can influence the outcome of computational modelling. In this study, we aim to identify nanodescriptor(s) and biological endpoint(s) capable of predicting the toxicity of titanium-di-oxide (TiO_2) NMs, and demonstrate how experimental variations determine the outcome of modelling using three case studies. We used TiO_2 *in vitro* data from our previously published study as case study 1 and two other external case studies (case study 2 and 3) performed under different exposure conditions (presence and/or absence of serum). Firstly, we identified the nanodescriptor(s) closely associated to biological endpoints. Secondly, we determined the strength of association of the identified nanodescriptor(s) with the respective biological endpoint. The results indicate that the experimental conditions influence the outcome of the computational modelling. Agglomerate size as a nanodescriptor was well associated with biological endpoints such as DNA damage and/or cytotoxicity. We conclude that, agglomerate size is an important nanodescriptor to assess the toxicological effects of TiO_2 NMs *in vitro*. However, the agglomeration state of NMs can be potentially influenced by *in vitro* exposure conditions and such influences could be just a confounder in broader contexts such as safety-by-design approaches, which require linking of material specific properties to the toxicological outcome.

Environmental significance

A better understanding of how the *in vitro* experimental conditions influencing the outcome of computational modelling in predicting the toxicity of titanium dioxide (TiO_2) nanomaterials (NMs) may lead to rational design of future *in vitro* experiments to generate and use the data for reliable toxicity prediction and in a broader context, such as safety-by-design of NMs for commercial, medical and environmental applications.

Introduction

Decades of nanotoxicological research revealed that because of their small size and enhanced surface reactivity, nanomaterials (NMs) can induce adverse effects in animals (*in vivo*) and in cell cultures (*in vitro*).[1–3] While animal models can closely mimic the response associated with real-life human exposure, current trends in nanotoxicology research show that evaluating NM hazards by animal testing is practically challenging because it is expensive, time-consuming and not ethical/legal in many countries. Thus, much emphasis is being given to the development of alternative testing strategies such as computational (predictive) models based on *in vitro* testing, for initial toxicity screening and to reduce animal testing.[4,5] Extensive efforts are needed to make progress in this direction.

Efforts have been made to predict the toxicity of NMs using different computational approaches such as multiple regression,[6] decision trees[7] and artificial neural networks.[8] A major drawback in many of these computational approaches

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is the selection of NM characteristics and probable multicollinearity, which can lead to bias in toxicity prediction. Because feature selection and machine learning (ML) approaches are data-driven and exploratory in nature, they are being used to select the characteristics of maximum relevance and to improve the accuracy in toxicity prediction.[9] Several other major issues need to be considered in computational toxicity prediction approaches. In a recent systematic review, Forest *et al.*[10] found that nearly 75% of the papers reporting computational approaches to predict the toxicity of NMs used only cytotoxicity/cell viability for evaluation and only a few used other biological endpoints such as oxidative stress and/or pro-inflammatory responses. Therefore, the predictability of selected biological endpoints in comparison to other endpoints is not always clear. While there are some consensus on the set of physicochemical properties (*e.g.*, size, shape, chemical composition and surface modification) that are essential to be evaluated in NM toxicological assessment,[11] the influence of experimental conditions in linking these characteristics to the biological responses is unknown. These discrepancies indicate that reliable prediction strategies are not yet possible to rapidly evaluate the hazardous potential of the NMs.

Titanium dioxide (TiO_2) NMs are widely produced and used in many practical applications such as in paints, food and in personal care products[12,13] and, exposure to these NMs *via* inhalation, dermal or oral routes is becoming more and more evident. Several *in vitro* studies showed that TiO_2 NMs can induce adverse effects such as cytotoxicity, oxidative stress, pro-inflammatory responses and genotoxicity[14] and therefore, it is essential to understand their toxicological behaviour in relation to their characteristics.

In this study, we aim to identify nanodescriptor(s) (*i.e.* physicochemical properties of NM) and biological endpoint(s) to predict the toxicity of TiO_2 NMs using multivariate modelling approaches. To have a view on the influence of different experimental conditions in predicting the toxicity of TiO_2 NMs, we composed three case studies with different datasets: the first case study (only *in vitro*) was built from data reported in Murugadoss *et al.*;[15] the two other case studies were composed from external data, studies performed with different TiO_2 NMs and at different *in vitro* experimental conditions. Using our case study 1, we performed a cluster analysis of experimental parameters such as NM concentration and cell type against all biological endpoints to identify their effect on the responses. Secondly, we aimed at identifying the nanodescriptors most associated with different biological endpoints using a feature selection approach. We then detected the strength of association between a given biological endpoint and the most associated nanodescriptor using linear and non-linear multivariate modelling. Similar analysis was repeated for case 2 and 3. Finally, to have a view on the overall impact, all case studies were combined and analyses were reiterated.

Methodology

Selection of studies

Studies/articles (i) reporting minimal characteristics such as constituent (primary) particle size, agglomerate size in stock and in cell culture medium, and (ii) at least reporting DNA damage and cell viability were taken into account for creating different case studies.

Case studies

Case study 1. This case study includes the *in vitro* data from Murugadoss *et al.*[15] In this study, cytotoxic effects induced by two TiO_2 NMs of identical chemical composition and shape (near-spherical) but with different constituent (primary) particle sizes (17 and 117 nm) was evaluated, and compared their *in vitro* toxicity in different agglomeration states (small and large agglomerates). First, the TiO_2 NMs in different agglomeration state (four dispersions in total) were comprehensively characterized for nanodescriptors (see Table 1) that might influence the toxicity under experimental conditions. Then, we evaluated the biological endpoints *in vitro* by exposing cell cultures to different dispersions. Results include the measurements of the effects in multiple cell types and at different concentrations on the cell metabolic activity (measured by WST-1 assay), cell viability (lactate dehydrogenase assay), barrier integrity (transepithelial/transendothelial electrical resistance [TEER]), oxidative stress (changes in total glutathione level), pro-inflammatory mediators (interleukin 8 [IL-8], IL-6, tumor necrosis factor α [TNF-α] and IL-1β proteins measured by ELISA) and DNA damage (alkaline comet assay). Cells were exposed to the TiO_2 NMs for 24 h in serum-free exposure conditions. Number of data rows (N) = 144. We are aware that a set of just two materials of the same composition in case study 1 naturally limits the variations of the descriptors, but we included this data in our study to validate our statistical approach by confirming the previously obtained results.

Case study 2. This case study includes data extracted from 11 articles that were systematically selected from EMBASE database (Fig. S1†). Case study 2 includes the measurements of cell viability (different colorimetric assays) and DNA damage (alkaline comet assay) induced by TiO_2 NMs with different constituent particle sizes, crystal phases and surface charge, in multiple cell types and at different concentrations (μg mL^{-1}). In these studies, cells were exposed to the TiO_2 NMs for 24 h. This case study also includes data from experiments performed with and without serum conditions. N = 49 (DNA damage) and 66 (cell viability).

Case study 3. This case study includes data extracted from a published study[16] performed in the ENPRA project (Risk assessment of engineered nanoparticles, European framework 7). The case study 3 includes measurements of cell viability and DNA damage (alkaline comet assay) induced by TiO_2 NMs with different constituent particle sizes and crystal phases, in multiple cell types and at different concentrations (μg mL^{-1}). The duration of exposure was 4 h

Table 1 Correlation matrix of nanodescriptors obtained from the characterization of agglomerated suspensions using different techniques

	Feret min	Feret max	MIC diameter	AEC diameter	Area	Aspect Ratio	*Z*-Average	*Z*-Average in CCM	PTA size	Zeta potential
Feret min	1.0000	0.9943	0.9508	0.9987	0.9420	0.3267	0.4181	−0.1732	0.4747	0.0226
Feret max	0.9943	1.0000	0.9157	0.9890	0.9434	0.4107	0.5013	−0.1007	0.4052	0.0585
MIC diameter	0.9508	0.9157	1.0000	0.9612	0.8807	0.0798	0.1600	−0.3585	0.5996	−0.0352
AEC diameter	0.9987	0.9890	0.9612	1.0000	0.9448	0.2880	0.3746	−0.2095	0.5006	0.0154
Area	0.9420	0.9434	0.8807	0.9448	1.0000	0.2072	0.3333	−0.2177	0.2821	0.3041
Aspect Ratio	0.3267	0.4107	0.0798	0.2880	0.2072	1.0000	0.9408	0.6137	−0.1466	−0.1117
***Z*-Average**	0.4181	0.5013	0.1600	0.3746	0.3333	0.9408	1.0000	0.6208	−0.2590	0.0869
***Z*-Average in CCM**	−0.1732	−0.1007	−0.3585	−0.2095	−0.2177	0.6137	0.6208	1.0000	−0.4795	0.0723
PTA size	0.4747	0.4052	0.5996	0.5006	0.2821	−0.1466	−0.2590	−0.4795	1.0000	−0.7467
Zeta potential	0.0226	0.0585	−0.0352	0.0154	0.3041	−0.1117	0.0869	0.0723	−0.7467	1.0000

and experiments were performed in the presence of serum (10%) conditions. N = 201 (DNA damage) and 135 (cell viability).

The raw datasets of case study 1, 2 and 3 (and all case studies combined) are provided in GitHub open repository https://github.com/Nilzkool/NanoQSARproject.

Nanodescriptors

Nanodescriptors in case study 1 – correlation based reduction. Certain nanodescriptors included in this case study are relatively similar, or based on similar measurements. Using the same information multiple times (multicollinearity) can lead to overfitting of data in a model and to skewed and/or biased results. Therefore, the correlation among the nanodescriptors is checked using cross correlation. The resulting correlation matrix is shown in Table 1. There was a very high correlation among size-based descriptors (multicollinearity) characterized by quantitative TEM [Feret min, Feret max, maximum inscribed circle (MIC) diameter, area equivalent circle (AEC) diameter and area]. From this set of descriptors, we selected minimum Feret diameter of agglomerates in further analysis as it was highly correlated with the other measures and can be considered as a representative estimate for the agglomerate size measured by TEM, and relevant in terms of the NM definition (external dimension).[17] The constituent (primary) particle size was not included in this analysis, as they could not be influenced by agglomeration. There was also a high correlation between the size, assessed by DLS as the

Table 2 TiO_2 nanodescriptors measured using different techniques (TEM, DLS and PTA). # – reported characteristics in the article; TEM – transmission electron microscopy; DLS – dynamic light scattering; PTA – particle tracking analysis

Case study	Techniques	Nanodescriptors
1	TEM	Constituent particle size (minimum Feret diameter)
	TEM (in stock dispersions)	Minimal external dimension (minimum Feret diameter) and elongation (aspect ratio) of agglomerates
	DLS (in stock and in cell culture medium)	Hydrodynamic size (*Z*-average)
	DLS (in stock)	Zeta potential
	PTA (in stock)	Hydrodynamic size (mean size)
2	TEM	Constituent particle size
	DLS (in stock and in cell culture medium)	Hydrodynamic size (*Z*-average)
	#	Crystal phase
3	TEM	Constituent particle size
	DLS (in stock and in cell culture medium)	Hydrodynamic size (*Z*-average)
	DLS (in stock)	Zeta potential
	#	Crystal phase
	#	Coating

Z-average, and the aspect ratio of agglomerates. Large agglomerates tend to be more elongated than the small agglomerates and this possibly explains the high correlation between the *Z*-average and aspect ratio. However, we used both nanodescriptors in further analyses as they describe two different characteristics such as the agglomerate size (*Z*-average) and its shape (aspect ratio), and are assessed using different techniques. Table 2 shows different nanodescriptors and the techniques used to assess them. For case studies 2 and 3, no correlation analysis was needed since a very low number of descriptors was presented and measured with different techniques.

Clustering of cell type and concentration effect

For case study 1, clustering and visualization of cell type and concentration effect against all biological responses were performed with 2D principal component analysis (PCA) (https://www.rdocumentation.org/packages/stats/versions/3.6.2/topics/prcomp) and non-linear t-distributed stochastic neighbor embedding (t-SNE) clustering (https://CRAN.R-project.org/package=Rtsne) approaches with Rstudio v1.1.463 (https://rstudio.com/).

Ranking of nanodescriptors and multivariate modelling

Fig. S2† shows the design for statistical analyses and modelling. Because feature selection and machine learning (ML) approaches are data-driven and exploratory in nature, they are being used to select the characteristics of maximum relevance and to improve the accuracy in prediction.[9] Therefore, we used a feature selection method (mRMR) in this study to identify nanodescriptors that are closely associated with biological responses. Firstly, datasets of each response variable (biological endpoint) in case study 1 was randomly split into a training (70%) and validation set (30%). In the training set, we first applied the minimal-redundancy-maximal-relevance (mRMR) technique[18] (https://cran.r-project.org/web/packages/mRMRe/vignettes/mRMRe.pdf) to rank the nanodescriptors associated with different biological endpoints (response variables). The nanodescriptors with positive mRMR scores represent the highest relevance with response variables, and nanodescriptors with negative mRMR values represent redundancy. Therefore, descriptors with positive mRMR scores were only used in modelling. Before modelling, the data were standardized by calculating the mean and standard deviation for each variable, and from each observed value of the respective variables, the mean was subtracted and divided by the standard deviation. Then machine learning approaches such as multiple linear regression (in-build testing in R studio) and a non-linear model called random forests regression[19] (https://www.rdocumentation.org/packages/stats/versions/3.6.2/topics/lm) was used to predict the biological endpoints in the training dataset (data not shown). Then the trained models were used to predict respective biological endpoints in the validation data set (30%) and in other case studies. All analyses involved use of Rstudio v1.1.463 (https://rstudio.com/). Results of validation data are in the Results section.

The codes for the entire workflow are provided in GitHub open repository https://github.com/Nilzkool/NanoQSARproject together with datasets.

Results

Case study 1

Clustering of cell type response and concentration effect. In case study 1, different cell types (human bronchial epithelial cells [16HBE14o-] human colon epithelial cells [Caco2] and human monocytes [THP-1]) and exposure concentrations (4, 64, 256 μg mL^{-1}) were used. To verify the effect of cell type and concentration used, we clustered all biological endpoints/response variables (see Table 3 for the list of response variables) by cell type (Fig. 1a and b) and by concentration (Fig. 1c and d) with use of linear 2D PCA and non-linear tSNE. We observed a clear differential response based on cell type with both approaches, whereas for concentration, the difference was less pronounced in t-SNE than PCA.

Ranking of nanodescriptors. Fig. S3† shows the ranking of nanodescriptors for different response variables and Table 3 shows the nanodescriptor(s) with a positive mRMR score. We discarded nanodescriptors with negative mRMR values because this represents redundancy. For case study 1, *Z*-average size in cell culture medium (CCM; *Z*-average in CCM) was most associated with all biological endpoints except metabolic activity and DNA damage, for which aspect ratio and *z*-average size in stock dispersions, respectively, were the most associated.

Multivariate modelling. Our clustering analysis of cell type and concentration for case study 1 revealed that toxicity induced by TiO_2 NMs was cell type- and to some extent concentration-dependent – this is not a surprise and has been documented before. Since we aim to identify nanodescriptors that predicts the toxicity of TiO_2 NMs, we started with a basic linear regression model with cell type and concentration as inputs (basic model) to predict a given response, and we added most associated nanodescriptor(s) to the model. Then we noted the corresponding changes in coefficient of determination (R^2) and mean absolute error (MAE) quantifying the goodness of fit. This procedure was repeated for non-linear RF modelling. Variable importance was assessed by the increase in R^2 and subsequent decrease in MAE. It is also important to mention here that our aim is not to build quantitative prediction models such as QSAR but only to identify the strength of association of nanodescriptors with biological endpoints. This also explain why we focussed on goodness of fit parameters R^2 and MAE. Table 4A and B summarize the effect on these statistical parameters for validation dataset when the most relevant descriptor was added to the basic model in linear and non-linear multivariate models, respectively.

Linear model (Table 4A). For case study 1, R^2 for the basic model (cell type + concentration) was high for response

Table 3 Nanodescriptors identified by mRMR approach that are associated closely to different response variables. *Z*-Average – hydrodynamic size measured by DLS; CCM-cell culture medium; mRMR-maximum relevance minimum redundancy

	Biological endpoints/response variables	Assays	Most relevant descriptor (mRMR score > 0)
Case study 1	Metabolic activity	WST-1 assay	Aspect ratio
	Cell viability	LDH assay	*Z*-Average in CCM
	Total glutathione (GSH) depletion	GSH assay	*Z*-Average in CCM
	Trans-epithelial electrical resistance (TEER)	Measurements by epithelial voltohmmeter	*Z*-Average in CCM
	IL-8 levels	ELISA	*Z*-Average in CCM
	IL-6 levels	ELISA	*Z*-Average in CCM
	TNF-α levels	ELISA	*Z*-Average in CCM
	IL-1β levels	ELISA	*Z*-Average in CCM
	DNA damage	Alkaline comet assay	*Z*-Average in stock dispersions

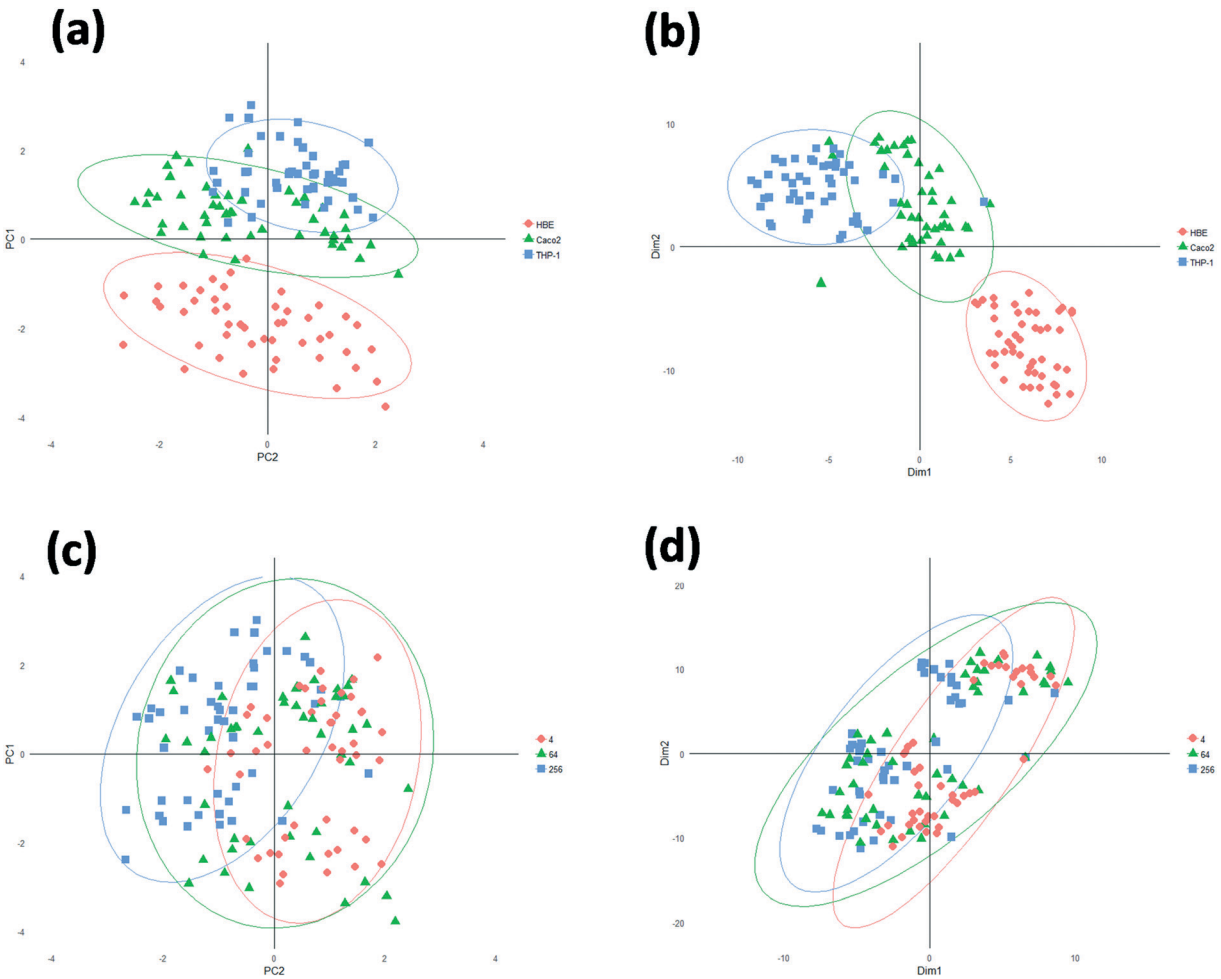

Fig. 1 Clustering of all biological responses against cell type (a and b) and concentration (c and d) using linear 2D PCA (a and c) and non-linear t-SNE (b and d). HBE, human bronchial epithelial cells; Caco2, human colon epithelial cells; THP-1, human monocytes. The concentrations are expressed as μg mL^{-1}.

variables such as IL-6 (R^2 = 0.958), IL-1β (0.904), transepithelial electrical resistance (TEER) (0.830) and IL-8 (0.777), indicating that the cell type and concentration alone captured the most variability for these response variables. Other response variables had low R^2 value, such as cell viability (0.469), glutathione depletion (0.454), DNA damage (0.320), metabolic activity (0.053) and TNF-α (0.00). Adding the most associated nanodescriptor (see Table 3) to these models did not affect R^2 and MAE (or only slightly).

RF model (Table 4B). In RF modelling, R^2 for the basic model was similar to the linear models [IL-6 (R^2 = 0.955), IL-1β (0.904), IL-8 (0.795), TEER (0.782); glutathione (0.595), cell viability (0.488), DNA damage (0.209), metabolic activity (0.019) and TNF-α (0.008)]. Adding the most associated

Table 4 Influence of statistical parameters that quantifies goodness of fit when the most associated nanodescriptor added to the basic model (cell type + concentration) in linear (A) and RF non-linear modelling (B). R^2 – coefficient of determination; MAE – mean absolute error

(A)	Biological endpoint (s)	Basic model	Validation dataset R^2	Validation dataset MAE	Model with TiO_2 descriptor	Validation dataset R^2	Validation dataset MAE
Case study 1	Metabolic activity	Cell type + Concentration	0.053	8.17	Cell type + concentration + Aspect ratio	0.115	8.10
	Cell viability	Cell type + Concentration	0.469	4.54	Cell type + concentration + *Z*-average in CCM	0.462	4.55
	GSH depletion	Cell type + Concentration	0.454	5.74	Cell type + concentration + *Z*-average in CCM	0.476	5.62
	TEER	Cell type + Concentration	0.830	610.24	Cell type + concentration + *Z*-average in CCM	0.832	608.61
	IL-8 levels	Cell type + Concentration	0.777	17.74	Cell type + concentration + *Z*-average in CCM	0.822	15.99
	IL-6 levels	Cell type + Concentration	0.958	6.94	Cell type + concentration + *Z*-average in CCM	0.958	6.97
	TNF-α levels	Cell type + Concentration	0.019	2.08	Cell type + concentration + *Z*-average in CCM	0.00	2.04
	IL-1β levels	Cell type + Concentration	0.904	3.39	Cell type + concentration + *Z*-average in CCM	0.905	3.42
	DNA damage	Cell type + Concentration	0.320	4.76	Cell type + concentration + *Z*-average	0.37	4.62

(B)	Biological endpoint	Basic model	Validation dataset R^2	Validation dataset MAE	Model with TiO_2 descriptor	Validation dataset R^2	Validation dataset MAE
Case study 1	Metabolic activity	Cell type + concentration	0.019	8.08	Cell type + concentration + Aspect ratio	0.020	8.27
	Cell viability	Cell type + concentration	0.488	4.17	Cell type + concentration + *Z*-average in CCM	0.515	4.00
	GSH	Cell type + concentration	0.595	4.70	Cell type + concentration + *Z*-average in CCM	0.652	4.20
	TEER	Cell type + concentration	0.782	759.35	Cell type + concentration + *Z*-average in CCM	0.856	628.88
	IL-8	Cell type + concentration	0.795	17.13	Cell type + concentration + *Z*-average in CCM	0.845	15.73
	IL-6	Cell type + concentration	0.955	6.96	Cell type + concentration + *Z*-average in CCM	0.969	5.72
	TNF-α	Cell type + concentration	0.036	2.05	Cell type + concentration + *Z*-average in CCM	0.008	2.19
	IL-1β	Cell type + concentration	0.904	4.45	Cell type + concentration + *Z*-average in CCM	0.895	3.73
	DNA damage	Cell type + concentration	0.209	5.31	Cell type + concentration + *Z*-average	0.670	3.63

nanodescriptor to these basic models did not affect R^2 or MAE except for DNA damage, for which adding *Z*-average in stock dispersions strongly affected R^2 (0.209 to 0.670), with a decrease in MAE (5.31 to 3.63).

These findings indicate that under the experimental conditions of case study 1, experimental parameters such as NM concentration and cell type were the well associated with several biological responses such as IL-6, IL-1β, IL-8, TEER and GSH while agglomerate size measured in stock dispersions was strongly associated with the DNA damage induced by TiO_2 NMs.

Case studies 2 and 3

Ranking of nanodescriptors. In case study 1, serum-free exposure conditions were used. To compare the findings with case study 1, we used two other external case studies (2 and 3) fed with data collected under different experimental conditions. Because we identified DNA damage as the only biological endpoint influenced by a NM descriptor (*Z*-average in stock dispersions) in case study 1, we primarily focused on DNA damage in the other two case studies. The datasets for case study 2 were extracted from 11 systematically selected studies, which were performed in the presence and/or in the absence of serum. However, when analysing these studies in detail for more endpoints, we found only cytotoxicity/cell viability reported in all the articles, with other endpoints such as oxidative stress and/or pro-inflammatory responses were rarely reported (see Table S1†). Therefore, we used DNA damage and cytotoxicity/viability results in case study 2 for further analysis. Datasets for case study 3 were from a single study (part of the ENPRA project) with testing performed only in the presence of serum.[16] DNA damage and cytotoxicity/cell viability were the only endpoints consistently reported in this study. Multiple cell types and concentrations were included in both case studies. Fig. S4A–D† shows the ranking of nanodescriptors for different biological endpoints, and Table 5A shows the nanodescriptor(s) closely associated with DNA damage and cell viability for these case studies. For case study 2, mRMR analysis revealed that constituent particle size and crystal phase were the only nanodescriptors associated with DNA damage and cell viability, respectively (among 4 nanodescriptors, see Table 2). Similar to case study 1, in case study 3, *Z*-average size in stock dispersions was most associated with DNA damage, but for cell viability, *Z*-average in CCM (among 5 nanodescriptors) was most associated.

Multivariate modelling. As observed for case study 1, adding the most associated descriptor to the basic linear model did not affect R^2 and MAE in any case study 2 and 3 (Table 5B).

However, for DNA damage, adding the constituent particle size and *Z*-average size (well associated descriptor for case studies 2 and 3, respectively) to the basic model in non-linear modelling contributed strongly to the increase in R^2 in case study 2 (0.014 to 0.658) and moderately in case study 3 (0.186 to 0.367) (Table 5C). A decrease in MAE was also observed in case study 2 (2.08 to 1.16) and case study 3 (0.57 to 0.49). For cell viability, in case study 3, *Z*-average in CCM increased the R^2 of the non-linear basic model (0.199 to 0.586), whereas

Table 5 Statistical analyses of other case studies 2 and 3. Most associated nanodescriptors identified by mRMR approach (A) and influence of statistical parameters that quantifies goodness of fit (R^2 and MAE) when the most associated nanodescriptor was added to the basic model (cell type + concentration) in linear (B) and RF non-linear modelling (C). R^2 – coefficient of determination; MAE – mean absolute error

(A)	Biological endpoints/response variables	Most relevant descriptor (mRMR score >0)
Case study 2	DNA damage	Constituent particle size
	Cell viability	Crystal phase
Case study 3	DNA damage	*Z*-Average
	Cell viability	*Z*-Average in CCM

(B)	Biological endpoint	Basic model	Validation dataset R^2	Validation dataset MAE	Model with TiO_2 descriptor	Validation dataset R^2	Validation dataset MAE
Case study 2	DNA damage	Cell type + concentration	0.471	1.22	Cell type + concentration + Constituent particle size	0.471	1.23
	Viability	Cell type + concentration	0.049	0.08	Cell type + concentration + Crystal phase	0.049	0.08
Case study 3	DNA damage	Cell type + concentration	0.246	0.57	Cell type + concentration + *Z*-average	0.257	0.56
	Viability	Cell type + concentration	0.006	0.04	Cell type + concentration + *Z*-average in CCM	0.085	0.04

(C)	Biological endpoint	Basic model	Validation dataset R^2	Validation dataset MAE	Model with TiO_2 descriptor	Validation dataset R^2	Validation dataset MAE
Case study 2	DNA damage	Cell type + concentration	0.014	2.08	Cell type + concentration + Constituent particle size	0.658	1.16
	Viability	Cell type + concentration	0.011	0.07	Cell type + concentration + Crystal phase	0.011	0.07
Case study 3	DNA damage	Cell type + concentration	0.186	0.57	Cell type + concentration + *Z*-average	0.367	0.49
	Viability	Cell type + concentration	0.199	0.04	Cell type + concentration + *Z*-average in CCM	0.586	0.03

crystal phase, the most associated descriptor in case study 2, did not affect R^2 of the non-linear basic model (Table 5C).

Altogether, these results indicate that DNA damage was affected by a nanodescriptor (agglomerate size in stock or constituent particle size) of TiO_2 NMs in case studies 2 and 3, whereas only in case study 3, cell viability/cytotoxicity was influenced by a nano-descriptor (agglomerate size in cell culture medium).

All case studies

To have a view on the overall influence on the outcome, all case studies were combined together (indicated as "all case studies") and similar analyses were performed. Among other nanodescriptors (constituent particle size, *Z*-average in stock and CCM, crystal phase and coating), agglomerate size and constituent particle size were determined as the most associated nanodescriptor for DNA damage and cytotoxicity, respectively (Fig. S4E and F† and Table 6A). As observed in all

Table 6 Statistical analyses of combined case studies. Most associated nanodescriptors identified by mRMR approach (A) and influence of statistical parameters that quantifies goodness of fit (R^2 and MAE) when the most associated nanodescriptor was added to the basic model (cell type + concentration) in linear (B) and RF non-linear modelling (C). R^2 – coefficient of determination; MAE – mean absolute error

(A)	Biological endpoints/response variables	Most relevant descriptor (mRMR score >0)
All case studies	DNA damage	*Z*-Average
	Cell viability	Constituent particle size

(B)	Biological endpoint	Basic model	Validation dataset R^2	Validation dataset MAE	Model with TiO_2 descriptor	Validation dataset R^2	Validation dataset MAE
All case studies	DNA damage	Cell type + concentration	0.256	0.04	Cell type + concentration + *Z*-average	0.273	0.04
	Viability	Cell type + concentration	0.108	0.07	Cell type + concentration + constituent particle size	0.104	0.07

(C)	Biological endpoint	Basic model	Validation dataset R^2	Validation dataset MAE	Model with TiO_2 descriptor	Validation dataset R^2	Validation dataset MAE
All case studies	DNA damage	Cell type + concentration	0.301	0.04	Cell type + concentration + *Z*-average	0.528	0.03
	Viability	Cell type + concentration	0.162	0.07	Cell type + concentration + constituent particle size	0.306	0.06

other case studies, adding the most associated nanodescriptor to the biological endpoint did not influence the R^2 and MAE in linear models (Table 6B). In RF modelling, R^2 and MAE was moderately influenced for DNA damage and cytotoxicity when adding the most associated nanodescriptor (*Z*-average and primary size, respectively) (Table 6C). Overall analysis of all case studies indicate that, as observed in case study 1 and 3, agglomerate size was the most associated nanodescriptor to DNA damage while the constituent particle size, in contrast to what was observed in case study 2, was more closely associated to cytotoxicity than DNA damage.

Discussion

In this study, we statistically analysed three independent sets of data (case studies) performed under distinct experimental conditions to identify nanodescriptors affecting the toxicity of TiO_2 NMs. Considering all case studies, agglomerate size was found to strongly influence the biological endpoints such as DNA damage and/or cytotoxicity in two case studies (case study 1 and 3) while the constituent particle size was found to influence the DNA damage only in one case study (case study 2).

Case study 1 was well controlled and systematically designed to determine the influence of agglomeration on toxicity and therefore, the association between agglomerate size and DNA damage could possibly be a bias due to the design of the experiment. In case study 3, the NMs agglomerate size found to be most relevant descriptor influencing both DNA damage and cytotoxicity, although the study was not designed to determine the influence of agglomeration on toxicity. It needs to be noted that agglomerate size is not a material specific property and can be influenced/driven by exposure conditions/situations such as pH, ionic strength and motion of the carrier medium.[20]

In case study 2, constituent particle size, a material specific property, was found to be the most relevant parameter. Studies in case study 2 were performed under different experimental conditions (with and without serum). It is known that the alterations in serum concentrations are shown to affect agglomeration stability, particle–cell interaction and toxicological outcome (genotoxicity) of TiO_2 NMs, both *in vitro*[21,22] and *in vivo*.[23] Moreover, there is wide variability in the dispersion protocol used in these studies (see Table S2†), which again could affect the agglomerate size and stability in stock and in exposure conditions. Altogether, when including studies performed in similar experimental conditions (as in case 1 & 3), agglomeration appeared in the analysis, but when including studies performed in varying experimental conditions in the analysis (different agglomeration conditions and different stability) the factor did not appear as a good predictor. This indicates that the models built from the data used in this study may be useful to predict the toxicity of NMs and associated NM parameters in a given experimental condition but may not be meaningful in a broader context.

We found that agglomerate size of TiO_2 NMs in stock dispersions (case studies 1 and 3) and in cell culture medium (case study 3) as a nanodescriptor closely associated with DNA damage and cytotoxicity, respectively. Agglomerate size in stock suspensions also found to be the most associated nanodescriptor to DNA damage when all case studies combined. These findings agree with another study that determined a strong positive correlation between TiO_2 agglomerate size and micronucleus frequency in human lymphocytes.[24] Likewise, other studies have also demonstrated that agglomerate size was found to be the most important factors in determining the cytotoxicity of NMs.[25,26] In *in vitro* studies, it is often discussed that large agglomerates tend to sediment faster, which could affect the toxicity due to higher biologically effective dose.[21] However, in our previous study, we determined that TiO_2 NM sedimentation was influenced rather by raw material and effective density of NMs than their size.[15] In addition, we observed that TiO_2 NMs were effectively taken up as agglomerates by the epithelial cells (HBE) and monocytes (THP-1) cells. Therefore, the association between agglomerate size and cellular uptake may be more relevant to understand the effect of agglomerate size on biological response, and more systematic research is needed in this aspect. We found several *in vitro* studies in which the agglomerate size is only assessed in stock dispersions or in cell culture medium, but not in both,[15,27–30] making it difficult to use these data in a toxicity prediction model. Furthermore, we and other authors found that large TiO_2 agglomerates induced stronger pulmonary responses *in vivo*[31,32] and systemic DNA damage in blood.[15] Therefore, agglomeration size could also be a relevant parameter to predict TiO_2 toxicity *in vivo*, although more and better data are needed to verify this.

Recently, effective density of NMs in *in vitro* exposure condition is recognised as an important parameter in determining their sedimentation, delivered dose and toxicity.[33] Depending on the nature of agglomerates formed by NMs, such as, soft or hard agglomerates, their effective density can vary. For instance, the softer the agglomerate the lower the effective density and hence, the lower the dose reaching the cells. In that case, soft agglomerates of large sizes can reach the cells relatively slower than that of hard agglomerates of small sizes. This indicate that, in addition to agglomerate size, effective density could also influence the cell dosimetry. VCM method is widely used to characterise the effective density of NMs as it is simple, cost effective, and experimentally validated for wide range of NMs. Therefore, we used VCM and estimated effective density in our study (case study 1).[34] However, the effective density was not reported in any of the studies that we used in case study 2 and 3. Therefore, we did not use the effective density in the analyses of case study 1 to make it comparable with case study 2 and 3. In the future, it is strongly recommended to estimate the effective density of NMs in exposure conditions, to not to miss the conceptual link between exposure conditions and observed toxicological effects.

Size and bio-available surface area, which are major determinants of biological interactions, cellular uptake and toxicity can be influenced by agglomerate and aggregate (AA) formation. Surface related properties such as optical and photocatalytic properties of TiO_2 and ZnO NMs are shown to be reduced by AA formation;[35,36] and *vice versa* de-agglomerated/de-aggregated NMs showed increased optical and photocatalytic properties. Spherical NMs, when agglomerated/aggregated, form different secondary structures such as chains and clusters, with different fractal dimensions and aspect-ratios.[37,38] In the case of soluble NMs such as ZnO and Ag, AA formation reduced the solubility of metallic ions in aqueous medium.[39,40] These information indicate that AA formation due to experimental variations can significantly affect the NM properties and hence the toxicological outcome.

Other issues. As DNA damage found to be better predictable endpoint for TiO_2, it is also essential to discuss the artifacts introduced by comet assay. Interaction of NMs, such as, decrease or increase of fluorescence of comet could also leads to bias in the scoring.[41] Moreover, studies have reported that TiO_2 NM tested in his study was not inherently toxic but their photoactivation leading to DNA damage.[42] Therefore, it is highly recommended to be critical in selecting the exposure concentration and to conduct the entire comet assay procedure in the dark to avoid potential artefacts induced by photoactivation.

The relationship between nanodescriptor(s) and toxicity/biological changes (response variable) of NMs was analysed in both linear and non-linear models to predict the biological endpoints induced by TiO_2 NM exposure. We found that nanodescriptors better accounted for the variability in the response variable in RF multivariate modelling than in linear models, which is in line with earlier studies reporting that non-linear models score better to predict cytotoxicity *in vitro* compared with linear models.[6,43] Moreover, Kimberly To T. *et al.*[44] reported that NM characteristics have non-linear dependence with developmental toxicity in zebrafish. These results suggest that non-linear rather than linear models could be more appropriate to predict the biological endpoints of NMs.

From our well-controlled study (case study 1), cell type and concentration explained most of the variability observed in several biological responses. It is well established that toxicity of NMs is cell type- and concentration-dependent, but in the literature, cell type- and concentration-dependent responses were often ignored in modelling approaches.[10] From our case study 1, it also appears that the influence of cell type and NM concentration is also biological endpoint dependent, and should therefore be included when building computational models to predict the toxicity of NMs.

In several computational studies, cytotoxicity/cell viability has been used as the (only) response variable to predict the *in vitro* toxicity of NMs.[6,43,45] However, some NMs, such as TiO_2, exhibit no or very mild cytotoxicity but strong DNA damage.[46–48] In this study, DNA damage was, in addition to concentration and cell-type, significantly affected by a nanodescriptor in all three case studies while cell viability/cytotoxicity popped-up in only one case study (case study 3). DNA damage also appears to have better predictability (higher R^2 value) than cytotoxicity/cell viability under the same experimental conditions (case study 1 and 2). The inclusion of multiple response variables can allow us to identify biological endpoints with better predictability and to make the predictive model more robust, but as evident from the Table S1,† studies reporting multiple endpoints are scarce.

How can we proceed from here to reliable toxicity prediction?. First step would be the standardization of dispersion protocols. This way one can utilize the data from the literature to perform a meta-analysis with minimal bias introduced by experimental conditions. In recent years, tremendous effort has been made in that direction,[49,50] but ideally general protocols are required that are applicable for many types of NMs. Exposure conditions should also be standardized and closely mimic the real exposure situation.

Secondly, systematic studies should be designed to establish in-depth understanding of the influence of material specific properties such as primary size, crystal phase, surface functionalization and modification on agglomeration in different experimental conditions. Alternatively, the same NM should be tested under different experimental condition such as with and without serum and NM characteristics such as catalytic property, release of ions *etc.* should be thoroughly characterized in each condition. This way, we can establish the scientific understanding of the link between material specific properties and agglomeration and therefore, better understanding on the association of material specific properties to the observed toxicity.

Conclusion

In conclusion, in the case of TiO_2, agglomerate size was identified as an important nanodescriptor to predict the biological/toxicological effects. However, agglomeration is strictly spoken not a material specific property and could therefore, be a potential confounding factor in broader context such as safety-by-design approaches. Further, in addition to constituent particle size, agglomerate size in stock and in media (at the start of the exposure) should be at least reported to consider the study as "usable or reliable" and at least two biological endpoints should be included to consider such nanotoxicity data as metadata. Furthermore, it is worth to mention that, in order to feed datasets in automised ML approaches, more focus should be given in the future to control/standardize dispersion protocols and exposure conditions.

Conflicts of interest

There are no conflicts to declare.

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