Protoplasma
DOI 10.1007/s00709-011-0320-3

REVIEW ARTICLE

The importance of a validated standard methodology to define *in vitro* toxicity of nano-TiO_2

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Received: 30 May 2011 / Accepted: 9 September 2011

Abstract Several *in vitro* studies on the potential toxicity of nano-TiO_2 have been published and recent reviews have summarised them. Most of these reports concluded that physicochemical properties of nanoparticles are fundamental to their toxicological effects. No published review has compared *in vitro* tests with similar test strategies in terms of exposure duration and measured endpoints and for this reason we have attempted to assess the degree of homogeneity among *in vitro* tests and to assess if they afford reliable data to support risk assessment. The responses in different *in vitro* tests appeared to be unrelated to primary particle size. The biologically effective concentrations in different tests can be seen to differ by as many as two orders of magnitude and such differences could be explained either by different sensitivities of cell lines to nanoparticles or by effect of the test media. Our review indicates that even when the *in vitro* tests measure the same biomarkers with the same exposure duration and known primary particle sizes, it is insufficient merely to use such data for risk assessment. In the future, validated standard methods should include a limited number of cell lines and an obligatory selection of biomarkers. For routine purposes, it is important that assays can be easily conducted, false negatives and false positives are excluded and unbiased interpretation of results is provided. Papers published to date provide an understanding of the mode on nano-TiO_2 action but are not suitable for assessment and management of risk.

Keywords TiO_2 nanoparticles · Risk assessment · Risk management · Nanotoxicity · Size-dependent effects

Handling Editor: Peter Nick

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Introduction

It has generally been assumed that *in vitro* toxicity tests designed for soluble chemicals are appropriate for nanomaterials (Park et al. 2009). *In vitro* testing is popular due to widely established methodologies, small set-ups with low costs, few ethical problems, ease of interpretation, large numbers of replicates and even miniaturization and automation (Hartung and Daston 2009). A major advantage of *in vitro* testing is replacement or reduction of the use of the laboratory animals but it has some disadvantages that are obscured in broad applications. Extrapolation of *in vitro* toxicology findings to humans can be difficult when the mode of action and/or metabolic conditions in the cell culture

Published online: 20 September 2011

Table 1 Published data on *in vitro* effects of nanosized TiO_2

Primary characteristic (crytal structure)	Supplier	Aggregate characteristics	Additional characterization	Cell line	Method	Exposure concentrations (μg/ml)	Lowest observed effect concentration (μg/ml)	Exposure time (h)	Reference
7, 20, 200 nm (A)	Ishihara Sangyo Kaisha	87, 160, 180 nm (DLS)	DLS, XRF	A549	MTT	16.4, 18.6, 30, 37.1, 41.1, 82.1, 100	**30** (7 nm(DLS 130 nm), 20 nm(DLS 160 nm), 200 nm(DLS 160 nm))	6	Masanori Horie et al. 2010
21 nm (75A)	Research Institute of Science and Technology University of Hertfordshire, England	368 nm	TEM, DLS	PC 12	MTT	0.008, 0.8, 8, 80	100	6	Liu et al. 2010
7 nm	Sigma-Aldrich	N.A.	HPPS	WIL2-NS	MTT	1000	130	6	Jing J. Wang et al. 2007
21 nm (75A)	Degussa	N.A.	N.A.	BEAS-2B	MTT	2, 40, 80	5	24	Eun-Jung Park et al. 2008
20 nm	Aldrich	N.A.	TEM, BET	CHO-K1	MTT	0.5, 1, 5, 10, 25, 50, 100	5	24	A.L. Di Virgilio et al. 2010
21 nm (75A)	Research Institute of Science and Technology University of Hertfordshire, England	368 nm	TEM, DLS	PC 12	MTT	0.008, 0.8, 8, 80	10	24	Liu et al. 2010
7, 20, 200 nm (A)	Ishihara Sangyo Kaisha	87, 160, 180 nm (DLS)	DLS, XRF	HaCaT	MTT	16.4, 18.6, 30, 37.1, 41.1, 82.1, 100	**30** (7 nm(DLS 130 nm), 20 nm(DLS 160 nm), 200 nm(DLS 160 nm))	24	Masanori Horie et al. 2010
15 nm	local vendor	N.A.	TEM	NIH3T3	MTT	0.0005, 0.005, 0.05, 0.5, 5, 50	50	24	Shing Huang et al. 2009
15 nm	local vendor	N.A.	TEM	HFW	MTT	0.0005, 0.005, 0.05, 0.5, 5, 50	50	24	Shing Huang et al. 2009
27 nm (A)	Sigma	28 nm (DLS)	EDS, DLS	BEAS-2B	MTT	5, 50, 100	50	24	Yongli Shi et al. 2010
30 nm (A)	Wako Chemicals	N.A.	N.A.	CHO	MTT	10, 25, 50, 100	100	24	T. Uchino et al. 2002
7 nm	Sigma-Aldrich	N.A.	HPPS	WIL2-NS	MTT	1000	130	24	Jing J. Wang et al. 2007
20 nm (80A)	Evonik	350 nm (sonicated), 900 nm (unsonicated) (DLS)	DLS, XRD, TEM	A549	MTT	0.3, 3, 30, 150, 300, 1000	150	24	Tedja et al. 2011
20 nm (80A)	Evonik	350 nm (sonicated), 900 nm (unsonicated) (DLS)	DLS, XRD, TEM	H1299	MTT	0.3, 3, 30, 150, 300, 1000	150	24	Tedja et al. 2011
10–20 nm (A), 50–60 nm (A)	gift (Su-Ping Qian)	N.A.	TEM	CHO	MTT	5, 10,20, 50, 100, 200, 500, 1000, 2000, 4000	**150** (10–20 nm), **175** (50–60 nm)	24	Rong R. Zhu et al. 2009
10–20 nm (A), 50–60 nm (A)	gift (Su-Ping Qian)	N.A.	TEM	T293	MTT	5, 10,20, 50, 100, 200, 500, 1000, 2000, 4000	**175** (10–20 nm), **225** (50–60 nm)	24	Rong R. Zhu et al. 2009
40 nm	Altair, Nanomaterials Inc.	N.A.	N.A.	BRL 3A	MTT	100, 200, 500, 1000	250	24	S.M. Hussain et al. 2005
23 nm (70A)	Degussa	N.A.	HRTEM, XRD	A549	MTT	100, 500, 1100	500	24	S.Wadhwa et al. 2011
5 nm (A)	Sunrise Chemical Co.	85 nm	TEM, PCS, ICP-AES, XRD	L929	MTT	1, 5, 10	600	24	Cheng-Yu Jin et al. 2008
7, 20, 200 nm (A)	Ishihara Sangyo Company	97, 135, 197 nm (DLS)	TEM, BET, DLS, XRD	HaCaT	MTT	40, 80	No observed effect up to **60**	24	Katsuhide Fujita et al. 2009
5, 40 nm (A)	Sigma-Aldrich, Inframat Advanced Materials LLC	N.A.	BET	MEF	MTT	up to 800	No observed effect up to **100** (5 nm), 20 (40 nm)	24	An Xu et al. 2009
≤ 25 nm (A)	Sigma-Aldrich	N.A.	N.A.	CHO-K1	MTT	5, 25, 50, 100, 200	No observed effect up to **200**	24	Wang et al. 2011
≤ 25 nm (A)	Sigma-Aldrich	950 nm	FEG-SEM, XRD, difuse reflectance, BET	HepG2	MTT	1, 10, 100, 250	Not observed up to **250**	24	Jana Petković et al. 2010
≤ 25 nm	Sigma-Aldrich	N.A.	TEM	HaCaT	MTT	25, 50, 75, 100, 250, 500, 750, 1000	No observed effect up to **1000**	24	Yoon-Hee Park et al. 2011
21 nm (75A)	Degussa	N.A.	N.A.	BEAS-2B	MTT	2, 40, 80	5	48	Eun-Jung Park et al. 2008
21 nm (75A)	Research Institute of Science and Technology	368 nm	TEM, DLS	PC 12	MTT	0.008, 0.8, 8, 80	10	48	Liu et al. 2010
5 nm (A)	Sunrise Chemical Co.	85 nm	TEM, PCS, ICP-AES, XRD	L929	MTT	1, 5, 10	60	48	Cheng-Yu Jin et al. 2008
7 nm	Sigma-Aldrich	N.A.	HPPS	WIL2-NS	MTT	1000	130	48	Jing J. Wang et al. 2007
≤ 25 nm (A)	Sigma-Aldrich	N.A.	N.A.	CHO-K1	MTT	5, 25, 50, 100, 200	200	48	Wang et al. 2011
3–7 nm (A)	Degussa Corporation,	N.A.	TEM, BET, XRD	HDF	MTT	250, 500, 1000, 1250, 10000	300	48	Christie M. Sayes et al. 2006

Table 1 (continued)

Primary characteristic (crytal structure)	Supplier	Aggregate characteristics	Additional characterization	Cell line	Method	Exposure concentrations (μg/ml)	Lowest observed effect concentration (μg/ml)	Exposure time (h)	Reference
	Hanau-Wolfgang								
3–7 nm (A)	Degussa Corporation, Hanau-Wolfgang	N.A.	TEM, BET, XRD	A549	MTT	250, 500, 1000, 1250, 10000	1500	48	Christie M. Sayes et al. 2006
≤ 25 nm (A)	Sigma-Aldrich	950 nm	FEG-SEM, XRD, difuse reflectance, BET	HepG2	MTT	1, 10, 100, 250	Not observed up to **250**	48	Jana Petković et al. 2010
≤ 25 nm	Sigma-Aldrich	N.A.	TEM	HaCaT	MTT	25, 50, 75, 100, 250, 500, 750, 1000	No observed effect up to **1000**	48	Yoon-Hee Park et al. 2011
6, 10, 50, 100(A), 21 nm (75A)	gift (Biswas, Bohkimi), Degussa, gift (Joshi)	479 (−20 mV), 216 (−14 mV), 749 (−13,7 mV), 1000 (−21,3 mV)	TEM, DLS, ζ pot.	HEL-30	LDH	5, 10, 25, 50, 100, 150	**5** (100 nm), **25** (10 nm, 50 nm), **100** (6,3 nm), no effect observed up to **150** (21 nm)	24	Laura K. Braydich-Stolle et al. 2009
20 nm (A), 1 μm (A)	Sigma Chemical Co., gift (dr. Shi Liyi)	385 nm (−12,5 mV), 25 μm (−20 mV)	TEM, DLS, ζ pot.	PC12	LDH	25, 50, 100, 200	**25** (20 nm)	24	Jie Wu et al. 2010
21–30 nm (A + R)	Sigma-Aldrich	(−) 10 mV	ζ pot., TEM, ICP-AES	H1299	LDH	50	50	24	Young Sook Lee et al. 2009
20 nm (A), 1 μm (A)	Sigma Chemical Co., gift (dr. Shi Liyi)	385 nm (−12,5 mV), 25 μm (−20 mV)	TEM, DLS, ζ pot.	PC12	LDH	25, 50, 100, 200	**200** (1 μm)	24	Jie Wu et al. 2010
25 nm (75A)	Degussa	576 nm	DLS, BET	Lung epithelial Type-I cell line R3/1	LDH	10, 200	No observed effect up to **200**	24	Xianglu Han et al. 2011
40 nm	Altair, Nanomaterials Inc.	N.A.	N.A.	BRL 3A	LDH	100, 200, 500, 1000	No effect up to **250**	24	S.M. Hussain et al. 2005
3–7 nm (A)	Degussa Corporation, Hanau-Wolfgang	N.A.	TEM, BET, XRD	HDF	LDH	250, 500, 1000, 1250, 10000	30	48	Christie M. Sayes et al. 2006
3–7 nm (A)	Degussa Corporation, Hanau-Wolfgang	N.A.	TEM, BET, XRD	A549	LDH	250, 500, 1000, 1250, 10000	300	48	Christie M. Sayes et al. 2006
5 nm (A)	Sunrise Chemical Co.	85 nm	TEM, PCS, ICP-AES, XRD	L929	LDH	1, 5, 10	600	48	Cheng-Yu Jin et al. 2008
21 nm (75A)	CEA, Degussa Corp., Sigma, Sigma	N.A.	N.A.	BEAS-2B	LDH	26, 65, 130	No effect up to **100**	48	A. Simon-Deckers et al. 2008
21 nm (75A)	CEA, Degussa Corp., Sigma, Sigma	N.A.	N.A.	BEAS-2B	LDH	26, 65, 130	No effect up to **100**	48	A. Simon-Deckers et al. 2008
20 nm (A), 1 μm (A)	Sigma Chemical Co., gift (dr. Shi Liyi)	385 nm (−12,5 mV), 25 μm (−20 mV)	TEM, DLS, ζ pot.	PC12	DCFH-DA	25, 50, 100, 200	**100** (20 nm)	6	Jie Wu et al. 2010
7, 20, 200 nm (A)	Ishihara Sangyo Kaisha	87, 160, 180 nm (DLS)	DLS, XRF	HaCaT	DCFH-DA	16.4, 18.6, 30, 37.1, 41.1, 82.1, 100	No observed effect up to **30** (7, 20, 200 nm)	6	Masanori Horie et al. 2010
20 nm (A), 1 μm (A)	Sigma Chemical Co., gift (dr. Shi Liyi)	385 nm (−12,5 mV), 25 μm (−20 mV)	TEM, DLS, ζ pot.	PC12	DCFH-DA	25, 50, 100, 200	No observed effect up to **200** (1 μm)	6	Jie Wu et al. 2010
21 nm (75A)	Research Institute of Science and Technology	368 nm	TEM, DLS	PC 12	DCFH-DA	0.008, 0.8, 8, 80	10	24	Liu et al. 2010
6, 10, 50, 100(A), 21 nm (75A)	gift (Biswas, Bohkimi), Degussa, gift (Joshi)	479 (−20 mV), 216 (−14 mV), 749 (−13,7 mV), 1000 (−21,3 mV)	TEM, DLS, ζ pot.	HEL-30	DCFH-DA	5, 10, 25, 50, 100, 150	**10** (21 nm), no effect observed up to **100** (6.3, 10, 50, 100)	24	Laura K. Braydich-Stolle et al. 2009
21 nm (75A)	Degussa	N.A.	N.A.	BEAS-2B	DCFH-DA	2, 40, 80	20	24	Eun-Jung Park et al. 2008
7, 20, 200 nm (A)	Ishihara Sangyo Kaisha	87, 160, 180 nm (DLS)	DLS, XRF	HaCaT	DCFH-DA	16.4, 18.6, 30, 37.1, 41.1, 82.1, 100	**30** (7 nm (DLS 90–100 nm), 7 nm (DLS 130–150 nm), 20 nm (DLS 150–160 nm), 200 nm (DLS 150–160 nm))	24	Masanori Horie et al. 2010
15 nm	local vendor	N.A.	TEM	NIH3T3	Trypan blue	10	10	24	Shing Huang et al. 2009
25–70 nm	Aldrich	N.A.	N.A.	TM3	Trypan blue	5, 10, 20, 40	20	24	Tomoko Komatsu et al. 2008
30–40 nm (90R)	Sigma-Aldrich	N.A.	N.A.	bmDC	Trypan blue	3, 100, 300	30	24	J. Palomaki et al. 2010
≤ 25 nm (A)	Sigma-Aldrich	N.A.	N.A.	CHO-K1	Trypan blue	5, 25, 50, 100, 200	100	24	Wang et al. 2011
30–40 nm (90R)	Sigma-Aldrich	N.A.	N.A.	RAW 264.7	Trypan blue	3, 100, 300	300	24	J. Palomaki et al. 2010

Table 1 (continued)

Primary characteristic (crytal structure)	Supplier	Aggregate characteristics	Additional characterization	Cell line	Method	Exposure concentrations (μg/ml)	Lowest observed effect concentration (μg/ml)	Exposure time (h)	Reference
≤ 25 nm (A)	Sigma-Aldrich	N.A.	TEM	Human nasal epithelia	Trypan blue	10, 25, 50, 100	No observed effect up to **100**	24	Stephan Hackenberg et al. 2010
≤ 20 nm (A)	Sigma-Aldrich	N.A.	TEM, XRD	BEAS-2B	Trypan blue	3.8, 19, 38, 76, 114, 228, 304, 380	No observed effect up to **320**	24	GCM Falck et al. 2009
15 nm	Local vendor	N.A.	TEM	NIH3T3	Trypan blue	10	10	48	Shing Huang et al. 2009
≤ 20 nm (A)	Sigma-Aldrich	N.A.	TEM, XRD	BEAS-2B	Trypan blue	3.8, 19, 38, 76, 114, 228, 304, 380	No observed effect up to **320**	48	GCM Falck et al. 2009

A (anatase), R (rutile) and A + R (anatse + rutile) represent different crystal forms of TiO_2. The number next to the letter represents the portion of that form of TiO_2 (e.g. 75A=75% of anatase). All exposure concentrations are expressed in micrograms per milliliter. All the lowest observed effective concentrations are expressed in micrograms per milliliter and are marked bold. Data in brackets (in lowest observed effective concentration column) represent characteristic of corresponding nanoparticles

model may not be relevant in humans. Chemicals may exert carcinogenic effects in humans via non-genotoxic mechanisms for which there are very few *in vitro* assays, and many *in vitro* models have mutations and increased cell proliferation absent in normal human cells. Additionally, many *in vitro* models do not have cellular detoxification pathways that are available to humans and *in vitro* assays study short-term exposures and immediate effects, while cancer develops in humans over a long exposure and latency period (Kirkland et al. 2007), (Thybaud et al. 2007).

Available *in vitro* tests have been rapidly adopted however for the assessment of the toxic potential of nanoparticles. The selection of test cell lines has typically been driven by the identity of possible target organs. The biomarkers used in nanotoxicity testing are the same as those used for other chemicals and focus on cell viability, membrane stability and lipid peroxidation. Genotoxicity, apoptosis and inflammation have also been examined.

A 2008 workshop held in Washington DC in October 2008, devoted to material characterization in nanotoxicology studies (http://www.characterizationmatters.org) suggested that nanotoxicity data of particles should be accompanied by the characteristics of nanomaterials such as particle size, shape, dissolution rate, agglomeration state, and surface area and chemistry (Oberdörster et al. 2005), (Sayes and Warheit 2008), (Powers et al. 2006), (Erickson 2008). Nanomaterials' characteristics can greatly influence their biological reactivity and are essential for proper interpretation of the findings (Warheit 2008). Recently, some comprehensive reviews have been published on the toxic potential of nano-TiO_2 relevant to human exposure (Aschberger et al. 2011), (Iavicoli et al. 2011) or environmental species exposure (Menard et al. 2011). The aim of these reviews was to provide an overview of the detrimental impact of nano-TiO_2 on organisms and to define risk assessment for human health and ecosystems, linking the observed effects to the exposure levels and evaluating the hazard associated. Some recommendations for future data generation were listed. Some of the conclusions are:

(a) Physical as well as chemical characteristics of particles are fundamental to their biological reactivity;

(b) Experimental conditions may modify the characteristics of particles in a suspension, and both primary and secondary characteristics of particles must be recorded in experimental studies;

(c) Hazards associated with nanoparticles should be evaluated in the light of the likely exposure levels;

(d) A predominant mechanism of nano-TiO_2 toxicity seems to be a ROS-driven effect that may lead to inflammation and potentially to geno- and cytotoxicity;

(e) Threshold mechanisms of toxicity are assumed to be operative with nano-TiO_2;

No published review compares the *in vitro* studies with similar test strategies in terms of exposure duration and endpoints measured. For this reason, the aim of the present review is to provide evidence if the degree of consistency among *in vitro* tests is sufficient to provide reliable data for risk assessment. We reviewed the studies that measured mitochondrial membrane cellular membrane stability, formation of intracellular reactive oxygen species and cell viability.

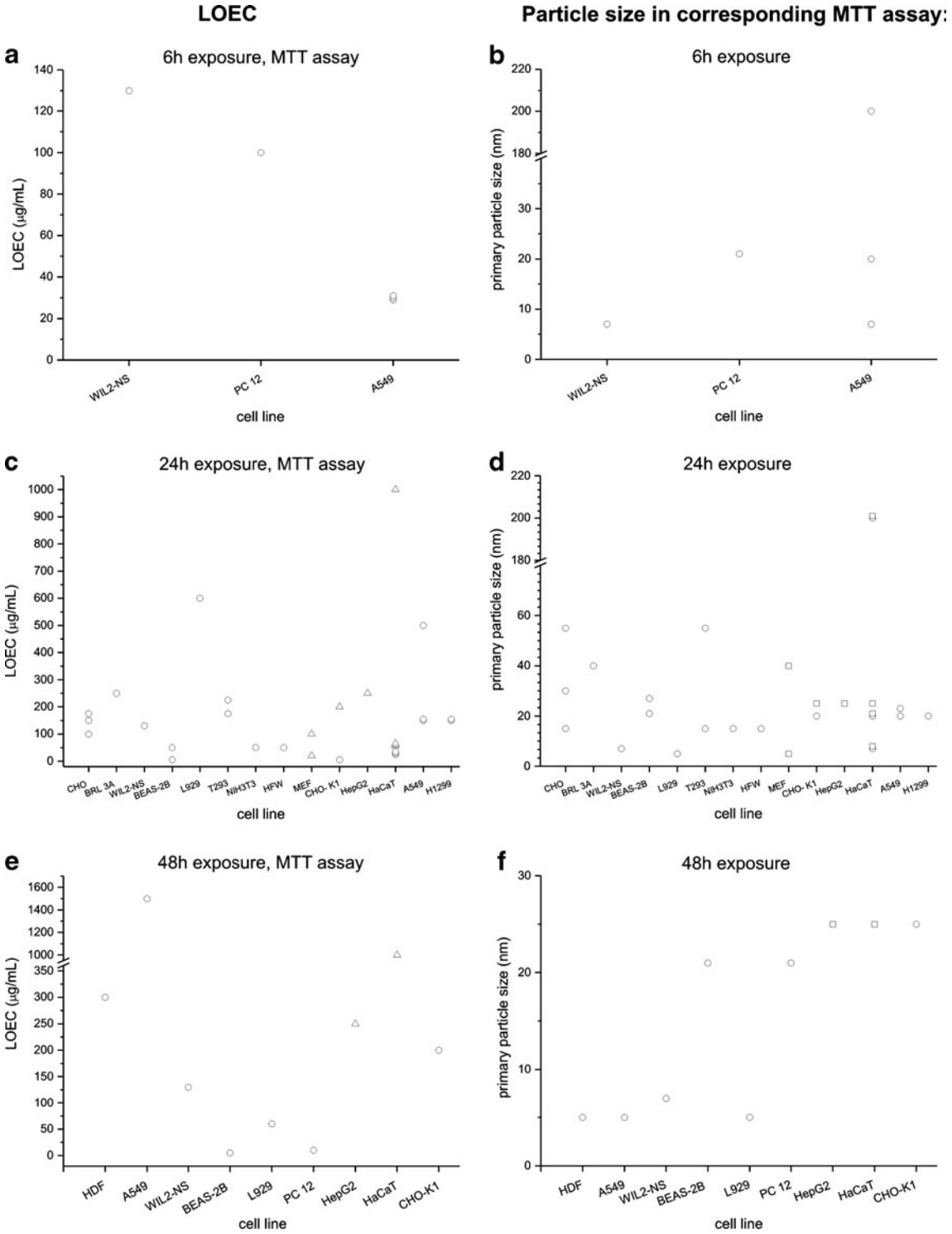

Fig. 1 **a**–**f** LOEC obtained from the MTT test in which the cells were exposed for 6 (**a**), 24 (**c**) or 48 h (**e**). Corresponding primary particle sizes tested in MTT test for 6 (**b**), 24 (**d**) and 48 h (**f**). *White circle* the value of either LOEC or primary particle size; *white triangle* below this concentration no response was observed; *white square* particle size where response was not recorded

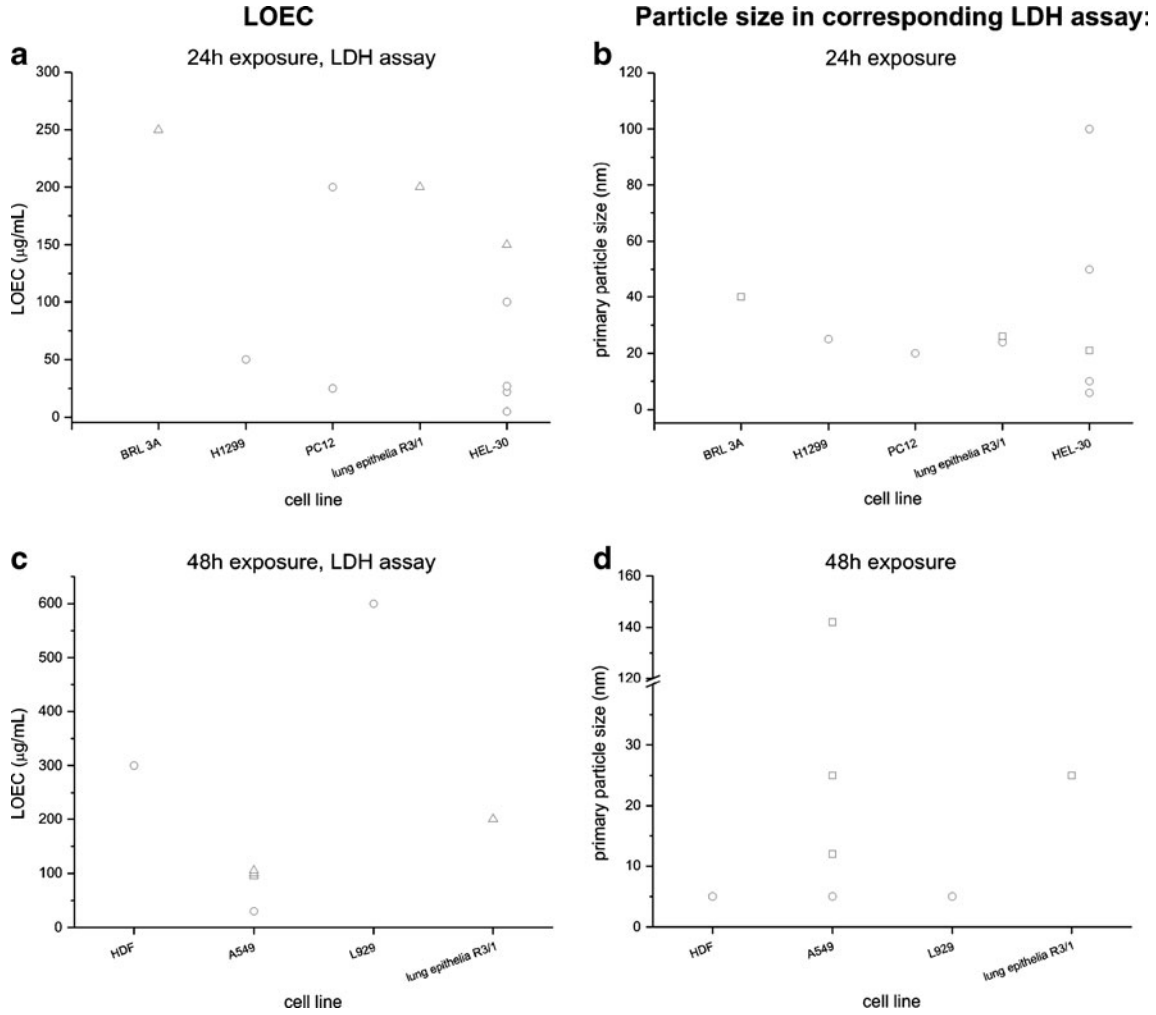

Fig. 2 **a**–**d** LOEC obtained from the LDH test in which the cells were exposed for 24 (**a**) or 48 h (**c**). Corresponding primary particle sizes tested in LDH test for 24 (**b**) and 48 h (**d**). *White circle* the value of either LOEC or primary particle size; *white square* below this concentration no response was observed; *white square* particle size where response was not recorded

In vitro studies reporting biomarker modifications after nano-TiO_2 exposure

The most frequently used biomarkers in *in vitro* tests of nano-TiO_2 include mitochondrial membrane stability (monitored by an MTT assay), cell membrane stability (LDH assay), intracellular reactive oxygen species formation (HDCF-DA) and viability (trypan blue assay). Occasionally, the same biological responses were interpreted differently. For example, the MTT assay, which assesses mitochondrial stability, is often described as a test for cell viability (Uchino et al. 2002).

To enable comparisons among the data, we have selected and graphically presented the lowest observed effect concentration (LOEC) for each test as reported in the literature. Our aims were:

(a) Elucidation of whether responses are related to the primary particle size and, when possible, the secondary particle sizes;
(b) Elucidation of whether responses are time dependent;
(c) Comparison of the sensitivity of cells;
(d) Comparison of the sensitivity of the response.

Changes in mitochondrial membrane stability tested by MTT assay were not related to primary particle size. One can also draw no firm conclusion on time dependency. Different cells generate very different LOEC data ranging from 5 up to 1,000 µg/ml (Table 1). It was assumed that similar exposure times to nano-TiO_2 would provoke generally similar changes in mitochondrial membrane stability. The discrepancy can be attributed either to differences in secondary characteristics of particles or in the sensitivity of cells to nanoparticles.

Lactate dehydrogenase (LDH) activity is used as an indicator of cell membrane integrity. Similar conclusions to those found in the case of affected mitochondrial membrane stability (Fig. 1a, b, c, d, e and f) could be drawn for cell membrane stability. In this case, however,

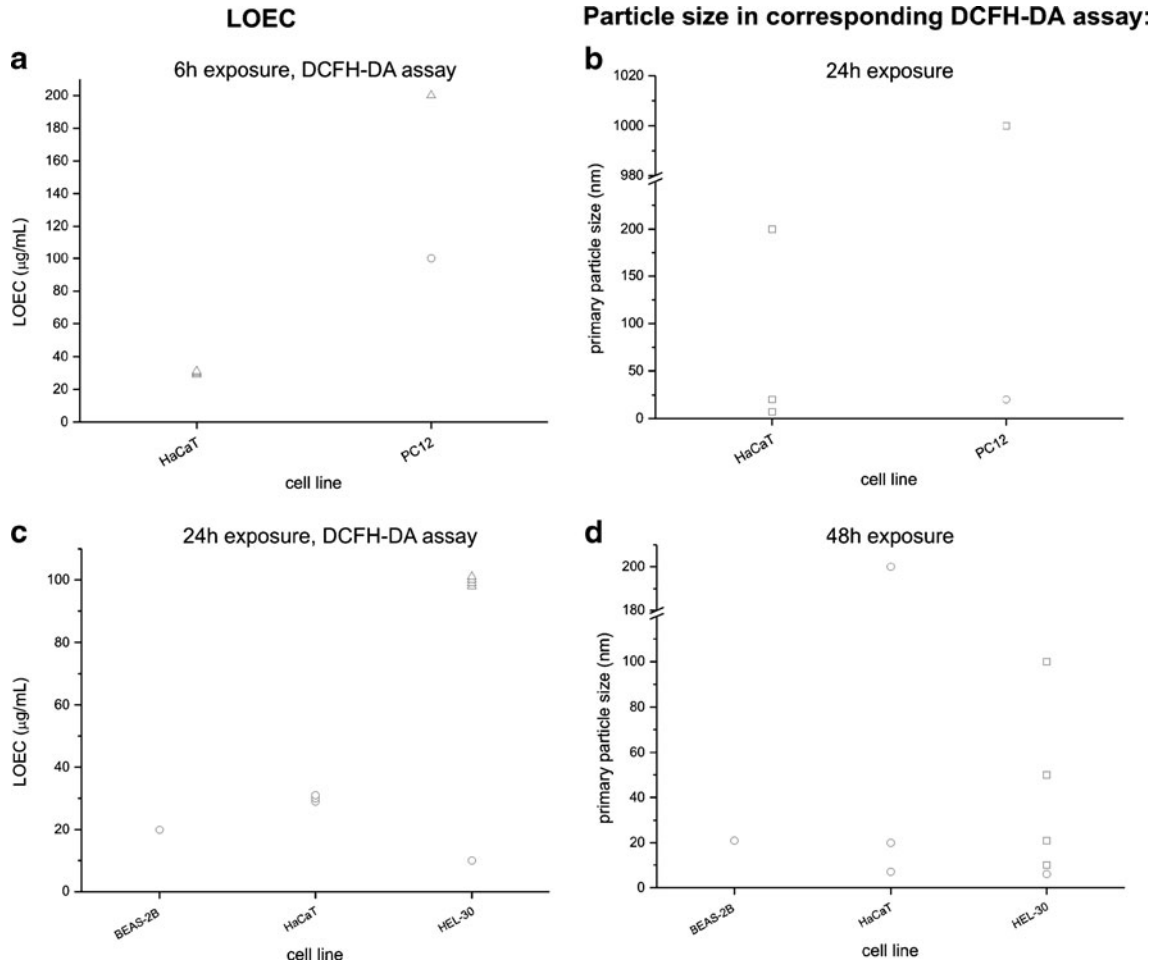

Fig. 3 **a**–**d** LOEC obtained from DCFH-DA assay in which cells were exposed for 6 (**a**) or 24 h (**c**). Corresponding primary particle sizes tested in DCFH-DA assay for 6 (**b**) and 24 h (**d**). *White circle* the value of either LOEC or primary particle size; *white square* no response up to this concentration was observed; *white square* particle size where response was not recorded

far fewer data are available to support any firm conclusions. Results of the LDH assay appear not to be related to primary particle size. Given the paucity of data, it is not possible to draw any conclusions concerning the

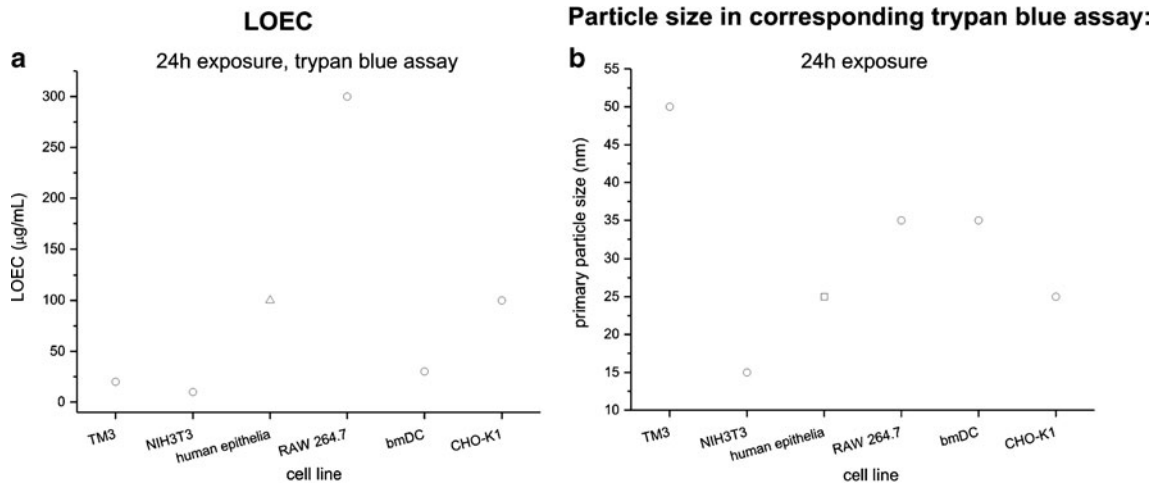

Fig. 4 **a** LOEC values obtained from trypan blue assay in which cells were exposed for 24 h. **b** Corresponding primary particle size determined with the trypan blue assay after 24 h exposure

time dependency of the effect (Fig. 2a, b, c and d). Different LOEC values obtained with different cell types are pronounced. Even when the LDH test was conducted on the same cell type (in this case, HEL-30 cells), different results were obtained.

The cell-based assay which uses 2′,7′-dichlorofluorescin-diacetate (DCFH-DA) is a useful indicator of reactive oxygen species (ROS) but is used less frequently in nanoparticle studies. Consequently, there is a paucity of data and no useful data comparisons could be made (Fig. 3a, b, c and d). A significant discrepancy in LOEC with the same type of cells (HEL-30 cells) is evident but the results appear to be unrelated to primary particle size.

The trypan blue exclusion test is based on the principle that live cells possess intact cell membranes that exclude certain dyes, such as trypan blue. Although few data are available, significant differences in test results among different cell lines are evident (Fig. 4a). The results are unrelated to primary particle size (Fig. 4b).

Discussion

In this review, we compared the *in vitro* studies that sought to evaluate the exposure of different cell lines to nano-TiO_2. The evaluations assessed similar biomarkers at the same exposure times. The biomarkers cited included mitochondrial and cell membrane stability, intracellular reactive oxygen species formation and viability. Responses in different *in vitro* tests were not related to primary particle size and were also not time dependent. The largest amount of data is available for mitochondrial membrane stability after exposure of different cells to nano-TiO_2. A comparison of these studies showed that the LOEC reported may differ by almost two orders of magnitude. Such discrepancies could be explained either by different sensitivities of cell lines to nano-TiO_2 or by interaction of the nanoparticles and the media. It is known that human cell lines show different sensitivities to the same chemical (Hensten-Pettersen and Helgeland 1981) and it has also been reported that cell line sensitivity varies with the assay technique used with no cell line being consistently more sensitive than others. Hensten-Pettersen and Helgeland (1981) also provided evidence that with soluble chemicals, not only the sensitivity of the cells, but the type of medium applied could affect the results obtained. When nanoparticles are suspended in different media, it should be expected that the medium will affect their agglomeration, determining the secondary size of particles and defining their biological reactivity (Murdock et al. 2008), (Meissner et al. 2009). Even if the characteristics of a suspension of nanoparticles, i.e. the secondary characteristics of particles are defined, it cannot be known to what extent the sensitivity of the cell lines and the incubation medium are responsible for the nanotoxicity.

On the basis of this review, we conclude that even when *in vitro* tests measure the same parameters with the same exposure durations and with known primary particle sizes, the resulting data are inadequate to support significant conclusions. Typically, results from very sensitive cells will be overestimated while when the medium interferes with the nanoparticles, the actual biological potential of the particles will be underestimated. The existing test results do not allow assessment of the extent to which the medium has modified the biological activity of the particles. The results of such studies should be interpreted together with likely exposure levels and finally, epithelial cells should be expected to be exposed to higher doses as those encountered by other cells.

For future studies aimed at risk assessment and management, validated standard methods are required. The existing *in vitro* studies on the effects of nano-TiO_2 are a valuable contribution to an understanding of the mode of action of nano-TiO_2 but they are inadequate for an evaluation of the hazards associated with nano-TiO_2 that would support risk assessment.

We suggest that only validated standard methods be used for generation of hazard information in the future. *In vitro* tests could be used for this purpose, but they have to be standardized in terms of exposure duration and parameters measured. In addition, toxicity data should be reported with reference to the primary and secondary characteristics of nanoparticles. Selection of biomarkers should include those defined with respect to different cellular compartments and processes. Based on our present knowledge of the interactions of nanoparticle with cells, assessment of cell and mitochondrial membrane stability, ROS generation, lipid peroxidation and viability appear to be good candidates with which to assess the effects of nanoparticles. Finally, for practical purposes, it is important that selected assay be easy to conduct, false negatives and false positives can be excluded and unbiased interpretation of results is possible.

Acknowledgments We would like to thank the Slovenian Research Agency (project number J1-9475), and G.W.A. Milne for editorial assistance.

Conflict of interest The authors declare that they have no conflict of interest.

References

Aschberger K, Micheletti C, Sokull-Kluttgen B, Christensen FM (2011) Analysis of currently available data for characterising the risk of engineered nanomaterials to the environment and human health—lessons learned from four case studies. Environ Int 37 (6):1143–1156

Braydich-Stolle LK, Schaeublin NM, Murdock RC, Jiang J, Biswas P, Schlager JJ, Hussain SM (2009) Crystal structure mediates mode of cell death in TiO_2 nanotoxicity. J Nanopart Res 11(6):1361–1374

Di Virgilio AL, Reigosa M, Arnal PM, de Mele MFL (2010) Comparative study of the cytotoxic and genotoxic effects of titanium oxide and aluminium oxide nanoparticles in Chinese hamster ovary (CHO-K1) cells. J Hazard Mater 177(1–3):711–718

Erickson BE (2008) Grassroots effort aims to improve quality of nanotoxicology studies. Chem Eng Tools 86(50):25–26

Falck GCM, Lindberg HK, Suhonen S, Vippola M, Vanhala E, Catalan J, Savolainen K, Norppa H (2009) Genotoxic effects of nanosized and fine TiO_2. Hum Exp Toxicol 28(6–7):339–352

Fujita K, Horie M, Kato H, Endoh S, Suzuki M, Nakamura A, Miyauchi A, Yamamoto K, Kinugasa S, Nishio K, Yoshida Y, Iwahashi H, Nakanishi J (2009) Effects of ultrafine TiO_2 particles on gene expression profile in human keratinocytes without illumination: involvement of extracellular matrix and cell adhesion. Toxicol Lett 191(2–3):109–117

Hackenberg S, Friehs G, Froelich K, Ginzkey C, Koehler C, Scherzed A, Burghartz M, Hagen R, Kleinsasser N (2010) Intracellular distribution, geno- and cytotoxic effects of nanosized titanium dioxide particles in the anatase crystal phase on human nasal mucosa cells. Toxicol Lett 195(1):9–14. doi:10.1016/j.toxlet.2010.02.022

Han X, Gelein R, Corson N, Wade-Mercer P, Jiang J, Biswas P, Finkelstein JN, Elder A, Oberdörster G (2011) Validation of an LDH assay for assessing nanoparticle toxicity. Toxicology In press

Hartung T, Daston G (2009) Are *in vitro* tests suitable for regulatory use? Toxicol Sci 111(2):233–237

Hensten-Pettersen A, Helgeland K (1981) Sensitivity of different human cell-lines in the biologic evaluation of dental resin-based restorative materials. Scand J Dent Res 89(1):102–107

Horie M, Nishio K, Fujita K, Kato H, Endoh S, Suzuki M, Nakamura A, Miyauchi A, Kinugasa S, Yamamoto K, Iwahashi H, Murayama H, Niki E, Yoshida Y (2010) Cellular responses by stable and uniform ultrafine titanium dioxide particles in culture-medium dispersions when secondary particle size was 100 nm or less. Toxicol *in vitro* 24(6):1629–1638

Huang S, Chueh PJ, Lin WY, Shih ST, Chuang SM (2009) Disturbed mitotic progression and genome segregation are involved in cell transformation mediated by nano-TiO_2 long-term exposure. Toxicol Appl Pharm 241(2):182–194

Hussain SM, Hess KL, Gearhart JM, Geiss KT, Schlager JJ (2005) *in vitro* toxicity of nanoparticles in BRL 3A rat liver cells. Toxicol *in vitro* 19(7):975–983

Iavicoli I, Leso V, Fontana L, Bergamaschi A (2011) Toxicological effects of titanium dioxide nanoparticles: a review of *in vitro* mammalian studies. Eur Rev Med Pharmacol Sci 15(5):481–508

Jin CY, Zhu BS, Wang XF, Lu QH (2008) Cytotoxicity of titanium dioxide nanoparticles in mouse fibroblast cells. Chem Res Toxicol 21(9):1871–1877

Kirkland DJ, Aardema M, Banduhn N, Carmichael P, Fautz R, Meunier JR, Pfuhler S (2007) *In vitro* approaches to develop weight of evidence (WoE) and mode of action (MoA) discussions with positive *in vitro* genotoxicity results. Mutagenesis 22 (3):161–175

Komatsu T, Tabata M, Kubo-Irie M, Shimizu T, Suzuki K, Nihei Y, Takeda K (2008) The effects of nanoparticles on mouse testis Leydig cells *in vitro*. Toxicol *in vitro* 22(8):1825–1831

Lee YS, Yoon S, Yoon HJ, Lee K, Yoon HK, Lee JH, Song CW (2009) Inhibitor of differentiation 1 (Id1) expression attenuates the degree of TiO_2-induced cytotoxicity in H1299 non-small cell lung cancer cells. Toxicology Lett 189(3):191–199

Liu SC, Xu LJ, Zhang T, Ren GG, Yang Z (2010) Oxidative stress and apoptosis induced by nanosized titanium dioxide in PC12 cells. Toxicology 267(1–3):172–177

Meissner T, Potthoff A, Richter V (2009) Physico-chemical characterization in the light of toxicological effects. Inhal Toxicol 21 (S1):35–39

Menard A, Jemec A, Drobne D (2011) Ecotoxicity of nanosized TiO_2. Review of in vivo data. Environ Pollut 159(3):677–684

Murdock RC, Braydich-Stolle L, Schrand AM, Schlager JJ, Hussain SM (2008) Characterization of nanomaterial dispersion in solution prior to *in vitro* exposure using dynamic light scattering technique. Toxicol Sci 101(2):239–253

Oberdörster G, Maynard A, Donaldson K, Castranova V, Fitzpatrick J, Ausman K, Carter J, Karn B, Kreyling W, Lai D, Olin S, Monteiro-Riviere N, Warheit DB, Yang H (2005) Principles for characterizing the potential human health effects from exposure to nanomaterials: elements of a screening strategy. Part Fibre Toxicol 2(8)

Palomaki J, Karisola P, Pylkkanen L, Savolainen K, Alenius H (2010) Engineered nanornaterials cause cytotoxicity and activation on mouse antigen presenting cells. Toxicology 267(1–3):125–131

Park EJ, Yi J, Chung YH, Ryu DY, Choi J, Park K (2008) Oxidative stress and apoptosis induced by titanium dioxide nanoparticles in cultured BEAS-2B cells. Toxicol Lett 180(3):222–229

Park MVDZ, Lankveld DPK, van Loveren H, de Jong WH (2009) The status of *in vitro* toxicity studies in the risk assessment of nanomaterials. Nanomedicine 4(6):669–685

Park YH, Jeong SH, Yi SM, Choi BH, Kim YR, Kim IK, Kim MK, Son SW (2011) Analysis for the potential of polystyrene and TiO_2 nanoparticles to induce skin irritation, phototoxicity, and sensitization. Toxicol *in vitro In press*

Petković J, Žegura B, Stevanović M, Drnovšek N, Uskoković D, Novak S, Filipič M (2010) DNA damage and alterations in expression of DNA damage responsive genes induced by TiO_2 nanoparticles in human hepatoma HepG2 cells. Nanotoxicology in press

Powers KW, Brown SC, Krishna VB, Wasdo SC, Moudgil BM, Roberts SM (2006) Research strategies for safety evaluation of nanomaterials. Part VI. Characterization of nanoscale particles for toxicological evaluation. Toxicol Sci 90(2):296–303

Sayes CM, Warheit DB (2008) An *in vitro* investigation of the differential cytotoxic responses of human and rat lung epithelial cell lines using TiO_2 nanoparticles. Int J Nanotechnol 5(1):15–29

Sayes CM, Wahi R, Kurian PA, Liu YP, West JL, Ausman KD, Warheit DB, Colvin VL (2006) Correlating nanoscale titania structure with toxicity: a cytotoxicity and inflammatory response study with human dermal fibroblasts and human lung epithelial cells. Toxicol Sci 92(1):174–185

Shi YL, Wang F, He JB, Yadav S, Wang H (2010) Titanium dioxide nanoparticles cause apoptosis in BEAS-2B cells through the caspase 8/t-Bid-independent mitochondrial pathway. Toxicol Lett 196(1):21–27

Simon-Deckers A, Gouget B, Mayne-L'Hermite M, Herlin-Boime N, Reynaud C, Carriere M (2008) *In vitro* investigation of oxide nanoparticle and carbon nanotube toxicity and intracellular accumulation in A549 human pneumocytes. Toxicology 253(1–3):137–146

Tedja R, Marquis C, Lim M, Amal R (2011) Biological impacts of TiO_2 on human lung cell lines A549 and H1299: particle size distribution effects. J Nanopart Res 13:3801–3813

Thybaud V, Aardema M, Clements J, Dearfield K, Galloway S, Hayashi M, Jacobson-Kram D, Kirkland D, MacGregor JT, Marzin D, Ohyama W, Schuler M, Suzuki H, Zeiger E (2007) Strategy for genotoxicity testing: hazard identification and risk assessment in relation to *in vitro* testing. Mutat Res Gen Toxicol Eng 627(1):41–58

Uchino T, Tokunaga H, Ando M, Utsumi H (2002) Quantitative determination of OH radical generation and its cytotoxicity induced by TiO_2-UVA treatment. Toxicol *in vitro* 16(5):629–635

Wadhwa S, Rea C, O'Hare P, Mathur A, Roy SS, Dunlop PSM, Byrne JA, Burke G, Meenan B, McLaughlin JA (2011) Comparative *in vitro* cytotoxicity study of carbon nanotubes and titania nanostructures on human lung epithelial cells. J Hazard Mater 191(1–3):56–61. doi:10.1016/j.jhazmat.2011.04.035

Wang JJ, Sanderson BJS, Wang H (2007) Cyto- and genotoxicity of ultrafine TiO_2 particles in cultured human lymphoblastoid cells. Mutat Res Gen Toxicol Eng 628(2):99–106

Wang S, Yu H, Wickliffe JK (2011) Limitation of the MTT and XTT assays for measuring cell viability due to superoxide formation induced by nano-scale TiO_2. Toxicol *in vitro In press*

Warheit DB (2008) How meaningful are the results of nanotoxicity studies in the absence of adequate material characterization? Toxicol Sci 101(2):183–185

Wu J, Sun JA, Xue Y (2010) Involvement of JNK and P53 activation in G2/M cell cycle arrest and apoptosis induced by titanium dioxide nanoparticles in neuron cells. Toxicol Lett 199(3):269–276

Xu A, Chai YF, Nohmi T, Hei TK (2009) Genotoxic responses to titanium dioxide nanoparticles and fullerene in gpt delta transgenic MEF cells. Part Fibre Toxicol 6: in press

Zhu RR, Wang SL, Chao J, Shi DL, Zhang R, Sun XY, Yao SD (2009) Bio-effects of nano-TiO_2 on DNA and cellular ultrastructure with different polymorph and size. Mat Sci Eng C Bio S 29(3):691–696