1
Titanium Dioxide Nanocomposites

Hans Arora, Caroline Doty, Ye Yuan, John Boyle, Katarina Petras, Bryan Rabatic, Tatjana Paunesku, and Gayle Woloschak

1.1
Introduction

In Nature, titanium dioxide exists in three primary phases – anatase, rutile, and brookite – with different sizes of crystal cells in each case [1]. The popularity of titanium dioxide in materials sciences began with the first photocatalytic splitting of water in 1972 [2]. However, in recent years TiO_2 has been used widely for the preparation of different types of nanomaterials, including nanoparticles, nanorods, nanowires, nanotubes, and mesoporous and nanoporous TiO_2-containing materials [3]. Regardless of scale, TiO_2 maintains its photocatalytic abilities, and in addition, nanoscale TiO_2 has a surface reactivity that fosters its interactions with biological molecules, such as phosphorylated proteins and peptides [4], as well as some nonspecific binding with DNA [5]. Nano-anatase TiO_2, which is smaller than 20 nm, has surface corner defects that alter the size of the crystal cell [6, 7] (Table 1.1).

The surface molecules of nanoscale TiO_2 particles are "on the corner" of the particle, and are forced by confinement stress into a pentacoordinated, square-pyramidal orientation. Such molecules have a propensity for stable nanoparticle conjugation to *ortho*-substituted bidentate ligands such as 3,4-dihydroxyphenethylamine (dopamine) [7, 8]. This binding with enediol ligands "heals" the surface corner defects and returns the surface TiO_2 molecules into an octahedral geometry. As a consequence, the stability of the chemical bonds formed on the nanoparticle surface precludes further modifications of the nanoparticle surface, which may aid in reducing nanoparticle aggregation and nonspecific interactions with cellular components [9, 10].

The methods used for the synthesis of TiO_2 nanoparticles have included sol, sol–gel, solvothermal, hydrothermal and other approaches [3], although new methods and modifications of the existing methods have been attempted with great frequency. Among such efforts are included the use of different dopants in the synthesis of TiO_2 nanocomposites, such as noble metals [11] (the use of core

Nanomaterials for the Life Sciences Vol.8: Nanocomposites. Edited by Challa S. S. R. Kumar

ISBN: 978-3-527-32168-1

Table 1.1 Phases of TiO_2.

Phase (Reference)	Crystal system	a (Å)	b (Å)	c (Å)
Rutile [1]	Tetragonal	4.594	4.594	2.959
Anatase [1]	Tetragonal	3.789	3.789	9.514
Brookite [1]	Orthorhombic	9.166	5.436	5.135
Anatase with corner defects [7]	–	3.96	3.96	2.7

materials such as iron oxide–silicon dioxide–titanium dioxide core-corona-shell nanoparticles [12]) and the use of different nanoparticle surface-coating molecules and photosensitizing dyes [5, 9, 10, 13–17].

Although, at present, no systematic nomenclature is used to codify nanostructures, a proposal has been made recently to develop a "nano nomenclature" [18], and it is hoped that this may aid in making any review (including the present chapter) more systematic. For example, if an attempt were made to apply this nomenclature to the 5 nm TiO_2 nanoparticles with DNA oligonucleotide and gadolinium–DOTA conjugated to its surface [13], the formula for this nanoconjugate would be 2-5B- TiO_2-(DNA,Gd, DOTA), where the "2" indicates a metallic nanoparticle, "5B" a size of 5 nm and a spherical shape, and TiO_2 the material of the particle and the (DNA,Gd, DOTA) molecules conjugated to the surface. Obviously, this designation would require a further determination of DNA sequence, as well as information such as the anticipated strength of the chemical bonds between the nanoparticle and the conjugated materials. With TiO_2 in particular, the nomenclature would also have to include information on the crystal polymorph of the nanoparticle, the presence of "corner defects" on the nanoparticle surface, and so on.

In this chapter, the most recent applications of nanoscale TiO_2 will briefly be summarized in: (i) Photocatalysis for chemical degradation and antimicrobial activity; (ii) phosphopeptide enrichment from biological materials *in vitro*; (iii) the uptake and effects of nanoscale TiO_2 and nanocomposites in cells; (iv) the use of TiO_2 and its composites for implants and tissue engineering; and (v) toxicology studies of TiO_2 nanomaterials in animals.

A comprehensive list of the nanoparticulate TiO_2 materials reviewed in the chapter is provided in Table 1.2.

1.2 Photocatalysis by TiO_2 Nanoparticles, Nanocomposites and Nanoconjugates for Chemical Degradation and Antimicrobial Activity

The photocatalytic activity of TiO_2 molecules has been widely studied and utilized in biological, chemical, and industrial applications. The term "photocatalytic" refers to the ability of a material to form electron-hole pairs upon absorbing elec-

Table 1.2 The nanoparticulate TiO_2 materials reviewed in this chapter.

Nanoparticle composition	Size	Method(s) used for sizing	Shape	Crystal structure (%)			Manufacturer	Surface area per mass ($m^2 g^{-1}$)	Dispersity	Reference
				Anatase	Rutile	Brookite				
TiO_2	25–70 nm						Sigma-Aldrich			
TiO_2	20 nm									[19]
TiO_2	21 nm			25	75	0	DeGussa-Hüls AG (Frankfurt, Germany)			[20]
TiO_2	<100 nm						Sigma-Aldrich			[21]
TiO_2	19–21 nm			0	100	0	DeGussa (Frankfurt, Germany)	50 ± 15		[22]
TiO_2	80–110 nm	TEM, XRD	Round	100		0	Self			[23]
TiO_2	<25 nm (10 nm via MFR)	TEM	Elongated and round	100		0	Sigma-Aldrich	145		[24]
TiO_2	<75 nm (40 nm via MFR)	TEM	Round	Mix	Mix	0	Sigma-Aldrich	40		[24]
TiO_2	20–30 nm			70	30	0	CAS no. 13463-67-7 (commercial)	48.6		[25]
TiO_2	21 nm (24.1 ± 2.8)	TEM		25	75	0	DeGussa ("Aeroxide" P25)	50 ± 15		[26]

Table 1.2 *Continued*

Nanoparticle composition	Size	Method(s) used for sizing	Shape	Crystal structure (%)			Manufacturer	Surface area per mass ($m^2 g^{-1}$)	Dispersity	Reference
				Anatase	Rutile	Brookite				
TiO_2	5 nm (3.5 ± 1)	TEM		100	0	0	Nanostructured and Amorphous Materials (Los Alamos, NM)	210 ± 10 (219 ± 3)		[27]
TiO_2	20.5 ± 6.7 nm	SEM	Round				Degussa (P25)	45.41		[28]
TiO_2	25–70 nm						Sigma-Aldrich			[29]
TiO_2	15 nm			100	0	0	Sigma-Aldrich	190–290		[30]
TiO_2	7, 20 nm						Sigma-Aldrich			[31]
TiO_2	3 nm	AFM		100	0	0	Self	299.1		[32]
TiO_2	20 nm	SEM		100	0	0	Shanghai Huijing Sub-Nanoscale New Material Co., Ltd., Shanghai, China	120 (105.0)		[32]
TiO_2	5 nm	XRD		100	0	0	Self			[33]
TiO_2	30 nm	TEM					Self			[34]

TiO_2	Diameter = 4–6 nm	TEM	Rods	0	100 (XRD)	0	Self	1.646		[35]
TiO_2	21 nm						DeGussa ("Aeroxide" P25) (New Jersey, USA)			[36]
TiO_2	20 nm			100	0	0				[37]
TiO_2	21 nm (20 nm)	TEM					DeGussa Korea (P25)	50		[38]
TiO_2	120 nm (hydrodynamic diameter)	TEM		80	20	0	DeGussa AG (New Jersey, USA)	50 ± 15	0.131–0.138 (polydispersity index)	[39]
TiO_2	21 nm			80	20	0	DeGussa ("Aeroxide" P25)			[40]
TiO_2	21 nm			80	20	0	DeGussa ("Aeroxide" P25)			[41]
TiO_2	34.2 ± 26.1 nm	TEM	Spherical	Mix	Mix	0	Sigma-Aldrich	18.6 ± 1.2		[42]
TiO_2	25–70 nm	FE-SEM		100	0	0	Sigma-Aldrich	18.6 ± 1.2		[43]
TiO_2	20–30 nm	TEM		70	30	0	CAS No. 13463-67-7	48.6		[44]
TiO_2	25, 80 nm	TEM					Hangzhou Dayang Nanotechnology Co. Ltd.			[45]

Table 1.2 *Continued*

Nanoparticle composition	Size	Method(s) used for sizing	Shape	Crystal structure (%)			Manufacturer	Surface area per mass ($m^2 g^{-1}$)	Dispersity	Reference
				Anatase	Rutile	Brookite				
TiO_2	80 nm (71.43 ± 23.53 nm)	SEM		0	100	0	Hangzhou Dayang Nanotechnology Co. Ltd.			[46, 47]
TiO_2	200 × 35 nm	TEM	Rods	100	0	0	Self	26.5		[48]
TiO_2	10 nm	TEM	Dots	100	0	0	Self	169.4		[48]
98% TiO_2 (core), 2% alumina (coating)	136.0 ± 35	HR-SEM		0	100	0	DuPont	18.2		[49, 50]
88% TiO_2 (core), 7% amorphous silica, 5% alumina (coating)	149.4 ± 50	HR-SEM		0	100	0	DuPont	35.7		[49, 50]
90% TiO_2 (core), 7% alumina, 1% amorphous silica (coating)	140.0 ± 44	HR-SEM		21	79		DeGussa (P25)	38.5		[49, 50]
N–TiO_2	NA	NA	NA	NA	NA	NA	NA	NA	NA	[51]

MnO_2/TiO_2	4.53–9.17 (pore size)	XRD	NA	100	0	0	NA	98.3–142.6	NA	[52]
Rare earth oxide-TiO_2	9	XRD and TEM	Square	100	0	0	NA	NA	NA	[53]
TiO_2-carbon nanotubes	9.6–11.5	XRD	TiO_2 spheres attached to carbon tubes	84.5–99.5		0.5–15.5	NA	75–156	NA	[54]
Al/TiO_2	25–45	XRD and TEM	NA	100			NA	134–361	NA	[55]
$Ag–TiO_2$	12.3–32.3	XRD and TEM	NA	All three phases	All three phases	All three phases	NA	NA	NA	[56]
$TiO_2–Ni/Fe_2O_4$	10–15 and 25–30	XRD	Sphere	100% for 10–15 nm		100% for 25–30 nm	NA	NA	NA	[57]
TiO_2/ montmorillonite	50–100	Dynamic light scattering	Sphere	100			NA	122–248	NA	[58]
Polypyrrole/TiO_2	15–20	XRD and TEM	Sphere	100			NA	NA	100% uniform dispersion	[59]
Carotenoid/TiO_2	7	NA	NA	100			Ishihara Co.	NA	NA	[60]

Table 1.2 *Continued*

Nanoparticle composition	Size	Method(s) used for sizing	Shape	Crystal structure (%)			Manufacturer	Surface area per mass ($m^2 g^{-1}$)	Dispersity	Reference
				Anatase	Rutile	Brookite				
CdS/TiO_2	8–10 × 150–300	XRD and TEM	Tube	NA	NA	NA	NA	214–245	NA	[61]
CNT/TiO_2	100–120	TEM	Tube	NA	NA	NA	NA	NA	NA	[62]
Ag/TiO_2	100	TEM	NA	100			NA	11.5	NA	[63]
$Au–TiO_2$	7–8	XRD and TEM	Sphere	100			NA	179.6–184.5	NA	[64]
$ZnO–TiO_2$	9	XRD	Hexagonal	100			NA	NA	NA	[65]
Polyaniline-AMTES-TiO_2	NA	XRD	NA	NA	NA	NA	NA	NA	NA	[66]
TiO_2/Ag	15–25	TEM	Rod	NA	NA	NA	NA	NA	NA	[67]
Au/TiO_2	10–15	XRD and TEM	Sphere	100			NA	NA	NA	[68]
Ag/TiO_2	16–20	XRD	Sphere	100	NA	NA	NA	NA	NA	[69]
Ag/TiO_2	10	XRD	NA	100	NA	NA	NA	157	NA	[70]
Ag/TiO_2 thin film	20	SEM	NA	NA	NA	NA	NA	NA	NA	[71]
$Ag–TiO_2$/Ag/a-TiO_2 thin film	35	SEM and TEM	Spheres in film	100			NA	NA	NA	[72]

Sn^{4-}/TiO_2	9	XRD and SEM	NA	100			NA	100	NA	[73]
$MWNT/TiO_2$	3–24 nm MWNT coated with 3 nm TiO_2	XRD	Needle-like structure	100			NA	172	NA	[74]
S/TiO_2	8.5–11.2	XRD and TEM	Sphere	100			NA	72.9–113.4	NA	[75]
Nd/TiO_2, W/TiO_2, Zn/TiO_2	6–8	XRD and TEM	Tetragonal	100			NA	NA	NA	[76]
EVOH-TiO_2	90	TEM	NA	100			NA	NA	NA	[77]
iPP/TiO_2	80	TEM	NA	100			NA	NA	NA	[78]
$Fe_3O_4@TiO_2$	NA	NA	NA	NA	NA	NA	NA	NA	NA	[79]
Au/TiO_2 film	5–10 nm Au particle in 250–300 nm TiO_2 film	TEM and SEM	Sphere	NA	NA	NA	NA	NA	NA	[80]
TiO_2	38 nm			100	0	0	Alfa Aesar			[81]
TiO_2	5 um						Sigma			[82]
$Fe_3O_4@TiO_2$ core-shell microspheres	Fe_3O_4 microspheres had diameter of 280 nm								10 mg/ml	[83]

Table 1.2 *Continued*

Nanoparticle composition	Size	Method(s) used for sizing	Shape	Crystal structure (%)			Manufacturer	Surface area per mass ($m^2 g^{-1}$)	Dispersity	Reference
				Anatase	Rutile	Brookite				
TiO_2	32 nm	Sintering	NA	60	40	0	Nanophase Technologies Corporations	NA	NA	[84]
TiO_2	49 mm	NA	NA	NA	NA	NA	Nanophase Technologies Corporations	NA	NA	[85]
TiO_2	32 nm	NA	NA	40	60	0	Nanophase Technologies Corporations	NA	NA	[86]
TiO_2	32 nm	Sintering	NA	NA	NA	NA	Nanophase Technologies Corporations	NA	NA	[87]
TiO_2	20, 26, 32, and 56 nm	Sintering	NA	90	10	0	Nanophase Technologies Corporations	NA	NA	[88]

tromagnetic radiation. TiO_2 is a wide gap semi band conductor with a band gap energy of 3.2 eV for the anatase crystal structure, and 3.0 eV for the rutile structure [87]. When TiO_2 absorbs photons of electromagnetic radiation with energy greater than its band gap, valence band electrons are promoted to the conduction band of the TiO_2 molecule, which leaves an electropositive hole in the valence band [2]. Thus, the absorption of electromagnetic radiation by TiO_2 produces electron-hole pairs ($e^- h^+$) that can be transferred through the material to the surface of the bulk TiO_2. At the surface, the charged electrons (e^-) are spatially separated from the electropositive holes (h^+), thus forming separate reducing and oxidizing centers [2]. When TiO_2 is in an oxygenated aqueous environment, the charged electrons can reduce O_2 to form superoxide ($O_2^{\bullet}$) whereas, the electropositive holes oxidize water to form hydroxyl radicals ($OH^{\bullet}$) [88]. Thus, the reductive and oxidative abilities of the electron-hole pairs can lead to the production of strong oxidizing agents applicable for many purposes, from chemical to microbial decontamination.

In the nanoparticle regime, TiO_2 preserves its photocatalytic properties; moreover, as the reaction efficiency increases in line with the surface-to-volume ratio of the material, it has been microparticle and nanoparticle formulations TiO_2 rather than the bulk material that have been used in biological, chemical, and industrial applications. Nevertheless, whilst the TiO_2-driven photocatalytic degradation of chemicals and microorganisms has been applied to decontamination and environmental purification, many drawbacks have emerged to prevent its even wider use. The first of the two main issues is centered around the high energy requirements for triggering a photocatalytic response (photoresponse). As the band gap of TiO_2 is 3.2 eV, the anatase crystal can only absorb photons of wavelengths shorter than 388 nm, primarily in the ultraviolet (UV) light spectrum. Hence, TiO_2 nanoparticles can only absorb approximately 2–3% of solar light energies, which makes the commercial applications of TiO_2 nanoparticles ineffective with natural light sources. The second major drawback to using TiO_2 nanoparticles in industrial applications is that the photocatalytic efficiency of TiO_2 is often low, due to charge recombination. During photocatalysis, the reactive electron and the electropositive hole of the electron-hole pairs can recombine within the material before they are transferred to its surface. However, when such recombination occurs, the catalytic efficiency of the nanoparticle decreases.

In an attempt to avoid difficulties associated with TiO_2 use, investigations have been made into the applications of TiO_2 nanocomposites and nanoconjugates. Hence, the primary goals for new TiO_2-based nanoscale materials are to:

- increase the photoresponse (the energetic range at which the nanoparticles can be excited) so that the TiO_2 nanoconjugates can be excited by energies in the visible light spectrum;
- increase the photocatalytic efficiency (the ability of a photocatalytic material to overcome charge recombination and allow separated charges to interact with molecules at the surface of the material).

The major strategies to attain these goals include: conjugation to a charge transfer catalyst; noble metal deposition; doping with metal and nonmetal ions;

blending with metal oxides; coating with photosensitizing dyes; compositing with polymers; and coupling with semiconductors. Although each of the modifications has different mechanisms by which they can either increase photoresponse or photocatalytic efficiency, all were found to aid the overall photocatalytic properties of TiO_2.

1.2.1 Methods for Evaluating Photocatalysis

Before providing descriptions of the different approaches used to increase the photocatalytic ability of TiO_2 nanocomposites, there is a need to outline the various methods generally used in its evaluation. The majority of such methods involve the breakdown of various molecules, typically dyes that change color in a predictable, dose-dependent manner in response to oxidation or reduction. An exception is the use of electron paramagnetic (spin) resonance (EPR), which provides a direct measurement of the production of reactive oxygen species (ROS). The most common dyes used for quantification of photocatalysis include methylene blue and methyl orange, in addition to assays for the degradation of phenol, formaldehyde, salicylic acid and various other oxidizable molecular targets. For example, methylene blue has a blue color in an oxidizing environment, but turns clear in a reducing environment. During photocatalysis, the dyes undergo decomposition that in turn causes a change in color which can be quantified spectrophotometrically, by measuring the absorption of the dye at a specific wavelength.

In contrast, EPR provides a direct measurement of the production of radicals (typically hydroxyl radicals) created by the oxidation of water by electropositive holes.

1.2.2 Different Types of TiO_2 Nanocomposites

1.2.2.1 Modifying TiO_2 with Charge-Transfer Catalysts

Charge-transfer catalysts (CTCs) are molecules that have the ability to trap reactive electrons (e^-) and electropositive holes (h^+) [89]. In the case of TiO_2, the addition of a CTC to the nanoparticle allows for a more efficient trapping of electropositive holes on the surface hydroxyl sites of the TiO_2 molecule [89]. The improved trapping ability of the nanocomposite decreases the charge recombination in TiO_2 and leads to an overall increase in photocatalytic efficiency. The most common CTCs used with TiO_2 are Al_2O_2, Al_2O_3 and SiO_2 [89].

1.2.2.2 Coating with Photosensitizing Dyes

The process of coating creates nanoconjugates rather than nanocomposites; however, these hybrid structures also often have improved photocatalytic abilities compared to the bare nanoparticles. The primary focus of adding a photosensitizing dye to a nanoparticle is most often not to reduce the charge recombination, but rather to change the photoresponse of the TiO_2 nanoparticle. For example,

alizarin can lower the photon energy required to excite the nanoparticles, thus decreasing the band gap energy of alizarin-coated TiO_2 nanoparticles to 1.4 eV, in the white light range [8]. In studies conducted by Konovalova *et al.*, the addition of carotenoids to TiO_2 led to the formation of ROS on the surface of nanoconjugates under red light irradiation [60]. One of the most effective photocatalysis reactions with TiO_2 was accomplished by the Gratzel laboratory, when a 10.6% solar light efficiency was achieved by using a dye-sensitized solar cell technology [90].

1.2.2.3 Noble Metal Deposition or Coupling

A noble metal is commonly defined as an element that can resist oxidation, even at high temperatures. Noble metals include rhenium, ruthenium, rhodium, palladium, silver, osmium, iridium, platinum, and gold; of these, the most commonly used in combination with TiO_2 nanoparticles are gold and silver. As noble metals are resistant to oxidation, they are thought to act as an electron sink, promoting the movement of reactive electrons away from the TiO_2 molecule onto the surface of the noble metal [67]. The noble metal surface then acts as a site where redox reactions occur, thus preventing charge recombination within the TiO_2 nanoparticle and increasing its photocatalytic reactivity.

In many studies, silver has been deposited onto TiO_2, primarily because it is more cost-effective than gold and platinum, but also because it has an intrinsic ability to prevent bacterial growth, as well as an effective photocatalytic ability at nanoscale [11]. Previously, silver has been added to TiO_2 nanoparticles, TiO_2 nanorods, and TiO_2 nanofilms. In fact, studies conducted by Li and colleagues have shown that silver-deposited TiO_2 anatase nanoparticles have an improved photoresponse compared to that of anatase TiO_2 nanoparticles, Degussa P25 TiO_2 nanoparticles, and mixed anatase–rutile TiO_2 nanoparticles [63]. The use of Ag–TiO_2 nanocomposite films has also been shown to have an increased photocatalytic reactivity compared to the nonmodified material. For example, UV-illuminated Ag–TiO_2 nanocomposite films are 6.3-fold more effective at photodegrading methyl orange than are UV-illuminated pure TiO_2 films [56].

The deposition of gold and platinum onto TiO_2 nanoparticles has also demonstrated an increase in the photocatalytic reactivity of TiO_2. Yu and coworkers have reported an improved photocatalytic reactivity of Au–TiO_2 nanocomposite microspheres compared to TiO_2 microspheres and Degussa P25 TiO_2 nanoparticles [64]. In addition, UV-illuminated TiO_2 nanofilms embedded with gold nanostructures have a better photonic efficiency than UV-illuminated pure TiO_2 films [80].

1.2.2.4 Doping and Grafting

The purpose of doping TiO_2 nanoparticles with metals is to create a heterojunction – a space which ranges from 10 to 100 nm in size, located between the surface of the doping metal and that of the TiO_2 nanoparticle. Within this space an interior electric field forms that aids in the separation of electron-hole pairs [91]. As a consequence, reduced electrons are driven to different surface sites away from the electropositive holes, which in turn results in a reduction of the charge

recombination. A special case of heterojunctions involves the use of carbon nanotubes (CNTs) with TiO_2 nanoparticles or TiO_2 nanofilms [91]. These arrays showed up to 99.1% degradation of phenol, compared to 78.7% degradation by pure TiO_2 nanotubes [91]. Similarly, multi-walled carbon nanotubes (MWCNTs) anchored to TiO_2 nanoparticles improved the photocatalytic degradation of methylene blue [54]. Ni-deposited, CNT-grafted TiO_2 nanocomposites have demonstrated photocatalytic reactivity both with UV irradiation and with exposure to an electric field of 500 V DC, with an enhanced photocatalytic degradation of NO gas molecules in comparison to pure TiO_2 nanoparticles [62]. Other molecules which have been used successfully for grafting include nitrogen, Ag_2S, and Pd–PdO [51, 92, 93]. Nitrogen-doped TiO_2-layered/isosterate nanocomposites showed an increased photocatalytic reactivity when illuminated with visible light between 380 and 500 nm [51].

UV-irradiated TiO_2 nanocomposites doped with rare earth oxides (oxides of Eu^{3+}, Pr^{3+}, Gd^{3+}, Nd^{3+} and Y^{3+}) showed a higher extent of degradation of partially hydrolyzed polyacrylamide (HPAM) [53].

1.2.2.5 Coupling with Semiconductors, Blending with Metal Oxides

The coupling of TiO_2 to a narrow-gap semiconductor material can result in an increase in photocatalytic reactivity, as well as an increase in photoresponse. When a narrow-gap semiconductor coupled to a TiO_2 nanoparticle is exposed to visible light, it produces reactive electrons that can travel through the semiconductor to the nonactivated TiO_2 nanoparticle [61]. This process extends the photoresponse of the TiO_2 to visible light wavelengths. Coupling TiO_2 to a semiconductor also decreases charge recombination, because the heterojunction space between the two semiconductors allows for a more efficient separation of reactive electrons and electropositive holes [61]. Common semiconductors and metal oxides coupled to TiO_2 include: CdS, WO_3 and $Cs_xH_{3-x}PW_{12}O_{40}$ [61, 89, 94, 95]. Zhu *et al.* have reported that the bamboo-like CdS/TiO_2-nanotube nanocomposites, when activated with visible light, demonstrated a methylene blue degradation of 83.7% compared to only 9.5% and 41.1% by TiO_2-nanotubes and pure CdS, respectively [61].

The metal oxides ZnO, MnO_2 and In_2O_3, when coupled to TiO_2 nanoparticles, have also been shown as efficient in increasing the photocatalytic capability of TiO_2 nanoparticles [52, 54, 96]. The addition of MnO_2 to TiO_2 nanoparticles broadens the excitation spectrum of TIO_2 to visible light ranges, as demonstrated by methylene blue degradation [52].

A different crystal structure of the TiO_2 may also have an effect on photoresponse. For example, under UV light conditions ZnO–TiO_2 nanotubes can oxidize rhodamine B with a higher efficiency than either pure TiO_2 nanoparticles, pure TiO_2 nanotubes, pure ZnO, or ZnO–TiO_2 nanoparticles [65].

1.2.2.6 Modifying with Polymers or Clays

The functionalization of TiO_2 nanoparticles with polymers with good conducting properties can be used to direct the charged electrons (e^-) and electropositive holes

(h^+) away from the surface of TiO_2. Moreover, the addition of polymers that allow for a large internal interface area between the polymer and the TiO_2 particle aids in charge segregation and also prevents charge recombination [59]. Similarly, the addition of clays can aid in charge segregation by providing a large internal interface between the clay and the TiO_2 molecule. In the past, a multitude of polymer/TiO_2 nanocomposites have been used on the basis of their ability to increase photocatalytic reactivity. Most notably, polypyrrole, kaolinite, polyaniline, poly amide and poly-lactic acid (PLA) have each been added to TiO_2 nanoparticles, and all have shown enhanced photocatalytic reactivity of the nanocomposites [59, 66, 97, 98]. For example, visible polypyrrole–TiO_2 nanocomposites degraded 95.54% of methyl orange compared to 40% degradation by visible light with pure TiO_2 nanoparticles [59]. Similarly, Shi-xiong *et al*. demonstrated the ability of UV- and solar light-irradiated anilinomethytriethoxysilane–TiO_2 nanoparticles composited with polyaniline (PANI/AMTES-TiO_2) to increase the photoresponse and reduce charge recombination [66].

Novel methods for developing TiO_2–clay nanocomposites have involved the use of heterocoagulation to create TiO_2–montmorillonite (MMT) nanocomposites, in which a silicate layer is used to support the TiO_2 nanoparticle. Kun and coworkers subsequently showed that these nanocomposites had a higher photocatalytic reactivity than pure TiO_2, due to the added catalytic activity of the silicate support layer of the nanocomposites [58].

1.2.3 Antimicrobial Uses of Nanocomposites

The antibacterial and decontamination applications of TiO_2 nanoparticles have undergone extensive investigation since 1985, when Matsunaga *et al*. first demonstrated the microbicidal effect of TiO_2 [99]. Subsequently, numerous studies have documented the bactericidal activity of TiO_2 nanoparticles, founded on their photocatalytic reactivity under UV-illumination [177]. The illumination of TiO_2 leads to the generation of ROS that oxidize membrane lipids and cause disruption to the outer and cytoplasmic membranes of the bacteria by lipid peroxidation; this leads in turn to the death of the bacterial cells [100].

Since the microbicidal ability of TiO_2 nanoparticles depends on their photoresponse and photocatalytic reactivity, all of the issues pertinent to the improvement of photocatalysis will aid with the killing of bacteria, viruses, and fungi. Again, the creation of nanocomposites to circumvent the partial photocatalytic reactivity of the TiO_2 due to charge recombination, and to increase the photoresponse of TiO_2, would permit nanocomposites to be used for sterilization with sunlight, in a most energy-effective way. Multiple additions can be made to TiO_2 nanoparticles, TiO_2 nanofilms and TiO_2 nanorods so as to create TiO_2 nanocomposites. These additions include noble metal deposition, doping with metal and nonmetal ions, compositing with a polymer, and the creation of core–shell magnetic nanoparticles. Currently, TiO_2 nanocomposites are used in multiple antimicrobial, antifungal, and waste decontamination applications, and can also be used to sterilize medical devices

such as catheters and dental implants [71, 101]. TiO_2 nanocomposites have also been tested for sterilization of food packaging and food preparation surfaces, to prevent the bacterial contamination of food [68, 102, 103]. However, perhaps the most often used application of TiO_2 nanocomposites in this area has been for the purification of drinking water and decontamination of waste water [88].

1.2.3.1 Antimicrobial Nanocomposites with Noble Metal Deposition

The most frequently used noble metals in antimicrobial applications are silver and gold. For example, gold-capped TiO_2 nanocomposites have a strong oxidizing ability and showed a 60–100% killing efficacy of *Escherichia coli* [68]. Likewise, silver has long been studied and recognized for its potential as an antimicrobial agent, with silver ions and nanoparticles having been shown capable of killing bacteria, viruses, and fungi [104]. Recently, Ag–TiO_2 nanocomposite powders, Ag–TiO_2 nanofilms, and Ag-deposited Ag–TiO_2 nanocomposite films were all shown to exhibit enhanced photocatalytic reactivities and bactericidal activities compared to TiO_2 nanoparticles and TiO_2 nanofilms. For example, when Zhang *et al*. used a one-pot sol–gel approach to produce 10 nm TiO_2 nanocomposites with a high Ag-loading ability, the nanocomposites showed a complete inhibition of *E. coli* growth at silver concentrations of only 2.4 $\mu g\,ml^{-1}$ [69]. The compositing of Ag into TiO_2 films has been met with similar success; as an example, Liu *et al*. used the Ag doping of a TiO_2 nanofilm to kill silver-resistant *E. coli* when the nanocomposite films were UV light-irradiated [70]. In this case, the bacterial survival rate on the nanocomposite was only 7%, compared to 53.7% on UV light-irradiated pure TiO_2 nanofilms [70]. Similarly, silicon catheters coated with Ag–TiO_2 nanofilms with embedded nanocomposites demonstrated a self-sterilizing effect, with a 99% sterilization of *E. coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* after UV illumination [71]. A similar doping of Ag–TiO_2 nanofilms with Ag–TiO_2 nanocomposite particles led, under solar light conditions, to a photocatalytic killing of *E. coli* that was 6.9-fold more effective than with TiO_2 nanofilms, and 1.35-fold more effective than with Ag/a-TiO_2 nanofilms [72]. Finally, UV-illuminated platinum nanoparticles embedded in a TiO_2 nanofilm demonstrated an increase in the photocatalysis-driven killing of *Micrococcus lylae* cells, compared to UV-illuminated pure TiO_2 nanofilms [105].

1.2.3.2 Antimicrobial Doped and Grafted Nanocomposites

The doping of TiO_2 nanoparticles with metals and nonmetals has been shown to be an effective way of increasing the photocatalytic reactivity of TiO_2. The applications for doped TiO_2 nanocomposites range from antimicrobial coatings on textiles, the inactivation of endospores, solid-surface antimicrobial coatings, and aqueous system-based biocides [74, 102, 106]. Another practical application of TiO_2 nanocomposites has been the use of tin (Sn^{4-})-doped TiO_2 nanofilms on glass surfaces, so as to confer a self-cleaning function [73]. In line with this, Sayikan *et al*. showed that Sn^{4-}-doped TiO_2 nanofilms on UV-illuminated glass surfaces had an antibacterial effect against both Gram-negative *E. coli* and Gram-positive *Staph. aureus*, whereas the TiO_2 films alone had no antibacterial effect [73, 75].

The grafting of MWCNTs to TiO_2 was used to inactivate bacterial endospores under UV light conditions, demonstrating a biocidal efficiency (LD_{90}) of 90% for the inactivation of *Bacillus cereus* endospores. Under the same conditions, pure TiO_2 nanoparticles showed no significant biocidal capabilities [74].

In addition to the nonmetal doping of TiO_2 nanoparticles, many groups have focused on the metal and metal-ion doping of TiO_2 nanoparticles. For example, when Venkatsubramanian *et al.* compared the antibacterial and photocatalytic reactivities of W^{4+}, Nd^{3+}, and Zn^{2+}-doped TiO_2 nanocomposites [76], the antibacterial activities of the nanocomposites were rated as follows: $W^{4+}/TiO_2 > Nd^{3+}/TiO_2 > Zn^{2+}/TiO_2 >$ pure TiO_2 nanoparticles [76]. It is believed that tungsten has the greatest effect on photocatalytic reactivity and antimicrobial activity due to its ability to reduce the band gap of TiO_2 and to aid in charge separation, which makes it highly photoresponsive and photocatalytically reactive [76]. Studies conducted by Lui Li-Fen *et al.* showed iron-doped TiO_2 nanocomposites to have a higher capacity for the UV photocatalytic disinfection of *E. coli* (20% survival) than did pure TiO_2 (40% survival) [102].

As an alternative, iron oxide–silicon dioxide–titanium dioxide core-corona-shell nanoparticles showed less photocatalytic reactivity than the pure Degussa P25 TiO_2; however, the ability to "recycle" such nanoconjugate constructs on the basis of their magnetic core outweighed the relative disadvantage of their lesser photocatalytic reactivity [12].

1.2.3.3 **Modifying with Polymers**

Polymers are commonly used as materials for food packaging, mainly because the addition of TiO_2 nanoparticles to polymer sheets can provide an antibacterial approach to sterilization. This was demonstrated by Cerrada *et al.*, who by incorporating TiO_2 nanoparticles into an ethylene–vinyl copolymer matrix (EVOH) were able to maintain an antimicrobial capacity at the interface of the TiO_2–EVOH nanocomposite; in fact, the UV irradiated TiO_2-EVOH killed 99.9% of all Gram-positive and Gram-negative bacteria tested [77]. In a similar fashion, TiO_2-embedded polymer oxide thin films also showed an enhanced antimicrobial activity. For this, TiO_2 was incorporated into an isotactic polypropylene (iPP) polymeric matrix, so as to create an iPP–TiO_2 thin film nanocomposite which, when illuminated with UV light, showed an 8- to 9-fold log increase in bactericidal effect against *P. aeruginosa* and *Enterococcus faecalis* when compared to pure TiO_2 nanoparticles [78].

1.2.3.4 **Creation of Magnetic Nanocomposites**

One major problem facing those industries that use TiO_2 nanoparticles to purify water has been to separate the TiO_2 from the system after use. The removal of TiO_2 from water decontamination applications is, in fact, often very difficult, and will involve the use of a slurry system. In order to overcome this problem, a core–shell magnetic nanoparticle was created that consisted of a magnetic core encapsulated by a TiO_2 shell. Thus, in a TiO_2–$NiFe_2O_4$ core–shell magnetic nanoparticle, the core would retain the magnetic properties, and the TiO_2 shell the photocatalytic

reactivity. When exposed to UV light, such nanoconjugates would cause a reduction in the growth of *E. coli* [57]. A similar Fe_3O_4@TiO_2 core–shell nanocomposite also exhibited antimicrobial activity. In practice, the nanocomposites were conjugated to an immunoglobulin G (IgG) antibody, which allowed the direct targeting of pathogenic bacteria such as *Staphylococcus saprophyticus*, *Streptococcus pyogenes*, methicillin-resistant *Staph. aureus*, and multi-antibiotic-resistant *S. pyogenes*. Whilst, after targeting, UV irradiation of the nanocomposites led to a reduction in bacterial survival compared to negative controls [79], the main benefit was that the Fe_3O_4 core allowed them to be separated from solution simply by applying a magnetic field [79].

With the advent of combining different materials with TiO_2, a multitude of light-activated antimicrobial applications has been developed. Typical strategies used to increase the antimicrobial activity of TiO_2 nanoparticles and nanofilms by increasing their photoresponse and photocatalytic reactivity have included noble metal deposition, doping with metal and nonmetal ions, compositing with a polymer, and the creation of core–shell magnetic nanoparticles. Clearly, these newly developed nanocomposites will continue to show promise as antimicrobial agents for both industrial and biological applications.

1.3 Use of TiO_2 Nanoparticles, Nanocomposites, and Nanoconjugates for Phosphopeptide Enrichment from Biological Materials In Vitro

In addition to its photocatalytic capabilities, TiO_2 nanoparticles also demonstrate a surface reactivity that has been harnessed for use in basic science research. As the nanoparticle surface has a high affinity for phosphate groups, this can lead to various nonspecific interactions between TiO_2 nanoparticles and biological materials, such as proteins and DNA [4, 5]. Currently phosphorylated proteins are of major interest in biomedical research, and the application of TiO_2 nanoparticles in this area will be discussed at this point.

Reversible phosphorylation is a critical cellular tool that is used to control key processes such as signal transduction, gene expression, cell cycle progression, cytoskeletal regulation, and apoptosis [81]. The most common phosphorylation targets in proteins are the amino acids serine, threonine and tyrosine, and almost 30% of all proteins in mammalian cells are phosphorylated at some point during their processing [107]. It is assumed that in diseases such as cancer, AIDS, diabetes and neuronal disorders, protein phosphorylation patterns and cell signaling networks have become disturbed, causing the negative health effects of the disease [108]. In recent years, it was shown that TiO_2 nanoparticles could be used to trap and identify phosphopeptides of interest [4], and for this purpose a range of TiO_2 columns has been created that are capable of enriching certain phosphopeptides from a complex peptide mixture. Moreover, this method is not only very effective but also introduces a greater efficiency into the subsequent steps of the total research process.

The most interesting phosphorylated proteins are usually of low abundance, such that the process of phosphorylation is typically sub-stoichiometric in nature. Yet, phosphorylation is also a highly dynamic event, and can occur at multiple sites on a protein when not all of the potential phosphorylation sites are fully occupied. Nonetheless, results with even a phosphopeptide-rich sample may be seriously affected by the presence of nonphosphorylated peptides (as demonstrated using mass spectrometry). As a result, it is very difficult to generate a sample that is suitable for mass spectrometric analysis, as the phosphorylated proteins must first be enriched and separated from their original complex sample. Although phosphorylated proteins are chemically stable, many enzymes can alter their phosphorylation status. For example, the human genome contains about 500 kinases and over 100 phosphatases; hence, when tissues or cells are lysed and samples are extracted, there is a high likelihood that further enzymatic reactions will occur and that the samples will be compromised. The benefit of TiO_2 materials in this respect is that they show a great specificity with regards to the types of oligomer that they bind, and so often are used in conjunction with the technique of immobilized metal affinity chromatography (IMAC). Notably, as TiO_2 can selectively adsorb organic phosphates, it is an ideal material for capturing phosphopeptides [4]. Moreover, TiO_2 has *amphoteric* properties, which allow it to behave as either a Lewis acid or a Lewis base, depending on the pH of the solution used to wash the TiO_2 material. Acidic conditions cause the TiO_2 to be positively charged and to exhibit anion-exchange properties [108]. Consequently, samples prepared for analysis on TiO_2 columns are often dissolved in an acidic solution so as to promote electrostatic binding between the positively and negatively charged groups [109], after which they can be desorbed under alkaline conditions [110]. An additional point is that nanosized materials have high surface area-to-volume ratios, which allows them to bind to a much greater number of targets [4]. One problem here is that TiO_2 can bind multi-phosphorylated peptides so strongly that their elution becomes difficult; consequently, it is more suited to the isolation of mono-phosphorylated peptides [109]. The overall process is very effective and about 50–75% more phosphopeptides will be detected if a TiO_2-enrichment stage precedes the mass spectrometry analysis [110].

TiO_2 nanocomposites can be synthesized via photopolymerization and adapted into stationary chromatography phases for use in either microchannels or micro tips. The composites are prepared by crosslinking TiO_2 nanoparticles with organic groups, which helps to prevent the loss of particles during washing through TiO_2 composite cartridges. In this way, a TiO_2-packed pipette tip may serve as an offline first-dimension separation step in a two-dimensional (2-D) chromatography system [107]. It has in fact been found that certain agents can improve the binding of phosphorylated peptides while blocking the attachment of nonphosphorylated peptides that are not of interest. Loading the samples in 2,5-dihydroxybenzoic acid (DHB) can reduce the binding of nonphosphorylated peptides to TiO_2, while maintaining the high binding affinity for phosphorylated peptides [110].

TiO_2 nanocomposites with a high loading capacity and high capture efficiency were formed by first silanizing nanoparticles with 3-mercaptopropyltrimethoxysi-

lane (MPTMS), and then photopolymerizing them in the presence of a diacrylate crosslinker [4]. Scanning electron microscopy (SEM) images of the nanocomposites revealed an agglomeration of particles, which helped them to be retained within the cartridge when used as a chromatographic packing material. Further investigations revealed that the TiO_2 nanocomposites had twice as much phosphate binding capacity, and a fivefold larger capture efficiency compared to 5 μm TiO_2 particles. Overall, the results of these studies indicated the need to identify a size balance for TiO_2 nanoparticles – they must not be so small as to be lost during the enrichment process, but not too large as to have any significant phenyl phosphate adsorption [4].

Titanium dioxide can also be incorporated into microspheres which consist of an iron oxide core and a titanium dioxide shell, where the iron core imparts magnetic properties on the sphere, and allows the material–target conjugate to be isolated from the solution simply by using a magnet. However, this ultimately will result in a trade-off between process efficiency and accuracy. It has been shown that magnetic core microspheres have an ill-defined structure, and a decreased selective affinity for phosphopeptides [81]. Li *et al.* attempted to overcome these problems by first synthesizing Fe_3O_4@C microspheres, and then attaching titanium via calcination to form the Fe_3O_4@TiO_2 microspheres. Following an enrichment with iron–titanium microspheres, all three potential phosphopeptides could be identified in the tryptic β-casein samples, whereas the non-enriched sample showed, very weakly, one of the potential peptides [81].

1.4 Uptake and Effects of Nanoscale TiO_2 and Nanocomposites in Cells

The prevalence of bulk TiO_2 in common household items such as toothpaste and sunscreens, as well as its importance in industrial syntheses, has led to many studies of how bulk and nanoscale TiO_2 interact with cells. In particular, intensive studies have been undertaken to determine how particle size [111], surface area, and surface chemistry can impact the ability of TiO_2 to enter cells. Some studies have also identified the phagocytic or endocytic pathways by which TiO_2 may enter eukaryotic cells, and how the surface modification or conjugation of biomolecules to its surface can impact on the uptake pathways and nanoparticle retention dynamics [9, 15, 112, 113]. A number of toxicology studies have been conducted to determine how nanoscale/ultrafine TiO_2 can induce inflammatory or apoptotic responses from immune and epithelial cells of the lung [112–117]. More recently, interest has been expressed in using the photocatalytic properties of TiO_2, coupled with the ability to conjugate biomolecules to nanoscale TiO_2, as a novel method for delivering therapeutic or diagnostic agents to malignant cells [9, 13, 16, 118].

The following section is organized according to the sequence of events culminating with the intracellular effects of TiO_2 nanocomposites. The uptake mechanisms important for the internalization of nanocomposites will first be discussed,

followed by details of the intracellular localization of internalized nanocomposites. Finally, the molecular and cellular responses of cells to internalized TiO_2 will be outlined.

1.4.1 Uptake of TiO_2 Nanomaterials

The precise mechanism by which TiO_2 crosses the selectively permeable barrier of the plasma membrane is a question that must be considered on a case-by-case basis, because different cells "favor" different uptake pathways for nanoparticles of different size, charge, and surface reactivity. On the other hand, different uptake pathways are associated with different intracellular fates of the nanomaterials internalized into cells. *Phagocytosis* is the predominant method of internalization employed by dedicated immune cells such as macrophages and neutrophils. *Endocytosis*, on the other hand, is used in almost every cell type, and can proceed through four distinct pathways: (i) clathrin-mediated endocytosis; (ii) caveolin-mediated endocytosis; (iii) macropinocytosis; and (iv) the clathrin/caveolin independent pathway. Not every cell possesses each one of these uptake mechanisms, however. In addition, *passive uptake* is a possible mechanism of cellular entry for different small molecules.

The predominant endocytic pathway in cells is that of clathrin-mediated endocytosis, which proceeds via the formation of clathrin-coated membrane invaginations that eventually pinch off to form clathrin-coated vesicles and *endosomes*. Endosomes formed from the clathrin pathway undergo acidification, and are eventually sorted for degradation in lysosomes. The pathways of caveolin-mediated endocytosis and macropinocytosis both have slower kinetics than the clathrin-mediated pathway, but the endosomes formed from these two pathways are not directed to the lysosomes. The final pathway is not well characterized, and is referred to simply as the clathrin/caveolin-independent pathway [119–121].

Studies using transmission electron microscopy (TEM) and energy dispersive X-ray spectroscopy (EDS) have shown that, while bulk TiO_2 was predominantly phagocytosed, TiO_2 nanoparticles were taken up via clathrin-coated pits [113]. Phagocytosis does not seem to be a major contributor to nanoparticle uptake, because the inhibition of phagocytosis (using cytochalasin D; cytD) in macrophages abolished the uptake of micrometer-sized particles, but not of 0.2 µm and 0.1 µm particles [112]. The study authors also noted that the intracellular nanoparticles were not membrane-enclosed, and concluded that non-phagocytic and non-endocytic mechanisms might also be responsible for their uptake. Others have proposed that nanoparticles would be sequestered in endosomes, the origin of which could be attributed to all three major pathways of endocytosis [113, 122]. Here, as in other nanoparticle uptake studies, there was a clear correlation between the nanoparticle localization in the cells, the "availability" of uptake pathways, and the cell type. As not all cell types have every endocytic mechanism [121], identical nanoparticles might be found in different cell compartments of near-isogenic cell lines [123].

Early endosomes formed by the clathrin, caveolin, and macropinocytic processes pursue a defined pathway that leads to the formation of late endosomes, followed by sorting within multivesicular bodies and, finally, fusion with degradative lysosomes [178, 179]. Notably, TiO_2 nanoparticles have been localized to endosomes as well as to multivesicular bodies [113].

Finally, nanoparticles can also penetrate epithelial cells (in particular) by the process of *transcytosis*, where particles are endocytosed from the apical surface of the cell, trafficked through the cytoplasm, and released from the basal cell surface. In an experimental set-up using Caco-2 cells that simulated intestinal epithelial cells *in vitro*, TiO_2 nanoparticles were able to pass through the cells by transcytosis, without disrupting the intercellular junctions or compromising cellular integrity [124].

1.4.2 Intracellular Localization of TiO_2 Nanomaterials

Non-functionalized TiO_2 nanoparticles are not found within the nucleus, nor in other subcellular organelles such as the mitochondria, endoplasmic reticulum, or Golgi apparatus [113]. In one study, nanoparticles were found to have aggregated in the perinuclear regions of cultured bronchial epithelial cells [116], whereas in another study they were seen to be enriched within the lysosomes of mouse fibroblast cells [125]. These differences might be attributed to the different uptake mechanisms that dominate in mouse fibroblasts and bronchial epithelial cells. In fibroblasts, where the nanoparticles were endocytosed, they ultimately appeared in the lysosomes. However, nanoparticles in the bronchial epithelial cells were not taken up by membrane-bound vesicles and could diffuse freely throughout the cytoplasm.

Recently, the surface reactivity of nanoscale TiO_2 has been used to functionalize nanoparticles with biomolecules in the form of dopamine-modified deoxyribonucleic acid (DNA) and peptide nucleic acid (PNA) oligonucleotides capable of hybridizing with cellular DNA (see also below) [5, 9, 10, 13–17]. The oligonucleotide-modified nanoconjugates which had been electroporated into the cells were shown to be retained inside the cells for up to several days post-transfection. Moreover, the bound oligonucleotides that had hybridized to nucleolar or mitochondrial sequences were seen to have aided nanoconjugate retention in the appropriate subcellular compartments such as the nucleolus and the mitochondria, respectively [9, 15]. It is important to note that electroporated nanoconjugates have free access to the cytoplasm, whereas endocytosed nanoconjugates must escape the endosome in order to reach subcellular locations.

The addition of other types of surface moieties can also be used to modulate the mechanisms of nanoparticle uptake. For example, the conjugation of specific antibodies to the nanoparticle surface fostered uptake by cells expressing antigenic cell-surface receptors, as shown in numerous other nanoparticle–cell combinations [118].

In most of these nanoparticle localization studies, TEM or X-ray fluorescence microscopy were used to interrogate the intracellular nanoparticle localization.

However, the addition of a fluorescent molecule such as Alizarin Red S to the nanoparticle as a surface-modifying moiety transformed the TiO_2 nanoconjugates into fluorescent nanoconjugates, suitable for investigations using fluorescence confocal microscopy and flow cytometry [10]. This type of surface modification may facilitate further investigations of nanoconjugate uptake, retention, and subcellular localization.

One unique application of surface-functionalized TiO_2 nanoconjugates is to surface-coat them both with a targeting moiety, such as a DNA oligonucleotide, and with dopamine-modified gadolinium (Gd)-based contrast agents. By using this approach, improved uptake and retention of Gd was obtained in targeted cell population harboring sequences that hybridized with the attached oligonucleotides [13, 16]. Such nanoconjugates would be suitable for magnetic resonance imaging (MRI) studies, and may yet find their place among biomedical diagnostics. When combined with potential therapeutic applications inherent to TiO_2 nanoparticles and nanoconjugates, this might represent a new approach towards the creation of so-called "theranostic nanomaterials," with combined applications in therapy and diagnostics.

1.4.3
Intracellular Interactions of TiO_2 Nanomaterials

Once they have been internalized, TiO_2 nanoparticles, nanocomposites and nanoconjugates can interact either passively or actively with cells. Passive interactions in this context are defined as cellular responses to nanomaterials as a foreign material. For the purpose of this section, active interactions will be defined as cellular events triggered by TiO_2-mediated photocatalysis, leading to the production of ROS and free electrons (e^-) and electropositive holes (h^+) within the intracellular milieu. Active interactions would also include such cases when the surface reactivity of the TiO_2 nanomaterial induced a cellular reaction. Whilst such interactions might have cytotoxic effects in cells, inasmuch as the activity can be targeted only to the population of cells that cause harm to the organism, such cytotoxicity might have therapeutic effects.

Several groups have shown that TiO_2 nanoparticles can induce DNA damage and apoptosis in cultured human peripheral blood lymphocytes [114, 115]. Indeed, not only has DNA damage been demonstrated (through single-cell gel electrophoresis) but also an accumulation of p53, a major regulator of DNA damage-induced cell cycle arrest. In addition, not only have increased levels of p38-MAPK and JNK (both of which are considered to be indirect activators of pro-apoptotic caspases) been demonstrated, but also an activation of caspase-8 [114, 115].

The toxicity of TiO_2 nanoparticles in an absence of photoactivation was also noted in numerous cell-based assays [126]. For this, TiO_2 nanoparticles of different sizes and crystal phases (or their mixtures) were used to treat cells *in vitro* at concentrations up to $10\,\mu g\,ml^{-1}$. In most cases, anatase TiO_2 was shown to induce some signs of cytotoxicity at concentrations above $5–10\,\mu g\,ml^{-1}$, though according to others the cytotoxic effects commenced only at concentrations above $100\,\mu g\,ml^{-1}$. In addition, the consensus was that among all particles smaller

than 1 μm in size, TiO_2 was the least toxic, especially when compared to Al_2O_3 and SiO_2.

In a more recent study, NIH 3T3 cells were maintained and treated with TiO_2 particles for 11 consecutive weeks. The nanoparticle sizes ranged between 2 and 30 nm, while within the treated cells increased numbers of multinucleated cells and micronuclei were found, as well as increased levels of polyploidy. In short, these data suggested that the TiO_2 nanoparticles might lead to chromosomal instability and cellular transformation [180]. However, the authors did not elaborate on any possible contribution of ambient light to their findings.

The introduction of potential stressors such as nanoparticles can trigger the expression of cellular stress signals and inflammation by activating leukocytes. The exposure of U937 human monocytes to TiO_2 nanoparticles led to an increased expression of matrix metalloproteases (MMPs)-2 and -9, both of which are involved in tissue remodeling and typically are secreted by monocytes that have been exposed to metal oxides [117]. Results from the same study suggested that nanoscale cobalt was a better activator of MMP-2 and MMP-9 than TiO_2 nanoparticles. Macrophage inhibiting factor (MIF), a pro-inflammatory cytokine, has also been shown to be upregulated in bronchial epithelial cells exposed to bovine serum albumin (BSA)-coated TiO_2 nanoparticles. Furthermore, the oxidative stress caused by the nanoparticles led to increased levels of cytoprotective proteins such as TALDO1, an enzyme that produces reducing equivalents.

One possible way to reduce the toxicity of TiO_2 nanoparticles might be through surface modifications to decrease the reactivity of the nanoparticle surface, and/or modulate its photocatalytic reactivity and photoresponse. One such surface modifier is glycidyl isopropyl ether (GIE), which has been used to mask the reactive surface of nanoscale TiO_2, without significantly affecting either the photocatalytic properties or the internalization of nanoconjugates [10].

1.4.4 Photocatalytic Uses of TiO_2 Nanomaterials to Induce DNA Cleavage and Cytotoxicity

The photocatalytic properties of TiO_2 nanomaterials have been discussed at length in previous sections, in addition to ways in which the efficiency of the process can be improved. Just as the degradation of methylene blue can be used to gauge the photocatalytic reactivity of TiO_2, the degradation of DNA has been used for the same purpose. In experiments conducted as long as 20 years ago, plasmid DNA degradation was used as a parameter of DNA degradation by photoactivated TiO_2 [181]. In a more recent study [182], the effects of 10–20 nm and 50–60 nm anatase and rutile TiO_2 on the formation of 8-hydroxydeoxyguanosine (8-OHdG) in an *in vitro* plasmid assay were found to decrease in the order 10–20 nm anatase > 50–60 nm anatase > 50–60 nm rutile. ROS generated at the surfaces of the nanomaterials as a result of TiO_2 photoactivation were seen to play a role in DNA cleavage [127–129]. Hence, the inclusion of ROS scavengers in TiO_2–DNA nanoconjugate cleavage experiments was shown to lead to a partial, if not complete, loss of DNA cleavage.

Similar to DNA *in vitro*, cellular DNA can sustain damage in cells that contain TiO_2 nanomaterials and have been exposed to UV light. Whilst cells are able to cope with a certain amount of ROS and can protect the integrity of their nuclear and mitochondrial genomes, it is possible to overwhelm the cellular antioxidant machinery and to induce DNA cleavage *in situ*. This in turn, can lead to cell cycle arrest, senescence, or cell death. As noted above, the overpowering of cells with ROS forms the basis of many current anti-cancer treatments, and may lead (potentially) to the therapeutic use of TiO_2 nanoparticles, nanoconjugates, and nanocomposites.

The earliest example of the anticancer use of TiO_2 was the triggering of photocatalysis in media-containing cultured cells [130], while several groups have used intercellular TiO_2 nanoparticles as photosensitizers to cause oxidative damage to malignant cells [131, 132]. In one study, bladder cells treated with TiO_2 were irradiated with UV-A light, after which an increased oxidative stress (as measured by major oxidative products) and increased apoptosis were identified [131].

Currently, most examples of the deliberate use of TiO_2 nanomaterials to induce cytotoxicity are based essentially on the same principle of action as the very first experiments, although the nanoparticles of today are generally smaller than those used in the past. In the following examples, the nanoparticles or nanorods used were less than 20 nm in size, which enabled an efficient nanoparticle uptake. In studies conducted at Cheon's laboratory [183], nanorods of 3.5×10 nm were used to treat A-375 melanoma cells. Having penetrated the cells, the nanorods were then excited by UV lamp illumination, such that the products of photocatalysis caused abundant apoptotic death at UV doses that normally would be harmless to this cell type. In a more recent example, TiO_2 nanoparticles were conjugated to a monoclonal antibody against interleukin (IL)-13αR, a receptor which is overexpressed in glioblastoma multiforme. This functionalization improved nanoparticle uptake by glioma cells (GBM and A172) in an antigen presentation-dependent fashion [118]. The cytotoxicity was found to be impaired by the addition of ROS quenchers, however, and particularly by those with singlet oxygen ($^1O_2$) and hydroxyl radical ($OH^{\bullet}$) traps.

While random DNA scission caused by ROS has its place in therapeutic approaches, it would be desirable to control this activity and to induce DNA degradation in specific locations in the genome; a preferred example would be at oncogene loci. In order to achieve such precision with the help of nanoparticles, several exploratory studies were conducted by Woloschak and coworkers [5]. As noted above, at the nanoscale, TiO_2 molecules on the surface of nanoparticles form "corner defects" that create a propensity for stable nanoparticle conjugation to *ortho*-substituted bidentate ligands such as 3,4-dihydroxyphenethylamine(dopamine) and 3,4-dihydroxyphenylacetic acid (dopac). These anchor molecules allow for further conjugation with new molecules via amide linkages; such covalent binding to the inorganic surface is energetically favorable because it allows the TiO_2 to regain the native octahedral geometry [7]. Dopamine is used as a linker for the attachment of oligonucleotides made from DNA and PNA, as well as peptides, antibodies and MRI contrast agents [5, 9, 10, 13–17]. When oligonucleotides are

bound to the nanoparticle it is possible for h^+ to be transferred from the TiO_2 nanoparticle through the dopamine linker onto the attached biomolecule. It has been shown that TiO_2 nanoparticles can act as an electron sink, thereby allowing an accumulation of h^+ on the attached DNA molecules, leading in turn to strand cleavage at the site where the electropositive holes accumulate. Paunesku *et al.* have shown that DNA oligonucleotides bound to TiO_2 nanoparticles can participate in enzyme reactions and retain the ability to hybridize with complementary DNA sequences. In the same study, the photoactivation of TiO_2 led to site-specific cleavage of the hybridized oligonucleotides [9]. The cleavage event is thought to occur by the accumulation of multiple h^+ at guanine residues, leading to the formation of guanine cation radicals that can react with neighboring water molecules. The attachment to the nanoparticle of a DNA strand that is complementary to a sequence of cellular DNA will then allow for a targeted cleavage of the genetic material. The results of several studies have indicated that cleavage efficiency is heavily dependent on the degree of nucleic acid strand hybridization. Indeed, Rajh and colleagues have demonstrated an *in vitro* DNA sequence-specific cleavage [133]. In a study conducted by Tachikawa *et al.*, the presence of mismatches between the oligonucleotide attached to the nanoparticle and the complementary oligonucleotide target was shown to modulate the extent of DNA cleavage [134]. To explain this phenomenon, the authors hypothesized that in a perfectly hybridized DNA–TiO_2 nanoconjugate the h^+ were more efficiently trapped on the nucleic acid moiety and did not undergo charge recombination with electrons on the surface of the nanoparticle. In a more recent study, the same group hypothesized that strand cleavage would most likely require both the transfer and accumulation of h^+ across the DNA strand, as well as the presence of free photogenerated ROS, by showing that cleavage could be quenched in the presence of ROS scavengers [135].

1.5
Use of Titania Oxide and Its Composites for Implants and Tissue Engineering

For many years, titanium and Ti-based alloys have been used extensively in permanent implants for orthopedic, dental, and prosthetic applications. More recently, however, titanium has been used on the nanoscale for implant surface modifications and tissue-engineering applications. With the details of the biological response to an implant placement having been elucidated at the subcellular level, nanotechnology has been utilized for the surface modification of titanium implants to maximize the natural tissue response, to achieve implant integrity, and to prevent implant failure. For tissue-engineering applications, TiO_2 has been integrated into bioactive glass composites for use as scaffolds for bone tissue generation. Tissue engineering has already superseded autologous bone grafts in the repair of fractures, bone defects, the resolution of long-bone nonunions, total joint revision surgery, repair of tumor resection, and spine fusion. Clearly, as technology improves and the techniques are refined, tissue-engineering applications will undoubtedly become much more prevalent.

1.5.1 Osseointegration

Osseointegration, as originally defined by Brånemark, is a direct structural and functional connection between ordered living bone and the surface of a load-carrying implant. An implant is considered to be osseointegrated when there is no progressive relative movement between the implant and the bone tissue [136, 137]. Clinically, osseointegration refers to the successful implant installation and healing, with the correct stress distribution when in function [138]. In order to achieve implant osseointegration, it is first necessary to achieve primary mechanical stability of the implant, as this will allow for biological fixation by the osteoid tissue and trabecular bone at an early stage [139]. Titanium serves as a substrate for bone formation, and provides a stable interface for the formation of new bone. Notably, TiO_2 is biologically inert and allows for hydroxyapatite (HAp) to form on its surface and act as a bonding layer for further bone development [140]. The healing process begins with an inflammatory response when the implant is inserted into the bone cavity. Within the first day, mesenchymal cells, pre-osteoblasts, and osteoblasts are recruited to the implant surface, and begin to produce collagen fibrils of osteoid tissue; this is followed initially by woven bone and then mature bone formation with trabeculae rich in capillaries. Trabecular bone is a calcified tissue that forms the initial architectural network within the space between the implant and bone, providing a high resistance to implant loading. Its architecture is a complex three-dimensional (3-D) network, with arches and bridges providing a biological scaffold for cell attachment and bone deposition on the implant surface. Thus, the development of novel methods to enhance implant function will depend heavily on knowledge of these processes.

1.5.2 Implantation Methods

Many of the novelties in implantation methodology have been built upon an established understanding of conventional implant–biological interactions and the recent development of nanotechnology, with emphasis placed on the modification of implant surfaces at the nanoscale level. Typical examples include nanograined surface modification, nanophase ceramics, and nano-rough poly-lactic-*co*-glycolic acid (PLGA)-coated-nanostructured titanium. Other developments include TiO_2-doped phosphate-based glasses for applications in bone tissue engineering. The use of nanotechnology in the realm of implant engineering is based on the fact that naturally occurring proteins are of nanoscale dimensions. Bone, for example, is a nanostructured material composed of proteins such as collagen type I, which contains linear fibrils that are 300 nm long and 0.5 nm in diameter, in addition to HAp crystals that range from 2 to 5 nm in thickness and from 20 to 80 nm in length [82, 141, 142]. It has been postulated that, because cells interact with nanostructured surface substrates in a biological setting, nanophase materials are able to duplicate the natural surface behavior of cells.

1.5.3
Osteoblast Adhesion

The initial osteoblast adhesion is a rapid response to implant placement, and involves short-term chemical interactions between the cells and the implant surface that initially involve ionic and van der Waals forces. The subsequent regenerative phase is a longer process, with continued cell migration to the adhesive site and the production of extracellular matrix (ECM) proteins, cell membrane proteins, and cytoskeleton proteins. This sequence of events comprises the natural healing process, and results from a signal cascade leading to the production of transcription factors and subsequent genetic induction [139].

It has been shown that osteoblast attachment, proliferation, viability, and morphology are promoted, together with an enhanced cytocompatability, on nanograined and nanophase materials such as alumina, titania, and HAp with surface pore sizes of less than 100 nm [82, 86, 142, 143]. Ceramics such as titania and alumina have long been used in implants on the basis of their favorable biocompatibility with osteoblasts. These ceramics have been produced via the sintering of TiO_2 powder at 600 °C to generate a surface roughness of less than 20 nm. Composites prepared in this manner have demonstrated an enhanced osteoblast function when compared to conventional ceramics [82, 86, 142]. Nanophase ceramics have the added benefit of physical properties that closely mimic those of physiological bone, thus promoting enhanced calcium and phosphate precipitation.

Ceramics with nanoscale topography demonstrate an increased specificity and selectivity for the adhesion of osteoblasts, with reduced adhesion of fibroblasts and endothelial cells, most likely because nanophase ceramics adsorb higher levels of vitronectin whereas conventional ceramics are more selective for laminin. Osteoblasts demonstrate increased adhesion and proliferation on nanoceramics, while endothelial cells show increased activity on conventional ceramics. Furthermore, the stereochemistry of vitronectin may make its adsorption more selective for the smaller pores of nanoceramic surfaces [82, 142].

1.5.4
Modification of Surface Chemistry

The modification of surface chemistry has been shown to increase the biological response at implant surfaces. Both, hydroxyl and carbonyl groups on the implant material surfaces demonstrate good support for osteoblasts, thus promoting adhesion, proliferation, and differentiation [144, 145]. It has been established that hydroxylated and hydrated titanium surfaces increase the amount of free surface energy, and also induce osteoblast differentiation; they have also been shown to be associated with the generation of an osteogenic microenvironment through the production of prostaglandin (PG) E_2 and transforming growth factor-beta (TGF-β) [146]. Studies of Ti polymers with surface pore sizes of 32 nm, when treated with NaOH, have provided evidence of the ability to simulate the surface

and chemical properties of bone and cartilage. The combined effects of surface nanotopography and chemical modulation serve to maximize the biological responses [83, 84].

1.5.5 Artificial Bone Substitute Materials

Various types of bioactive ceramics, glasses, and glass ceramics have been developed for use as artificial bone substitute materials. These neither damage healthy tissue, nor pose any viral or bacterial risk to patients, and can be supplied at any time, in any quantity. Examples include Bioglass® in the Na_2O–CaO–SiO_2–P_2O_5 system [147], sintered HAp; $Ca_{10}(PO_4)_6(OH)_2$ [148], sintered β-tricalcium phosphate (TCP) ($Ca_3(PO_4)_2$ [149], HAp/TCP bi-phase ceramics [150], and apatite-containing glass ceramics [151]. Phosphate glasses represent a unique class of biomaterials that are biodegradable, biocompatible, and for which the rate of degradation can be modulated from days to several months. The primary advantage of using bioactive glasses is that there is no need for surgical removal once their function has been fulfilled. Rather, they are broken down and harmlessly expelled from the body. *In vivo* studies, which included a bone-healing model of marrow ablation of the rat tibia, have shown that filling the intramedullary space with bioactive glass microspheres results in new bone formation and a high turnover of the local bone [152]. During the primary biological response to bioactive glass, relatively undifferentiated mesenchymal tissue surrounds the microspheres. Initially, the tissue differentiates into an immature woven bone structure, followed by bone remodeling and the formation of lamellar bone (though this is limited by the presence of the bioactive glass microspheres). As the microspheres begin the dissolution process, new bone is continually formed in their place. It has been proposed that there is a balance between the gradual dissolution of bioactive glass matrix, and the synthesis of new bone on its surface [153]. The rate of dissolution is controlled by increasing the covalent character of the bonds within the glass structure; this is achieved by doping the degradable composition with modified metal oxides that have a greater field strength, such as Fe_2O_3, CuO, Al_2O_3, and TiO_2 [154–158]. The ability to modulate the properties of bioactive glass allows for many potential applications as reinforcing agents for composites, and also as scaffolds for tissue engineering [159].

In addition to bone regeneration, TiO_2 nanoparticles have been used recently to create an extracorporeal culture system for hepatocytes that would mimic the *in vivo* microenvironment. For this, TiO_2 nanoparticles and nanorods of 25–75 nm in size were dispersed uniformly on the surface of the 120–300 μm pores of a highly porous chitosan structure. When the attachments of hepatic cell line HL-7702 to a chitosan scaffold and to a scaffold decorated by nanoparticles were compared, the overall difference between the two cell systems was insignificant. The most notable difference was the fact that the TiO_2/chitosan structures contained more spheroids, whereas the chitosan-only structures contained more single HL-7702 cells [160].

1.6 Toxicology Studies of TiO_2 Nanomaterials in Animals

Although no whole-animal studies appear to have been conducted with nanoconjugates and nanocomposites of TiO_2, many have described the toxicity of TiO_2 itself. Neither have there been any demonstrations of the diagnostic and/or therapeutic applications of TiO_2. In considering the potential toxicity of TiO_2 *in vivo*, it can be assumed that there will be no light exposure and activation of TiO_2 photocatalysis of cellular components; however, there is still a need to consider the surface reactivity of TiO_2 nanomaterials, their ability to bind to proteins and nucleic acids, and a general propensity of nanomaterials to interact with molecules within a similar size range [161]. In addition to the fact that biological responses caused by nanoscale materials may be very different from those caused by bulk materials [39, 162], the increased surface reactivity of TiO_2 at the nanoscale may, potentially, lead to an aggregation of nanoparticles, and to the triggering of immune responses, the clogging of vessels and ducts, and an accumulation in organs associated with the filtration of blood and lymph. At present, the conditions that either increase or decrease the rate of aggregation of TiO_2 nanomaterials have not yet been widely studied in biological systems, in part because there are so many factors to consider, such as crystal structure and size, pH, and the ionic strength of the colloid. Moreover, in a study of microscale TiO_2 particles, anatase TiO_2 was found to produce more ROS than its rutile counterpart, adding crystal polymorphology as yet another potential factor to be controlled when evaluating the toxicity of TiO_2 nanoparticles *in vivo* [163].

It has been argued that the total surface area-per-unit-mass is the best predictor of TiO_2 toxicity *in vivo*, whereas others have maintained that this characteristic is not indicative of toxicity at all [164]. Whilst photocatalytic reactivity of TiO_2 *in vivo* is unlikely, concern has been expressed that ROS may be generated at the particle surface when TiO_2 nanoparticles interact with biological processes, potentially causing oxidative damage to adjacent tissues.

The expected clearance of TiO_2 nanomaterials is via the reticuloendothelial system (RES) and, because of their small size, they would be expected to locate to the liver, spleen, lymph nodes, and kidneys. In the field of cancer diagnostics and therapeutics, nanoscale molecules would potentially accumulate in tumors because of the poorly developed and largely fenestrated blood vessels of the neovasculature. Poor lymphatic drainage would reduce the clearance of these nanoscale structures from the tumor. This phenomenon, which is referred to as the enhanced permeability and retention (EPR) effect, is a mainstay of most so-called "passive targeting" approaches to concentrate nanoparticles within tumors.

While little doubt exists regarding the clearance route of TiO_2 nanomaterials, there are many possible routes for nanoparticle entry into the organism, including inhalation, intratracheal instillation, oral administration, oral, nasal and bronchial lavage, and intraperitoneal and intravenous delivery. Not all of these approaches have been used in animals, or were used only sporadically. In the interest of focus and brevity, the following sections are organized in terms of the most common

routes of entry used to study TiO_2 nanoparticle toxicity in mammalian models; moreover, only nanomaterials <100 nm in size were considered.

1.6.1 Inhalation and Intratracheal Instillation

The vast majority of studies of the effects of TiO_2 nanomaterials in mammals have focused on their interactions with the respiratory system. Such studies have been conducted in two general ways:

- *Inhalation* requires the use of nebulizers or other devices to create an aerosol of TiO_2 nanoparticles, and occasionally a closed housing unit to fit the test subjects. Knowledge of the animal's respiratory rate, lung volume, and controlling for exposure time and air flow rate allows the relatively accurate measurement of the quantity of material to which the subject is being, or has been, exposed.
- *Intratracheal instillation* not only closely simulates environmental conditions but is also relatively inexpensive, as it can be conducted with a syringe and the dosage of test material much more easily defined. Intratracheal instillation also avoids the possibility of nanoparticles being absorbed through the skin, which is a concern during inhalation [32].

1.6.1.1 Intratracheal Instillation

Results on the long-term toxicity of TiO_2 nanoparticles administered via intratracheal instillation have varied greatly. One group used mice to compare 3 nm and 20 nm nanoparticles, varying the concentrations from 0.4, 4, and 40 mg kg^{-1} body weight. After three days, the 3 nm particles showed a dose-dependent toxicity comparable to that of the 20 nm nanoparticles, which had only about one-third of the total surface area, indicating that size and surface area were not factors of toxicity with this experimental set-up and delivery route. In both cases, the nanoparticles caused slight increases in total protein, albumin, alkaline phosphatase, acid phosphatase and lactate dehydrogenase (LDH) in the bronchoalveolar lavage fluid (BALF), and were present in macrophages on histological examination [32]. However, another one-week study showed 5 nm anatase nanoparticles to cause more severe pulmonary toxicity than 21 nm and 50 nm particles at 5 and 50 mg kg^{-1}, respectively, but relatively no toxicity at 0.5 mg kg^{-1}. Inflammatory infiltrates and interstitial thickening were observed on histological examination. At the highest dose (50 mg kg^{-1}), it was suggested that the 5 nm anatase nanoparticles inhibited the phagocytic ability of alveolar macrophages, although no mechanism was suggested [165]. Several studies of this type have used 21 nm nanoparticles (80/20, anatase/rutile) that were purchased commercially. It appeared that a single dose (5, 20, 50 mg kg^{-1}) of these nanoparticles could cause a dose-dependent increase in a number of pro-inflammatory and T-cell-derived (Th1- and Th2-type) factors in the BALF at one day post-treatment, although ultimately these particles might exert chronic inflammatory damage on the lungs via a Th2 mediated pathway

[38]. Over one to two weeks, 21 nm rutile particles were found to induce pulmonary emphysema, to disrupt the septal walls of the alveoli, and to cause epithelial wall apoptosis – altogether a much more drastic response than had been observed by others with comparable doses (3–16 mg kg^{-1} body weight) [22]. A longer-duration study in rats (1, 7, and 42 days) at lower doses showed an increased ROS production (by immunohistochemical staining), and persistence of inflammatory and cytotoxic factors, when compared to fine TiO_2 particles. In this case, it was suggested that the smaller size of the nanoparticles had allowed them to pass more easily into the interstitium, as they were also found in much greater frequency in adjacent lymph nodes. However, by controlling for equal surface area exposure, TiO_2 nanoparticles were found to be only slightly more immunogenic and cytotoxic than their fine-particle counterparts – a finding which conflicted with conclusions drawn by others [40]. A comparison between 10 nm TiO_2 anatase "dots", 200 × 35 nm anatase rods, and 300 nm rutile particles administered in a single intratracheal instillation at either 1 or 5 mg kg^{-1} to rats, concluded that the different particle types were no more cytotoxic or immunogenic in the lung than their counterparts, and indicating that surface area was not a factor in pulmonary toxicity [48]. However, others considered the surface area and particle size to be more important determinants of toxicity than the mass of material administered [166].

1.6.1.2 **Inhalation**

Studies using inhalation as an exposure method also showed varied results. In a comparison of the inhalation of ultrafine versus fine TiO_2 nanoparticles, the ultrafine material caused a more significant microvascular dysfunction [36]. Comparisons between nanoparticles (20 nm) and pigment-grade particles (250 nm) of TiO_2 in rats during inhalation exposure over three months (6 hours per day, five days per week) showed mildly severe focal alveolitis after six months. However, at one year post-exposure the levels of alveolitis had largely returned to those of the untreated controls, although more alveolar macrophages persisted. One group concluded that, pending cessation, chronic exposure to TiO_2 nanoparticles over a limited period of time had no significantly toxic lasting effects on the lungs [19]. Rats were shown to develop a much more severe inflammatory response to 21 nm TiO_2 nanoparticles than mice, but the fibroproliferative and epithelial changes in lungs were accompanied by increased macrophage and neutrophil numbers in the BALF, particularly at an aerosol concentration of 10 mg m^{-3}. Over the course of one year post-treatment, however, there was a dose-dependent decrease in toxicity markers, even at this concentration [20]. A study of 2–5 nm particles at an aerosol concentration of 0.77 and 7.22 mg m^{-3} showed moderate inflammatory responses in lung sections at up to two weeks after exposure, although by the third week only minimal lung toxicity or inflammation was observed, indicating a return to baseline [27]. In most cases, alveolar macrophages and neutrophils persisted in the BALF, but levels of inflammatory cytokines and markers had generally returned to normal [167].

Despite the copious data generated on the topic of TiO_2 nanoparticle toxicity, it is not entirely clear whether the nanoparticle dispersity, dose, form, or some other

factor can be used as the definitive predictor for determining lung toxicity. It is possible that a combination of factors may be the true determinant to evaluate the effects on the mammalian respiratory system, although differing responses to the same treatments have also been found across different species. What does appear to be true is that, in low doses (whether via intratracheal instillation or inhalation), TiO_2 nanoparticles are relatively nontoxic and do not carry any lasting effects. Higher doses, however, tend to be retained and may increase inflammatory mediators, macrophage and neutrophil infiltration, and possibly emphysema-like reactions in the lungs. Alveolar macrophages may be impaired in their ability to phagocytose these nanoparticles if their capacity is overloaded, resulting in a decreased clearance [168]. From this, it is apparent that it is important to continue studying the effects of TiO_2 nanoparticles on the lungs, and to develop acceptable standards for inhalation exposure to TiO_2 nanoparticles, not only from a single encounter but also over extended periods of time, in order to simulate chronic low-dose exposure conditions.

1.6.2 Dermal Exposure

Recent reviews of nanoparticle dermal toxicity have included an overview of studies conducted with TiO_2 nanoparticles [169], though all of these utilized microfine TiO_2 that mostly originated from sunscreen lotions. One study that included 10/15 and 20 nm TiO_2 nanoparticles showed that, in human subjects, exposure to such materials led to an accumulation of TiO_2 no deeper than the outermost layers of the striatum corneum [170]. Other studies [171] with 10.1, 5.2 and 3.2 nm TiO_2 nanoparticles of different crystal structure demonstrated some cytotoxicity and inflammation at and above concentrations of 100 mg ml^{-1}, in conjunction with the anatase crystal phase.

1.6.3 Ingestion and Oral Lavage

The accumulation of TiO_2 nanomaterials in the environment might eventually become distributed in the food and water supply of natural wildlife and, eventually, of humans. It has been suggested that nanosized materials can cross the intestinal epithelium and exert effects on other organs in the body, notably the spleen and kidneys [172]. An interesting study using simulated intestinal epithelial cells *in vitro* (cell line Caco2) showed that TiO_2 nanoparticles at concentrations above 100 µg ml^{-1} would accumulate at the apical surface of the cells, pass through the junctions, and traverse through the cells by transcytosis [124].

In a subsequent study, male and female mice were administered a single large oral dose (5 g kg^{-1} body weight) of a TiO_2 nanoparticle suspension via a syringe [45]. The TiO_2 nanoparticles were 25 and 80 nm in size, and any effects were compared to a dose of 155 nm TiO_2 particles over the course of two weeks. The animals were sacrificed after two weeks and the internal organs analyzed for their titanium content. Any histopathological changes were also evaluated, and blood

serum collected to monitor any changes in biochemical markers of organ-specific damage or inflammation. No major changes were found in the body weights of all treatment groups compared to controls, although female mice showed a significant increase in liver weight relative to total body weight. The alanine aminotransferase : aspartate aminotransferase (ALT/AST) ratio, which serves as a marker of liver damage, was significantly increased in the 25 nm nanoparticle-treated group compared to controls, but possible reasons for this gender-specific effect were not discussed. Pathological changes were identified around the central vein of the liver, and hepatocyte necrosis was evident. Serum levels of LDH and alpha-hydroxybutyrate dehydrogenase (markers of cardiovascular damage) were increased in the 25 nm- and 80 nm-nanoparticle treated mice, compared to control and 155 nm-nanoparticle treated mice. Although the 80 nm group showed the highest increases, no pathological changes were identified on histological examination of the heart. The 25 nm group also showed increased blood urea nitrogen (BUN) levels, but no significant changes in creatine kinase. Histology showed proteinaceous liquid in the renal tubules of the 80 nm mice, with significant swelling in the renal glomerulus of the 155 nm-treated group, but no obvious pathology in the 25 nm-treated group. In fact, no abnormal histological changes were found in any of the tissues of mice treated with 25 nm TiO_2 nanoparticles. An examination of the heart, lungs, testicles/ovaries, and spleen showed no overt pathology on histological examination. The titanium content analyses showed an accumulation in the liver, kidneys, spleen, and lungs (in decreasing order), which confirmed the expectation of an accumulation in the RES with eventual excretion via the kidneys [45]. Overall, the 80 nm TiO_2 nanoparticles were found to be more toxic to mice after oral ingestion, particularly to the liver, while 25 nm TiO_2 nanoparticles had no appreciable effects on the liver compared to untreated and 155 nm TiO_2 particle-treated controls. The 25 nm nanoparticles were deposited in the spleen, kidneys, and lungs of test subjects, although no overt pathology was identified. Some kidney dysfunction was noted, possibly due to the high load of TiO_2 being excreted.

1.6.4 Intravenous Injection

The majority of studies of the toxic effects of TiO_2 and TiO_2-containing nanomaterials have focused on mimicking environmental exposure (inhalation, ingestion, etc.). However, in order to study the pharmacokinetics of TiO_2 nanomaterials more directly, it is necessary to recreate a situation of 100% bioavailability, namely intravenous injection [25]. In this way, information obtained from studies in which other routes of administration have been evaluated can be placed into context, and also aid in extrapolating the data acquired from animal models to the human situation [25]. Studies have also been conducted with TiO_2 nanomaterials as a tool for cancer diagnosis and therapy [10, 13, 16], further supporting the need for a thorough evaluation of the effects of TiO_2 nanomaterials in the bloodstream. Unfortunately, very few studies have focused on the potential toxicity of TiO_2

nanomaterials in mammals in the setting of intravenous injection. It is to be expected that TiO_2 nanomaterials would be scavenged by the RES [173]. When bulk anatase TiO_2 (0.2–0.4 um) was administered intravenously (via a tail vein) to female Sprague-Dawley rats, no overt pathology was observed on histological examination. However, over a 24 h period, bulk TiO_2 was localized primarily to the celiac lymph nodes, liver and spleen, in decreasing order. After one year, accumulation in the celiac lymph nodes still far exceeded (over 18-fold) that in the mediastinal lymph nodes, where the next largest accumulation of TiO_2 was found, followed by the spleen, liver, and lungs. Accumulation in the celiac lymph nodes was deemed appropriate, as these particular lymph nodes filter lymph from the liver, indicating that the majority of the TiO_2 had traversed the liver before their deposition in the celiac lymph nodes [173]. Further investigations of the intravenous administration of TiO_2 have been conducted based on the results of this study.

A study in male Wistar rats used a single bolus injection of TiO_2 nanoparticles into the tail vein, at a concentration of 5 mg kg^{-1} body weight (0.5%). These nanoparticles were 20–30 nm in diameter, and had a surface area of 48.6 $m^2 g^{-1}$. In 200–300 g rats this translates to 1–1.5 mg TiO_2 per rat; the equivalent average dose in humans would be a single bolus of 350–450 mg TiO_2. The rats were sacrificed at 1, 14, and 28 days post-injection, and the TiO_2 content analyzed in blood cells, plasma, kidneys, spleen, brain, lymph nodes, liver, and lungs. Only the liver, spleen, lungs, and kidneys showed detectable levels of TiO_2 accumulation (in decreasing order), whilst only the liver retained TiO_2 at the final 28-day time point [25]. However, bearing in mind that the nanoparticles were smaller and did not aggregate, a much more efficient renal clearance could have been expected [174].

For nanoparticles of 20–30 nm diameter, persistent liver accumulation is to be expected when considering the principles behind the EPR effect and the profuse fenestrations of the liver. Just as nanoparticle treatments take advantage of the poorly formed and unnaturally fenestrated blood supply of tumors, those organs designed to filter the blood may also selectively acquire untargeted nanomaterials, regardless of their nature. Although a further analysis of cytokines and enzymes was conducted, there was no indication of any inflammation or cytotoxicity. Thus, in rats at 100% bioavailability, a dose level of 5 mg kg^{-1} body weight of TiO_2 nanomaterial showed neither toxic nor adverse effects [25].

One key component that was not addressed in this study was the aggregation potential of TiO_2 nanomaterials under biological conditions. A menagerie of components would be involved in mimicking an *in vitro* model of TiO_2 nanomaterial interactions with biological conditions. While pretreatment must always involve a disaggregation step (most commonly by prolonged sonication), when the nanomaterials had been administered the biological milieu (blood, peritoneum, lung) would immediately enhance the aggregation status of TiO_2 [39, 162]. By using dynamic light scattering (DLS) techniques it is possible to assess the aggregation status of TiO_2 nanomaterials under different conditions. Likewise, the use of EDS allows the presence of TiO_2 to be detected in tissue sections. DLS experiments *in vitro* showed a dramatic increase in aggregation (as characterized by

hydrodynamic diameter) when the TiO_2 nanoparticles were moved from pure water to saline. In this case, three dilutions were evaluated: 100×, 1000×, and 10 000×. Whilst there was no appreciable difference between aggregation status (hydrodynamic size) in Milli-Q H_2O versus 10 mM NaCl, the *Z*-avg when nanoparticles were solvated in phosphate-buffered saline (PBS) rose dramatically, by more than 20-fold. Although the pH of Milli-Q H_2O and 10 mM NaCl were equivalent at each dilution (6.83–5.35), the pH of PBS ranged from 7.43 to 7.4. The effects of controlled pH changes at constant ionic strength were not evaluated in this study [39, 162]. This change was attributed not so much to the increase in pH as to the increase in ionic strength of the surrounding solution. It was postulated that the electric double layer surrounding the surface of the charged nanoparticles would be diminished in the presence of an increasing ionic strength of the solution, thus screening the electrostatic repulsion that would hinder aggregation in low-ionic strength solutions. For this reason, the electrostatic repulsive forces would be overcome by van der Waals attractions and the nanoparticles would aggregate [39, 162].

These same nanoparticles were injected either intravenously (560 mg kg^{-1}) or subcutaneously (5600 mg kg^{-1}) into female Balb/c mice, which were sacrificed at three days post-treatment and their organs examined histopathologically. By using a combination of transmission electron microscopy (TEM) and scanning electron microscopy (SEM)-EDS, TiO_2 aggregates were identified in the liver, kidneys, lungs, lymph nodes, and spleen in the setting of intravenous injection, while none were identified in sections of the heart and brain. This indicated that TiO_2 nanoparticles of this size do not accumulate appreciably in the heart, nor are they able to cross the blood–brain barrier. Liver sections showed the greatest accumulation of TiO_2 aggregate material, with the larger aggregates accumulating within the vacuoles of Kupffer cells (the monocytes of the liver), and smaller aggregates being distributed more widely between the sinusoidal spaces and the liver parenchyma. The uptake by Kupffer cells was expected, based on their role as phagocytic cells in the RES [39, 162]. While there was occasional evidence of macrophage activation and lymphocyte infiltration, there was no overt cellular degeneration of inflammation in response to infiltration and the deposition of TiO_2 aggregates. Lung sections showed a profuse distribution of large (up to 200 μm) aggregates along the alveolar walls, accompanied by an activation of intravascular macrophages, although no material was found in the alveolar lumens or the intravascular macrophages themselves. Fewer aggregates were found in the kidneys, lymph nodes, and spleen; however, beyond the colocalization of a few phagocytic cells, no obvious pathology was evident in tissue sections of these organs.

Subcutaneous injection revealed aggregates only in the liver, lymph nodes, and spleen histological sections from test subjects; however it should be noted that subsequent experiments (not described) demonstrated the presence of TiO_2 also in the kidneys. Overall, these experiments highlighted the dependence of the organ distribution of TiO_2 nanoparticles on the route of administration, and the change in aggregation potential in solutions of increasing ionic strength as applicable to a biological model [39, 162].

The administration of TiO_2 nanomaterials by intravenous injection permits the study of systemic effects in the setting of 100% bioavailability, while bypassing any potential route-specific organ pathologies associated with more natural environmental exposure. The biodistribution of injected nanomaterials in male Wistar rats follows the expected clearance by the RES, with TiO_2 found to accumulate primarily in the liver, lymph nodes and spleen at up to 28 days after injection, with a decrease in TiO_2 levels over time [25]. The aggregation of TiO_2 nanoparticles due to changes in the ionic strength of the surrounding milieu may perhaps affect the rate of clearance of TiO_2 once a certain size limit has been reached, although at low doses (up to $560\,mg\,kg^{-1}$ body weight) hematoxylin and eosin staining of histological sections and an analysis of various circulating markers of inflammation and cellular degeneration showed no obvious pathological changes compared to controls [25, 39, 162]. Taken together, the results of these studies indicated a relative lack of toxicity of low-dose TiO_2 nanomaterials in mammals within the setting of intravenous injection, and supported the safety of using TiO_2 nanomaterials in the development of novel diagnostics and treatments [13, 16].

1.6.5 Intraperitoneal Injection

As an alternative to intravenous injection, the intraperitoneal route has been selected as a means of studying the *in vivo* transportation of TiO_2 nanomaterials to various organs, and thus any pathological changes that might arise following the systemic administration of TiO_2 nanomaterials. As the majority of reported applications of TiO_2 have involved concern as to potential adverse environmental effects, toxicology studies have focused on common routes of exposure such as ingestion, inhalation, and dermal absorption [168]. The use of TiO_2 nanomaterials as a diagnostic and therapeutic tool has only recently entered the realm of applications [5, 9, 10, 13–17, 118]. As with most cancer diagnostics and therapeutics, these applications of TiO_2 nanomaterials are likely to involve intravenous administration so as to ensure 100% bioavailability. Whilst, in the laboratory setting, it may be commonplace to use intraperitoneal injections (e.g., the use of streptozotocin to eliminate insulin-producing cells to reproduce a Type 1 diabetes mellitus model [175]), the correlation to environmental or therapeutic exposure in this setting is limited. The effects of intraperitoneal injections can vary, depending on the side at which the material was injected (left or right flank, i.e., liver side or spleen side), or the skill of the individual performing the injection (was the injection consistently accurate, with no damage to the internal organs?). The injected material may adhere to internal organs and viscera as it has complete access to the peritoneal cavity, whereas alternative routes of exposure (e.g., inhalation, ingestion, intravenous injection) would require the material to pass through any combination of layers and types of tissue before gaining access to the serosal surfaces of the organs in the peritoneum. Whilst it has been suggested that particles in the nanoscale regime can readily pass through various types of tissue, it is unlikely that this passive diffusive capacity can approach the availability of an intraperitoneal

injection [176]. In addition, the volume of material being injected may have significant effects on the outcome, considering that the introduction of a large volume into the peritoneal cavity does not have any reasonable corollary when considering the effects of environmental exposure. A much more appropriate approach to studying the transportation of TiO_2 nanomaterials *in vivo*, and the possible effects on various organ systems without the concern of a rate-limiting interaction between nanomaterial and organ system of exposure, would be that of intravenous injection. Nevertheless, toxicology studies of TiO_2 nanomaterials administered via intraperitoneal injection have been conducted, albeit with varying results.

One such study of intraperitoneal injection in mice used 80–110 nm anatase TiO_2 nanoparticles, prepared in house using a sol–gel method under acidic conditions. The mice were injected with different dosages (0, 324, 648, 972, 1296, 1944, or 2592 mg kg^{-1} body weight) of TiO_2 nanomaterial, and the histopathology was evaluated at 7 and 14 days post-treatment. Blood samples were analyzed for markers of hepatotoxicity and nephrotoxicity at 24 and 48 h post-treatment. The markers included ALT, AST, alkaline phosphatase (ALP), and BUN. It should be noted that, given the body mass of an average human, these dosages translate to upwards of 180–190 g of TiO_2 nanomaterials in a single bolus injection into the peritoneum. From a clinical perspective, it may not be reasonable to interpret this experiment as an adequate representation of potential environmental or even clinically relevant exposure, but rather as an example of acute toxicity at extreme doses. The animals' behavioral patterns and physical characteristics were observed, when it was noted that all mice showed signs of passivity, loss of appetite, tremor and lethargy compared to controls; however, these signs gradually disappeared in the mice receiving lower dosages (324, 648, and 1296 mg kg^{-1}). The mice receiving high doses (1944 and 2592 mg kg^{-1}) also showed signs of anorexia, diarrhea, lethargy, tremor, weight loss, and lusterless skin [23]. Unfortunately, no scaled rating system was used to qualify or quantify these findings, and the biochemical assays for serum parameters showed wide variations in ALT and AST levels at different treatment dosages. While an effect may be present, it would be incorrect to consider that a definitive concentration-dependence of AST and ALT could be discerned from these data. The authors noted no significant effects on BUN (kidney function) over time, and asserted that TiO_2 nanoparticles injected into the peritoneum had a greater impact on the liver than on the kidneys [23].

The titanium contents of tissues were evaluated using inductively coupled plasma-mass spectrosmetry (ICP-MS) in tissues removed at 24, 48, 168, or 336 h post-injection. The spleen, heart, lungs, kidneys, and liver were digested and the titanium content was examined over time. Accumulation in the spleen was seen to be dose-dependent, but not so in other tissues. An examination of the data presented, however, showed a large decrease in titanium content from 24 to 48 h, with little change from 48 h to 7 days, but then another increase between 7 and 14 days. Whilst this discrepancy was unexplained, it is possible that over longer time points the organ distribution of TiO_2 nanoparticles changed. There was also a potential for matter injected into the peritoneum to adhere to the serosal surfaces of the peritoneal organs, and TiO_2 nanoparticles cannot be ruled out as an excep-

tion. While studies focusing on alternative routes of administration need not be concerned with this possibility, studies of the intraperitoneal injection of TiO_2 nanomaterials would do well to account for this surface accumulation, which might skew any inferences of the systemic distribution of TiO_2. The liver titanium content was significantly less than that of the spleen at all time points, which contrasted with other studies using intraperitoneal injection [33]. Again, given the propensity for injected material to adhere to the peritoneal surfaces, the side of the peritoneum at which the injection was made could represent a major factor in the location of the greatest TiO_2 accumulation, and this was a clear shortcoming of studies where this method of administration was applied. The total body titanium content was not assessed, and neither was the titanium content of fecal matter and urine collected and analyzed. An evaluation of waste for titanium content would strengthen studies of how mammals clear TiO_2 nanoparticles, although at the time of this writing such studies have yet to be conducted. Mild increases were found in the titanium contents of the lungs and kidneys, but no titanium was detected in the heart [23].

Despite the relatively large splenic content of titanium, the histological examination of target organs showed a greater pathology in the liver, followed in order of decreasing severity by the spleen, kidneys, and lungs. The high-dose groups showed hepatic fibrosis around the central vein, apoptotic bodies, and minor fatty change in the liver, accompanied by swelling in the renal glomerulus and dilatation and an accumulation of protein-rich liquid in the renal tubules. Spleen sections showed massive inflammation and neutrophil infiltration in the high-dose groups, and alveolar septal thickening and mild neutrophil infiltration was found in the lungs. No histological changes were found in the heart. In addition, 8% (5/60) of the injected mice that died were in the high-dose groups. Given that these changes were found only in the highest-dose groups (1944 and 2592 $mg\,kg^{-1}$), these data infer a relatively low toxicity of TiO_2 nanoparticles when injected at low doses. However the dose-dependence of the biochemical parameters and histological changes at low doses cannot be stated accurately from these data [23].

One important point made by Liu *et al.* was that the majority of studies, regardless of the route of administration, tended to focus on a single large bolus injection or the instillation of TiO_2 nanomaterials, whereas ecological exposure would more likely be chronic [33]. In order to study these chronic effects, Liu and coworkers designed a study that included multiple, smaller exposures to 5 nm anatase TiO_2 nanoparticles, and for comparison administered commercially available bulk rutile TiO_2 particles with an average grain size of 15–20 μm [33]. For this study, female ICR mice were injected intraperitoneally daily for 14 days, and then sacrificed. The titanium contents of the liver, kidneys, spleen, lungs, brain and heart were analyzed using ICP-MS and evaluated according to the organ coefficient (the ratio of organ tissue to body weight). A comprehensive list of biochemical parameters was also assessed to evaluate the effects of treatment on organ function and on carbohydrate and lipid metabolism.

The liver, kidneys, and spleen all showed an increase in net weight at doses of 50, 100, and 150 $mg\,kg^{-1}$, but these were equivalent to increases in the organ

weights of animals treated with 150 mg kg^{-1} bulk TiO_2. The lungs and brain showed a decrease in net organ weight, indicating possible degenerative damage. All of the organs tested showed a dose-dependent increase in titanium content, although interestingly the bulk TiO_2-treated mice showed titanium contents closer to those of the 100 mg kg^{-1}-treated group, which suggested that 5 nm anatase TiO_2 nanoparticles might enter the organs more easily than bulk TiO_2.

Biochemical parameters evaluated in the liver showed increases at higher doses (notably of ALA and alkaline phosphatase), followed by more moderate increases in leucine acid peptide, pseudocholinesterase, and total protein. Increases were also noted in the livers of bulk TiO_2-treated animals, though to a lesser extent than with equivalent concentrations of nanoparticle-treated animals. Similarly, serum markers of kidney function showed decreases (notably uric acid and BUN), with lesser decreases in bulk TiO_2-treated animals. These data indicate an increased toxicity of nanoscale TiO_2 to the liver and kidneys compared to equivalent concentrations of bulk TiO_2 particles. On evaluating markers of metabolic equilibrium, glucose, total cholesterol, triglycerides and high-density lipoprotein were found to have increased (but with no effect on low-density lipoprotein) at higher doses of TiO_2, indicating metabolic toxicity of TiO_2 nanoparticles. Whilst not evaluated in this study, an accumulation of titanium in the pancreas might suggest the cause of this change in carbohydrate and lipid metabolism. It was concluded that, in the higher doses tested (100 and 150 mg kg^{-1}), repeated exposure to TiO_2 nanoparticles caused toxicity and inflammation in the liver, kidneys and myocardium, in addition to a disruption of carbohydrate and lipid metabolism, compared to bulk TiO_2 particles. Hence, it was suggested that the size, crystal structure (anatase versus rutile) and route of administration might represent significant factors in the toxicity of TiO_2 nanoparticles, though lower concentrations were relatively nontoxic [33].

1.6.6 Subcutaneous Administration

In one (relatively rare) study in pregnant mice, the transfer of subcutaneously injected nanoparticles was examined, and the subsequent effects on the genital and cranial nerve systems were evaluated. (Note: This study is included as an example of TiO_2 nanoparticle administration that does not mimic environmental exposure. Moreover, as the site of injection was not indicated, the role of possible TiO_2 nanoparticle migration and proximity of the site to the target organs are again drawn into question.)

Pregnant Slc:ICR mice were administered 25–70 nm anatase TiO_2 nanoparticles subcutaneously, with each mouse receiving 100 µl of a 1 mg ml^{-1} TiO_2 in saline plus 0.05% Tween 80 surfactant (to prevent nanoparticle aggregation) [39, 162]. The TiO_2 doses were administered at 3, 7, 10, and 14 days post-coitum, and the male offspring sacrificed at 4 days or 6 weeks of age.

Histological sectioning and immunohistochemical staining for caspase-3 (a marker of apoptosis) showed significant toxicity relating to TiO_2 nanoparticle

exposure in the testes, epididymes, and seminal vesicles. At both time points, TiO_2 nanoparticles were detected in the Leydig cells, Sertoli cells and spermatids in the testis. There was, in addition, an obvious disorganization and disruption of the normal morphology of the seminiferous tubules, and fewer mature spermatozoa were observed, indicating a severe toxic effect in the male reproductive organs. Sperm motility was notably decreased, and the mitochondria of spermatozoa collected from TiO_2 nanoparticle-treated animals showed significant damage. The mean body weight of TiO_2 nanoparticle-exposed offspring was only 88% of that of their nonexposed counterparts. An inspection of the olfactory bulb and cerebral cortex of the brain of these mice at six weeks showed the presence of crescent-shaped cells (a known feature of apoptosis) and numerous caspase-3-positive cells in the TiO_2 nanoparticle-treated mice. Whilst previously, TiO_2 nanoparticles have been shown not to accumulate in the brain, it was postulated that because the blood–brain barrier was not yet fully developed at the time of injection, the TiO_2 could pass from mother to fetus, so as to exert pathological effects on the brain and interfere with normal fetal central nervous system development. Although, in other studies, the effects on the male reproductive organs were not examined, the blood–testis barrier (much like the blood–brain barrier) may not have been fully developed at the time of treatment [43].

While studies of the intraperitoneal or subcutaneous injection of TiO_2 nanoparticles do not translate readily into comparable situations of natural environmental or therapeutic exposure, they do confirm the results obtained after intravenous injection. At low doses, TiO_2 nanoparticles injected into the peritoneum of mammals proved to be relatively nontoxic and to accumulate predominantly in the RES, with minimal pathological changes to those organs where accumulation had occurred. However, high doses result in significant TiO_2 nanoparticle aggregate accumulation and toxicity to the liver, spleen and kidneys, as was evident from the fluctuations in serum levels of biochemical markers and pathological changes.

1.7 Conclusions

TiO_2 nanoparticles, nanocomposites and nanoconjugates show variable degrees of photocatalytic reactivity, photoresponse and surface reactivity, all of which influence their interactions with biological systems. Whilst it is difficult – and perhaps even impossible – to arrive at a consensus based on currently available information, several conclusions have come to the fore:

1. Anatase TiO_2 nanoparticles – especially those below 20 nm in size, where this crystal phase predominates – have the most pronounced surface reactivity, as evidenced by their interactions with phosphoproteins, cells in culture, and tissues *in vivo*. This surface reactivity in and of itself can cause derangements in cells and organisms, depending on the nanoparticle concentration. According to the most conservative estimates, anatase TiO_2 induces some signs of

cytotoxicity at concentrations above 5–10 $\mu g\,ml^{-1}$, although more relaxed estimates claim that 10-fold higher concentrations still pose no risk of cytotoxicity. Any therapeutic or diagnostic treatment with anatase TiO_2 nanoparticles must take these dose limits into consideration in order to protect healthy tissues from such cytotoxic effects. Consequently, modifications of the nanoparticle surface will be essential in order to prevent nanoparticles from aggregating and behaving as macroparticles, which are subject to different *in vivo* clearance mechanisms than nanoparticles. With aggregates, a greater accumulation can be expected, for example in the Kupffer cells of the liver and in alveolar macrophages, and in turn an extended retention in the liver, lungs and spleen and a relatively slow clearance of aggregates via the kidneys. Generally, nano-anatase TiO_2 nanomaterials will show good renal clearance, provided that their surface is rendered "inert" with respect to any crossreaction with tissues and other nanoparticles.

2. The photoresponse and photocatalytic reactivity of TiO_2 nanomaterials may be harnessed to deliberately induce cell ablation. This capacity depends, as in chemical degradation and microbicidal activity, on the ability to illuminate and activate nanoparticles or nanocomposites. Composites of TiO_2 will likely be better agents than pure TiO_2 for these applications, and the main obstacles during the course of the therapeutic use of such nanomaterials will be the ability to target the nanomaterials to the cells in question and to deliver light of the correct wavelength so as to induce cytotoxicity only in the desired cells and tissue locations.

In the meantime, TiO_2 nanoparticles, nanocomposites and nanoconjugates will continue to be used for different industrial purposes, from water purification to self-sterilizing surgical devices.

References

1 Naicker, P.K., Cummings, P.T. *et al.* (2005) Characterization of titanium dioxide nanoparticles using molecular dynamics simulations. *Journal of Physical Chemistry B*, **109** (32), 15243–9.

2 Fujishima, A. and Honda, K. (1972) Electrochemical photolysis of water at a semiconductor electrode. *Nature*, **238** (5358), 37–8.

3 Chen, X. and Mao, S.S. (2006) Synthesis of titanium dioxide (TiO2) nanomaterials. *Journal of Nanoscience and Nanotechnology*, **6** (4), 906–25.

4 Liang, S.S., Makamba, H. *et al.* (2006) Nano-titanium dioxide composites for the enrichment of phosphopeptides. *Journal of Chromatography A*, **1116** (1–2), 38–45.

5 Brown, E.M.B., Paunesku, T. *et al.* (2008) Methods for assessing DNA hybridization of peptide nucleic acid-titanium dioxide nanoconjugates. *Analytical Biochemistry*, **383** (2), 226–35.

6 Chen, L.X., Rajh, T. *et al.* (1997) *Journal of Physical Chemistry B*, **101**, 10688.

7 Rabatic, B.M., Dimitrijevic, N.M. *et al.* (2006) Spacially confined corner defects induce chemical functionality of TiO_2 nanorods. *Advanced Materials*, **18**, 1033–7.

8 Rajh, T., Chen, L.X. *et al.* (2002) Surface restructuring of nanoparticles: an efficient route for ligand-metal oxide

crosstalk. *Journal of Physical Chemistry B*, **106** (41), 10543–52.
9 Paunesku, T., Rajh, T. *et al.* (2003) Biology of TiO_2-oligonucleotide nanocomposites. *Nature Materials*, **2** (5), 343–6.
10 Thurn, K.T., Paunesku, T. *et al.* (2009) Labeling TiO_2 nanoparticles with dyes for optical fluorescence microscopy and determination of TiO_2-DNA nanoconjugate stability. *Small*, **5** (11), 1318–25.
11 Cozzoli, P.D., Comparelli, R. *et al.* (2004) Photocatalytic synthesis of silver nanoparticles stabilized by TiO_2 nanorods: a semiconductor/metal nanocomposite in homogeneous nonpolar solution. *Journal of the American Chemical Society*, **126** (12), 3868–79.
12 Yao, K.F., Peng, Z. *et al.* (2009) Preparation and photocatalytic property of TiO_2-Fe_3O_4 core-shell nanoparticles. *Journal of Nanoscience and Nanotechnology*, **9** (2), 1458–61.
13 Endres, P.J., Paunesku, T. *et al.* (2007) DNA-TiO_2 nanoconjugates labeled with magnetic resonance contrast agents. *Journal of the American Chemical Society*, **129** (51), 15760–+.
14 Paunesku, T., Stojicevic, N. *et al.* (2003) Intracellular localization of titanium dioxide-biomolecule nanocomposites. *Journal De Physique IV*, **104**, 317–19.
15 Paunesku, T., Vogt, S. *et al.* (2007) Intracellular distribution of TiO_2-DNA oligonucleotide nanoconjugates directed to nucleolus and mitochondria indicates sequence specificity. *Nano Letters*, **7** (3), 596–601.
16 Paunesku, T., Ke, T. *et al.* (2008) Gadolinium-conjugated TiO_2-DNA oligonucleotide nanoconjugates show prolonged intracellular retention period and T_1-weighted contrast enhancement in magnetic resonance images. *Nanomedicine: Nanotechnology, Biology and Medicine*, **4** (3), 201–7.
17 Wu, A., Paunesku, T. *et al.* (2008) Titanium dioxide nanoparticles assembled by DNA molecules hybridization and loading of DNA interacting proteins. *NANO*, **3** (1), 27–36.
18 Gentleman, D.J. and Chan, W.C. (2009) A systematic nomenclature for codifying engineered nanostructures. *Small*, **5** (4), 426–31.
19 Baggs, R.B., Ferin, J. *et al.* (1997) Regression of pulmonary lesions produced by inhaled titanium dioxide in rats. *Veterinary Pathology*, **34** (6), 592–7.
20 Bermudez, E., Mangum, J.B. *et al.* (2004) Pulmonary responses of mice, rats, and hamsters to subchronic inhalation of ultrafine titanium dioxide particles. *Toxicological Sciences*, **77** (2), 347–57.
21 Blaise, C., Gagne, F. *et al.* (2007) Ecotoxicity of selected nano-materials to aquatic organisms. *13th International Symposium on Toxicity Assessment, Toyama, Japan*.
22 Chen, H.W., Su, S.F. *et al.* (2006) Titanium dioxide nanoparticles induce emphysema-like lung injury in mice. *FASEB Journal*, **20** (13), 2393–5.
23 Chen, J.Y., Dong, X. *et al.* (2009) In vivo acute toxicity of titanium dioxide nanoparticles to mice after intraperitoneal injection. *Journal of Applied Toxicology*, **29** (4), 330–7.
24 Drobne, D., Jemec, A. *et al.* (2009) In vivo screening to determine hazards of nanoparticles: nanosized TiO_2. *Environmental Pollution*, **157** (4), 1157–64.
25 Fabian, E., Landsiedel, R. *et al.* (2008) Tissue distribution and toxicity of intravenously administered titanium dioxide nanoparticles in rats. *Archives of Toxicology*, **82** (3), 151–7.
26 Federici, G., Shaw, B.J. *et al.* (2007) Toxicity of titanium dioxide nanoparticles to rainbow trout (*Oncorhynchus mykiss*): gill injury, oxidative stress, and other physiological effects. *Aquatic Toxicology*, **84** (4), 415–30.
27 Grassian, V.H., O'Shaughnessy, P.T. *et al.* (2007) Inhalation exposure study of titanium dioxide nanoparticles with a primary particle size of 2 to 5 nm. *Environmental Health Perspectives*, **115** (3), 397–402.
28 Griffitt, R.J., Hyndman, K. *et al.* (2009) Comparison of molecular and histological changes in zebrafish gills exposed to metallic nanoparticles. *Toxicological Sciences*, **107** (2), 404–15.

29 Heinlaan, M., Ivask, A. *et al.* (2008) Toxicity of nanosized and bulk ZnO, CuO and TiO_2 to bacteria *Vibrio fischeri* and crustaceans *Daphnia magna* and *Thamnocephalus platyurus*. *Chemosphere*, **71** (7), 1308–16.

30 Jemec, A., Drobne, D. *et al.* (2008) Effects of ingested nano-sized titanium dioxide on terrestrial isopods (*Porcellio scaber*). *Environmental Toxicology and Chemistry*, **27** (9), 1904–14.

31 Lee, S.W., Kim, S.M. *et al.* (2009) Genotoxicity and ecotoxicity assays using the freshwater crustacean *Daphnia magna* and the larva of the aquatic midge *Chironomus riparius* to screen the ecological risks of nanoparticle exposure. *Environmental Toxicology and Pharmacology*, **28** (1), 86–91.

32 Li, J.A., Li, Q.N. *et al.* (2007) Comparative study on the acute pulmonary toxicity induced by 3 and 20 nm TiO_2 primary particles in mice. *Environmental Toxicology and Pharmacology*, **24** (3), 239–44.

33 Liu, H.T., Ma, L.L. *et al.* (2009) Biochemical toxicity of nano-anatase TiO_2 particles in mice. *Biological Trace Element Research*, **129** (1–3), 170–80.

34 Lovern, S.B., Strickler, J.R. *et al.* (2007) Behavioral and physiological changes in *Daphnia magna* when exposed to nanoparticle suspensions (titanium dioxide, nano-C-60, and C(60)HxC(70)Hx. *Environmental Science and Technology*, **41** (12), 4465–70.

35 Nemmar, A., Melghit, K. *et al.* (2008) The acute proinflammatory and prothrombotic effects of pulmonary exposure to rutile TiO_2 nanorods in rats. *Experimental Biology and Medicine*, **233** (5), 610–19.

36 Nurkiewicz, T.R., Porter, D.W. *et al.* (2008) Nanoparticle inhalation augments particle-dependent systemic microvascular dysfunction. *Particle and Fibre Toxicology*, **5**, Article no. 1.

37 Oberdorster, G., Ferin, J. *et al.* (1994) Correlation between particle size, in vivo particle persistence, and lung injury. *Environmental Health Perspectives*, **102** (Suppl. 5) (96), 173–9.

38 Park, E.J., Yoon, J. *et al.* (2009) Induction of chronic inflammation in mice treated with titanium dioxide nanoparticles by intratracheal instillation. *Toxicology*, **260** (1–3), 37–46.

39 Patri, A., Umbreit, T. *et al.* (2009) Energy dispersive X-ray analysis of titanium dioxide nanoparticle distribution after intravenous and subcutaneous injection in mice. *Journal of Applied Toxicology*, **22**, 22.

40 Sager, T.M., Kommineni, C. *et al.* (2008) Pulmonary response to intratracheal instillation of ultrafine versus fine titanium dioxide: role of particle surface area. *Particle and Fibre Toxicology*, **5**, Article no. 17.

41 Sager, T.M. and Castranova, V. (2009) Surface area of particle administered versus mass in determining the pulmonary toxicity of ultrafine and fine carbon black: comparison to ultrafine titanium dioxide. *Particle and Fibre Toxicology*, **6**, Article no. 15.

42 Scown, T.M., van Aerle, R. *et al.* (2009) High doses of intravenously administered titanium dioxide nanoparticles accumulate in the kidneys of rainbow trout but with no observable impairment of renal function. *Toxicological Sciences*, **109** (2), 372–80.

43 Takeda, K., Suzuki, K.I. *et al.* (2009) Nanoparticles transferred from pregnant mice to their offspring can damage the genital and cranial nerve systems. *Journal of Health Science*, **55** (1), 95–102.

44 van Ravenzwaay, B., Landsiedel, R. *et al.* (2009) Comparing fate and effects of three particles of different surface properties: nano-TiO_2, pigmentary TiO_2 and quartz. *Toxicology Letters*, **186** (3), 152–9.

45 Wang, J.X., Zhou, G.Q. *et al.* (2007) Acute toxicity and biodistribution of different sized titanium dioxide particles in mice after oral administration. *Toxicology Letters*, **168** (2), 176–85.

46 Wang, J.X., Chen, C.Y. *et al.* (2008) Potential neurological lesion after nasal instillation of TiO_2 nanoparticles in the anatase and rutile crystal phases. *Toxicology Letters*, **183** (1–3), 72–80.

47 Wang, J.X., Liu, Y. *et al.* (2008) Time-dependent translocation and potential impairment on central nervous system by intranasally instilled TiO_2

nanoparticles. *Toxicology*, **254** (1–2), 82–90.
48 Warheit, D.B., Webb, T.R. *et al.* (2006) Pulmonary instillation studies with nanoscale TiO_2 rods and dots in rats: toxicity is not dependent upon particle size and surface area. *Toxicological Sciences*, **91** (1), 227–36.
49 Warheit, D.B., Hoke, R.A. *et al.* (2007) Development of a base set of toxicity tests using ultrafine TiO_2 particles as a component of nanoparticle risk management. *Toxicology Letters*, **171** (3), 99–110.
50 Warheit, D.B., Webb, T.R. *et al.* (2007) Pulmonary toxicity study in rats with three forms of ultrafine-TiO_2 particles: differential responses related to surface properties. *Toxicology*, **230** (1), 90–104.
51 Matsumoto, T., Iyi, N. *et al.* (2007) High visible-light photocatalytic activity of nitrogen-doped titania prepared from layered titania/isostearate nanocomposite. *Catalysis Today*, **120** (2), 226–32.
52 Xue, M., Huang, L. *et al.* (2008) The direct synthesis of mesoporous structured MnO_2/TiO_2 nanocomposite: a novel visible-light active photocatalyst with large pore size. *Nanotechnology*, **19** (18), Article no. 185604.
53 Li, J.H., Yang, X. *et al.* (2009) Rare earth oxide-doped titania nanocomposites with enhanced photocatalytic activity towards the degradation of partially hydrolysis polyacrylamide. *Applied Surface Science*, **255** (6), 3731–8.
54 Wang, Q., Yang, D. *et al.* (2007) Synthesis of anatase titania-carbon nanotubes nanocomposites with enhanced photocatalytic activity through a nanocoating-hydrothermal process. *Journal of Nanoparticle Research*, **9**, 1087–96.
55 Yang, C.S., Wang, Y.J. *et al.* (2009) Photocatalytic performance of alumina-incorporated titania composite nanoparticles: surface area and crystallinity. *Applied Catalysis A: General*, **364** (1–2), 182–90.
56 Yu, J.G., Xiong, J.F. *et al.* (2005) Fabrication and characterization of Ag-TiO_2 multiphase nanocomposite thin films with enhanced photocatalytic activity. *Applied Catalysis B: Environmental*, **60** (3–4), 211–21.
57 Rana, S., Rawat, J. *et al.* (2005) Anti-microbial active composite nanoparticles with magnetic core and photocatalytic shell: TiO_2-$NiFe_2O_4$ biomaterial system. *Acta Biomaterialia*, **1** (6), 691–703.
58 Kun, R., Mogyorosi, K. *et al.* (2006) Synthesis and structural and photocatalytic properties of TiO_2/montmorillonite nanocomposites. *Applied Clay Science*, **32** (1–2), 99–110.
59 Li, S.Y., Chen, M.K. *et al.* (2009) Preparation and characterization of polypyrrole/TiO_2 nanocomposite and its photocatalytic activity under visible light irradiation. *Journal of Materials Research*, **24** (8), 2547–54.
60 Konovalova, T.A. (2004) Generation of superoxide anion and most likely singlet oxygen in irradiated TiO_2 nanoparticles modified by carotenoids. *Journal of Photochemistry and Photobiology*, **162**, 1–8.
61 Zhu, J.H., Yang, D. *et al.* (2008) Synthesis and characterization of bamboo-like CdS/TiO_2 nanotubes composites with enhanced visible-light photocatalytic activity. *Journal of Nanoparticle Research*, **10** (5), 729–36.
62 Kuo, C.S., Tseng, Y.H. *et al.* (2007) Synthesis of a CNT-grafted TiO_2 nanocatalyst and its activity triggered by a DC voltage. *Nanotechnology*, **18**, Article no. 465607.
63 Li, G.H. and Gray, K.A. (2007) The solid-solid interface: explaining the high and unique photocatalytic reactivity of TiO_2-based nanocomposite materials. *Chemical Physics*, **339**, 173–87.
64 Yu, J.G., Yue, L. *et al.* (2009) Hydrothermal preparation and photocatalytic activity of mesoporous Au-TiO_2 nanocomposite microspheres. *Journal of Colloid and Interface Science*, **334** (1), 58–64.
65 Wang, L.S., Xiao, M.W. *et al.* (2009) Synthesis, characterization, and photocatalytic activities of titanate nanotubes surface-decorated by zinc oxide nanoparticles. *Journal of Hazardous Materials*, **161** (1), 49–54.
66 Min, S.X., Wan, F. *et al.* (2009) Preparation and photocatalytic activity of

PANI/AMTES-TiO_2 nanocomposite materials. *Acta Physico-Chimica Sinica*, **25** (7), 1303–10.

67 Cozzoli, P.D., Fanizza, E. *et al.* (2004) Role of metal nanoparticles in TiO_2/Ag nanocomposite-based microheterogeneous photocatalysis. *Journal of Physical Chemistry B*, **108** (28), 9623–30.

68 Fu, G.F., Vary, P.S. *et al.* (2005) Anatase TiO_2 nanocomposites for antimicrobial coatings. *Journal of Physical Chemistry B*, **109** (18), 8889–98.

69 Zhang, H.J. and Chen, G.H. (2009) Potent antibacterial activities of Ag/TiO_2 nanocomposite powders synthesized by a one-pot sol-gel method. *Environmental Science and Technology*, **43** (8), 2905–10.

70 Liu, Y., Wang, X.L. *et al.* (2008) Excellent antimicrobial properties of mesoporous anatase TiO_2 and Ag/TiO_2 composite films. *Microporous and Mesoporous Materials*, **114** (1–3), 431–9.

71 Yao, Y., Ohko, Y. *et al.* (2008) Self-sterilization using silicone catheters coated with Ag and TiO_2 nanocomposite thin film. *Journal of Biomedical Materials Research Part B - Applied Biomaterials*, **85B** (2), 453–60.

72 Akhavan, O. (2009) Lasting antibacterial activities of Ag-TiO_2/Ag/a-TiO_2 nanocomposite thin film photocatalysts under solar light irradiation. *Journal of Colloid and Interface Science*, **336** (1), 117–24.

73 Sayilkan, F., Asilturk, M. *et al.* (2009) Photocatalytic antibacterial performance of Sn(4+)-doped TiO(2) thin films on glass substrate. *Journal of Hazardous Materials*, **162** (2–3), 1309–16.

74 Lee, S.H., Pumprueg, S. *et al.* (2005) Inactivation of bacterial endospores by photocatalytic nanocomposites. *Colloids and Surfaces B: Biointerfaces*, **40** (2), 93–8.

75 Yu, J.C., Ho, W. *et al.* (2005) Efficient visible light induced photocatalytic disinfection on sulfur-doped nanocrystalline titania. *Environmental Science and Technology*, **39**, 1175–9.

76 Venkatasubramanian, R., Srivastava, R.S. *et al.* (2008) Comparative study of antimicrobial and photocatalytic activity in titania encapsulated composite nanoparticles with different dopants. *Materials Science and Technology*, **24** (5), 589–95.

77 Cerrada, M.L., Serrano, C. *et al.* (2008) Self-sterilized EVOH-TiO_2 nanocomposites: interface effects on biocidal properties. *Advanced Functional Materials*, **18** (13), 1949–60.

78 Kubacka, A., Ferrer, M. *et al.* (2009) Boosting TiO_2-anatase antimicrobial activity: polymer-oxide thin films. *Applied Catalysis B: Environmental*, **89** (3–4), 441–7.

79 Chen, W.-J., Tsai, P.-J. *et al.* (2008) Functional Fe_3O_4/TiO_2 core/shell magnetic nanoparticles as photokilling agents for pathogenic bacteria. *Small*, **4** (4), 485–91.

80 Bannat, I., Wessels, K. *et al.* (2009) Improving the photocatalytic performance of mesoporous titania films by modification with gold nanostructures. *Chemistry of Materials*, **21** (8), 1645–53.

81 Li, Y., Xu, X.Q. *et al.* (2008) Novel Fe_3O_4@TiO_2 core-shell microspheres for selective enrichment of phosphopeptides in phosphoproteome analysis. *Journal of Proteome Research*, **7** (6), 2526–38.

82 Webster, T.J., Ergun, C. *et al.* (2000) Specific proteins mediate enhanced osteoblast adhesion on nanophase ceramics. *Journal of Biomedical Materials Research*, **51** (3), 475–83.

83 McManus, A.J., Doremus, R.H. *et al.* (2005) Evaluation of cytocompatibility and bending modulus of nanoceramic/polymer composites. *Journal of Biomedical Materials Research Part A*, **72** (1), 98–106.

84 Kay, S., Thapa, A. *et al.* (2002) Nanostructured polymer/nanophase ceramic composites enhance osteoblast and chondrocyte adhesion. *Tissue Engineering*, **8** (5), 753–61.

85 Webster, T.J. and Smith, T.A. (2005) Increased osteoblast function on PLGA composites containing nanophase titania. *Journal of Biomedical Materials Research Part A*, **74** (4), 677–86.

86 Webster, T.J., Siegel, R.W. *et al.* (1999) Osteoblast adhesion on nanophase ceramics. *Biomaterials*, **20** (13), 1221–7.

87 Mills, A. and Le Hunte, S. (1997) An overview of semiconductor

photocatalysis. *Journal of Photochemistry and Photobiology A: Chemistry*, **108**, 1–35.

88 Blake, D.M., Maness, P.C. *et al.* (1999) Application of the photocatalytic chemistry of titanium dioxide to disinfection and the killing of cancer cells. *Separation and Purification Methods*, **28** (1), 1–50.

89 Yang, H.M., Shi, R.R. *et al.* (2005) Synthesis of WO_3/TiO_2 nanocomposites via sol-gel method. *Journal of Alloys and Compounds*, **398** (1–2), 200–2.

90 Gratzel, M. (2004) Conversion of sunlight to electric power by nanocrystalline dye-sensitized solar cells. *Journal of Photochemistry and Photobiology A: Chemistry*, **164**, 3–14.

91 Yu, H.T., Quan, X. *et al.* (2008) TiO_2-carbon nanotube heterojunction arrays with a controllable thickness of TiO_2 layer and their first application in photocatalysis. *Journal of Photochemistry and Photobiology A: Chemistry*, **200** (2–3), 301–6.

92 Neves, M.C., Monteiro, O.C. *et al.* (2008) From single-molecule precursors to coupled Ag_2S/TiO_2 nanocomposites. *European Journal of Inorganic Chemistry*, **2008** (28), 4380–6.

93 Su, H.L., Dong, Q. *et al.* (2008) Biogenic synthesis and photocatalysis of Pd-PdO nanoclusters reinforced hierarchical TiO_2 films with interwoven and tubular conformations. *Biomacromolecules*, **9** (2), 499–504.

94 Xiao, M.W., Wang, L.S. *et al.* (2009) Synthesis and characterization of WO_3/titanate nanotubes nanocomposite with enhanced photocatalytic properties. *Journal of Alloys and Compounds*, **470** (1–2), 486–91.

95 Yu, M.D., Guo, Y. *et al.* (2008) A novel preparation of mesoporous $Cs_xH_{3-x}PW_{12}O_{40}/TiO_2$ nanocomposites with enhanced photocatalytic activity. *Colloids and Surfaces A: Physicochemical and Engineering Aspects*, **316** (1–3), 110–18.

96 Shchukin, D., Poznyak, S. *et al.* (2004) TiO_2-In_2O_3 photocatalysts: preparation, characterisations and activity for 2-chlorophenol degradation in water. *Journal of Photochemistry and Photobiology A: Chemistry*, **162** (2–3), 423–30.

97 Lei, S.M., Gong, W.Q. *et al.* (2006) Preparation of TiO_2/kaolinite nanocomposite and its photocatalytical activity. *Journal of Wuhan University of Technology: Materials Science Edition*, **21** (4), 12–15.

98 Luo, Y.B., Li, W.D. *et al.* (2009) Preparation and properties of nanocomposites based on poly(lactic acid) and functionalized TiO_2. *Acta Materialia*, **57** (11), 3182–91.

99 Matsunaga, T., Tomoda, T. *et al.* (1985) Photoelectrochemical sterilization of microbial cells by semiconductor powder. *FEMS Microbiology Letters*, **29**, 211–16.

100 Maness, P.C., Smolinski, S. *et al.* (1999) Bactericidal activity of photocatalytic TiO(2) reaction: toward an understanding of its killing mechanism. *Applied and Environmental Microbiology*, **65** (9), 4094–8.

101 Mo, A.C., Xu, W. *et al.* (2007) Antibacterial activity of silver-hydroxyapatite/titania nanocomposite coating on titanium against oral bacteria. *Bioceramics*, **19** (Pts 1 and 2) (330–332), 455–8.

102 Liu, L.F., Barford, J. *et al.* (2007) Non-UV based germicidal activity of metal-doped TiO_2 coating on solid surfaces. *Journal of Environmental Sciences: China*, **19**, 745–50.

103 Mahltig, B., Gutmann, E. *et al.* (2007) Solvothermal preparation of metallized titania sols for photocatalytic and antimicrobial coatings. *Journal of Materials Chemistry*, **17** (22), 2367–74.

104 Sheel, D.W., Brook, L.A. *et al.* (2008) Biocidal silver and silver/titania composite films grown by chemical vapour deposition. *International Journal of Photoenergy*, Article no. 168185.

105 Wang, X.C., Yu, J.C. *et al.* (2005) A mesoporous Pt/TiO_2 nanoarchitecture with catalytic and photocatalytic functions. *Chemistry*, **11** (10), 2997–3004.

106 Kangwansupamonkon, W., Lauruengtana, V. *et al.* (2009) Antibacterial effect of apatite-coated titanium dioxide for textiles applications. *Nanomedicine-Nanotechnology Biology and Medicine*, **5** (2), 240–9.

107 Mazanek, M., Mituloviae, G. *et al.* (2007) Titanium dioxide as a chemo-affinity solid phase in offline phosphopeptide chromatography prior to HPLC-MS/MS analysis. *Nature Protocols*, **2** (5), 1059–69.
108 Yu, L.R., Zhu, Z.Y. *et al.* (2007) Improved titanium dioxide enrichment of phosphopeptides from HeLa cells and high confident phosphopeptide identification by cross-validation of MS/MS and MS/MS/MS spectra. *Journal of Proteome Research*, **6** (11), 4150–62.
109 Ashman, K. and Villar, E.L. (2009) Phosphoproteomics and cancer research. *Clinical and Translational Oncology*, **11** (6), 356–62.
110 Paradela, A. and Albar, J.P. (2008) Advances in the analysis of protein phosphorylation. *Journal of Proteome Research*, **7** (5), 1809–18.
111 Karlsson, H.L., Gustafsson, J. *et al.* (2009) Size-dependent toxicity of metal oxide particles – a comparison between nano- and micrometer size. *Toxicology Letters*, **188** (2), 112–18.
112 Geiser, M., Rothen-Rutishauser, B. *et al.* (2005) Ultrafine particles cross cellular membranes by nonphagocytic mechanisms in lungs and in cultured cells. *Environmental Health Perspectives*, **113** (11), 1555–60.
113 Singh, S., Shi, T.M. *et al.* (2007) Endocytosis, oxidative stress and IL-8 expression in human lung epithelial cells upon treatment with fine and ultrafine TiO_2: role of the specific surface area and of surface methylation of the particles. *Toxicology and Applied Pharmacology*, **222** (2), 141–51.
114 Kang, S.J., Kim, B.M. *et al.* (2009) Titanium dioxide nanoparticles induce apoptosis through the JNK/p38-caspase-8-Bid pathway in phytohemagglutinin-stimulated human lymphocytes. *Biochemical and Biophysical Research Communications*, **386** (4), 682–7.
115 Kong, S.J., Kim, B.M. *et al.* (2008) Titanium dioxide nanoparticles trigger p53-mediated damage response in peripheral blood lymphocytes. *Environmental and Molecular Mutagenesis*, **49** (5), 399–405.
116 Park, E.J., Yi, J. *et al.* (2008) Oxidative stress and apoptosis induced by titanium dioxide nanoparticles in cultured BEAS-2B cells. *Toxicology Letters*, **180** (3), 222–9.
117 Wan, R., Mo, Y.Q. *et al.* (2008) Matrix metalloproteinase-2 and-9 are induced differently by metal nanoparticles in human monocytes: the role of oxidative stress and protein tyrosine kinase activation. *Toxicology and Applied Pharmacology*, **233** (2), 276–85.
118 Rozhkova, E.A., Ulasov, I. *et al.* (2009) A high-performance nanobio photocatalyst for targeted brain cancer therapy. *Nano Letters*, **9** (9), 3337–42.
119 Johannes, L. and Lamaze, C. (2002) Clathrin-dependent or not: is it still the question? *Traffic*, **3** (7), 443–51.
120 Kirkham, M. and Parton, R.G. (2005) Clathrin-independent endocytosis: new insights into caveolae and non-caveolar lipid raft carriers. *Biochimica et Biophysica Acta*, **1746** (3), 349–63.
121 Mosesson, Y., Mills, G.B. *et al.* (2008) Derailed endocytosis: an emerging feature of cancer. *Nature Reviews Cancer*, **8** (11), 835–50.
122 Hussain, S., Boland, S. *et al.* (2009) Oxidative stress and proinflammatory effects of carbon black and titanium dioxide nanoparticles: role of particle surface area and internalized amount. *Toxicology*, **260** (1–3), 142–9.
123 Barua, S. and Rege, K. (2009) Cancer-cell-phenotype-dependent differential intracellular trafficking of unconjugated quantum dots. *Small*, **5** (3), 370–6.
124 Koeneman, B.A., Zhang, Y. *et al.* (2009) Toxicity and cellular responses of intestinal cells exposed to titanium dioxide. *Cell Biology and Toxicology*, [Epub ahead of print].
125 Jin, C.Y., Zhu, B.S. *et al.* (2008) Cytotoxicity of titanium dioxide nanoparticles in mouse fibroblast cells. *Chemical Research in Toxicology*, **21** (9), 1871–7.
126 Suh, W.H., Suslick, K.S. *et al.* (2009) Nanotechnology, nanotoxicology, and neuroscience. *Progress in Neurobiology*, **87** (3), 133–70.
127 Hirakawa, K., Mori, M. *et al.* (2004) Photo-irradiated titanium dioxide catalyzes site specific DNA damage via

generation of hydrogen peroxide. *Free Radical Research*, **38** (5), 439–47.
128 Serpone, N., Salinaro, A.E. *et al.* (2006) Beneficial effects of photo-inactive titanium dioxide specimens on plasmid DNA, human cells and yeast cells exposed to UVA/UVB simulated sunlight. *Journal of Photochemistry and Photobiology A: Chemistry*, **179** (1–2), 200–12.
129 Wamer, W.G., Yin, J.J. *et al.* (1997) Oxidative damage to nucleic acids photosensitized by titanium dioxide. *Free Radical Biology and Medicine*, **23** (6), 851–8.
130 Cai, R.X., Kubota, Y. *et al.* (1992) Induction of cytotoxicity by photoexcited TiO_2 particles. *Cancer Research*, **52** (8), 2346–8.
131 Chihara, Y., Fujimoto, K. *et al.* (2007) Anti-tumor effects of liposome-encapsulated titanium dioxide in nude mice. *Pathobiology*, **74** (6), 353–8.
132 Kubota, Y., Shuin, T. *et al.* (1994) Photokilling of T-24 human bladder-cancer cells with titanium-dioxide. *British Journal of Cancer*, **70** (6), 1107–11.
133 Liu, J., de la Garza, L. *et al.* (2007) Photocatalytic probing of DNA sequence by using TiO_2/dopamine-DNA triads. *Chemical Physics*, **339**, 154–63.
134 Tachikawa, T., Asanoi, Y. *et al.* (2008) Photocatalytic cleavage of single TiO_2/DNA nanoconjugates. *Chemistry*, **14** (5), 1492–8.
135 Tachikawa, T. and Majima, T. (2009) Single-molecule fluorescence imaging of TiO_2 photocatalytic reactions. *Langmuir*, **25** (14), 7791–802.
136 Branemark, P.I. (1959) Vital microscopy of bone marrow in rabbit. *Scandinavian Journal of Clinical and Laboratory Investigation*, **11** (Suppl. 38), 1–82.
137 Branemark, R., Branemark, P.I. *et al.* (2001) Osseointegration in skeletal reconstruction and rehabilitation: a review. *Journal of Rehabilitation Research and Development*, **38** (2), 175–81.
138 Adell, R., Lekholm, U. *et al.* (1981) A 15-year study of osseointegrated implants in the treatment of the edentulous jaw. *International Journal of Oral Surgery*, **10** (6), 387–416.
139 Franchi, M., Fini, M. *et al.* (2005) Biological fixation of endosseous implants. *Micron*, **36** (7–8), 665–71.
140 Lindgren, M., Astrand, M. *et al.* (2009) Investigation of boundary conditions for biomimetic HA deposition on titanium oxide surfaces. *Journal of Materials Science: Materials in Medicine*, **20** (7), 1401–8.
141 Rho, J.Y., Kuhn-Spearing, L. *et al.* (1998) Mechanical properties and the hierarchical structure of bone. *Medical Engineering and Physics*, **20** (2), 92–102.
142 Webster, T.J., Ergun, C. *et al.* (2000) Enhanced functions of osteoblasts on nanophase ceramics. *Biomaterials*, **21** (17), 1803–10.
143 Misra, R.D., Thein-Han, W.W. *et al.* (2009) Cellular response of preosteoblasts to nanograined/ultrafine-grained structures. *Acta Biomaterialia*, **5** (5), 1455–67.
144 Ramires, P.A., Cosentino, F. *et al.* (2002) In vitro response of primary rat osteoblasts to titania/hydroxyapatite coatings compared with transformed human osteoblast-like cells. *Journal of Materials Science: Materials in Medicine*, **13** (8), 797–801.
145 Sailaja, G.S., Ramesh, P. *et al.* (2006) Human osteosarcoma cell adhesion behaviour on hydroxyapatite integrated chitosan-poly(acrylic acid) polyelectrolyte complex. *Acta Biomaterialia*, **2** (6), 651–7.
146 Zhao, G., Schwartz, Z. *et al.* (2005) High surface energy enhances cell response to titanium substrate microstructure. *Journal of Biomedical Materials Research Part A*, **74** (1), 49–58.
147 Meng, D., Ioannou, J. *et al.* (2009) Bioglass®-based scaffolds with carbon nanotube coating for bone tissue engineering. *Journal of Materials Science: Materials in Medicine*, **20** (10), 2139–44.
148 Jarcho, M., Kay, J.F. *et al.* (1977) Tissue, cellular and subcellular events at a bone-ceramic hydroxylapatite interface. *Journal of Bioengineering*, **1** (2), 79–92.
149 Paderni, S., Terzi, S. *et al.* (2009) Major bone defect treatment with an osteoconductive bone substitute. *Musculoskeletal Surgery*, **93** (2), 89–96.
150 Daculsi, G., LeGeros, R.Z. *et al.* (1990) Formation of carbonate-apatite crystals

after implantation of calcium phosphate ceramics. *Calcified Tissue International*, **46** (1), 20–7.

151 Kitsugi, T., Yamamuro, T. *et al.* (1986) Bone bonding behavior of three kinds of apatite containing glass ceramics. *Journal of Biomedical Materials Research*, **20** (9), 1295–307.

152 Valimaki, V.V., Yrjans, J.J. *et al.* (2005) Combined effect of BMP-2 gene transfer and bioactive glass microspheres on enhancement of new bone formation. *Journal of Biomedical Materials Research Part A*, **75** (3), 501–9.

153 Valimaki, V.V. and Aro, H.T. (2006) Molecular basis for action of bioactive glasses as bone graft substitute. *Scandinavian Journal of Surgery*, **95** (2), 95–102.

154 Abou Neel, E.A. and Knowles, J.C. (2008) Physical and biocompatibility studies of novel titanium dioxide doped phosphate-based glasses for bone tissue engineering applications. *Journal of Materials Science: Materials in Medicine*, **19** (1), 377–86.

155 Abou Neel, E.A., Ahmed, I. *et al.* (2005) Effect of iron on the surface, degradation and ion release properties of phosphate-based glass fibres. *Acta Biomaterialia*, **1** (5), 553–63.

156 Abou Neel, E.A., Chrzanowski, W. *et al.* (2008) Effect of increasing titanium dioxide content on bulk and surface properties of phosphate-based glasses. *Acta Biomaterialia*, **4** (3), 523–34.

157 Ahmed, I., Collins, C.A. *et al.* (2004) Processing, characterisation and biocompatibility of iron-phosphate glass fibres for tissue engineering. *Biomaterials*, **25** (16), 3223–32.

158 Neel, E.A., Ahmed, I. *et al.* (2005) Characterisation of antibacterial copper releasing degradable phosphate glass fibres. *Biomaterials*, **26** (15), 2247–54.

159 Prabhakar, R.L., Brocchini, S. *et al.* (2005) Effect of glass composition on the degradation properties and ion release characteristics of phosphate glass–polycaprolactone composites. *Biomaterials*, **26** (15), 2209–18.

160 Zhao, L., Chang, J. *et al.* (2009) Preparation and HL-7702 cell functionality of titania/chitosan composite scaffolds. *Journal of Materials Science: Materials in Medicine*, **20**, 949–57.

161 Dobrovolskaia, M.A., Patri, A.K. *et al.* (2009) Interaction of colloidal gold nanoparticles with human blood: effects on particle size and analysis of plasma protein binding profiles. *Nanomedicine: Nanotechnology, Biology, and Medicine*, **5** (2), 106–17.

162 Patri, A., Umbreit, T. *et al.* (2009) Energy dispersive X-ray analysis of titanium dioxide nanoparticle distribution after intravenous and subcutaneous injection in mice. *Toxicology*, **230** (1), 90–104.

163 Olmedo, D.G., Tasat, D.R. *et al.* (2008) Biological response of tissues with macrophagic activity to titanium dioxide. *Journal of Biomedical Materials Research Part A*, **84** (4), 1087–93.

164 Tran, C.L., Buchanan, D. *et al.* (2000) Inhalation of poorly soluble particles. II. Influence Of particle surface area on inflammation and clearance. *Inhalation Toxicology*, **12** (12), 1113–26.

165 Liu, R., Yin, L.H. *et al.* (2009) Pulmonary toxicity induced by three forms of titanium dioxide nanoparticles via intra-tracheal instillation in rats. *Progress in Natural Science*, **19** (5), 573–9.

166 Oberdorster, G. (2001) Pulmonary effects of inhaled ultrafine particles. *International Archives of Occupational and Environmental Health*, **74** (1), 1–8.

167 Yokohira, M., Kuno, T. *et al.* (2008) Lung toxicity of 16 fine particles on intratracheal instillation in a bioassay model using F344 male rats. *Toxicologic Pathology*, **36** (4), 620–31.

168 Handy, R.D. and Shaw, B.J. (2007) Toxic effects of nanoparticles and nanomaterials: implications for public health, risk assessment and the public perception of nanotechnology. *Health Risk & Society*, **9** (2), 125–44.

169 Crosera, M., Bovenzi, M. *et al.* (2009) Nanoparticle dermal absorption and toxicity: a review of the literature. *International Archives of Occupational and Environmental Health*, **82** (9), 1043–55.

170 Schulz, J., Hohenberg, H. *et al.* (2002) Distribution of sunscreens on skin. *Advanced Drug Delivery Reviews*, **54**, S157–63.

171 Sayes, C.M., Wahi, R. *et al.* (2006) Correlating nanoscale titania structure with toxicity: a cytotoxicity and inflammatory response study with human dermal fibroblasts and human lung epithelial cells. *Toxicological Sciences*, **92** (1), 174–85.

172 Chen, Z., Meng, H. *et al.* (2006) Acute toxicological effects of copper nanoparticles in vivo. *Toxicology Letters*, **163** (2), 109–20.

173 Huggins, C.B., Froehlich, J.P. *et al.* (1966) High concentration of injected titanium dioxide in abdominal lymph nodes. *The Journal of Experimental Medicine*, **124** (6), 1099–106.

174 Longmire, M., Choyke, P.L. *et al.* (2008) Clearance properties of nano-sized particles and molecules as imaging agents: considerations and caveats. *Nanomedicine*, **3** (5), 703–17.

175 Paik, S.G., Fleischer, N. *et al.* (1980) Insulin-dependent diabetes mellitus induced by subdiabetogenic doses of streptozotocin: obligatory role of cell-mediated autoimmune processes. *Proceedings of the National Academy of Sciences of the United States of America*, **77** (10), 6129–33.

176 Garnett, M.C. and Kallinteri, P. (2006) Nanomedicines and nanotoxicology: some physiological principles. *Occupational Medicine*, **56** (5), 307–11.

177 Kikuchi, Y., Sunada, K. *et al.* (1997) Photocatalytic bactericidal effect of TiO_2 thin films: Dynamic view of the active oxygen species responsible for the effect. *Journal of Photochemistry and Photobiology A - Chemistry* **106** (1–3), 51–6.

178 Le Roy, C. and Wrana, J.L. (2005) Clathrin- and non-clathrin-mediated endocytic regulation of cell signalling. *Nature Reviews of Molecular and Cell Biology*, **6** (2), 112–26.

179 Hillaireau, H. and Couvreur, P. (2009) Nanocarriers' entry into the cell: relevance to drug delivery. *Cellular and Molecular Life Sciences*, **66** (17), 2873–96.

180 Huang, S., Chueh, P.J. *et al.* (2009) Disturbed mitotic progression and genome segregation are involved in cell transformation mediated by nano-TiO_2 long-term exposure. *Toxicology and Applied Pharmacology*, **241** (2), 182–94.

181 Cai, R.X., Kubota, Y. *et al.* (1992) Induction of cytotoxicity by photoexcited TiO_2 particles. *Cancer Research*, **52** (8), 2346–8.

182 Zhu, R.R., Wang, S.L. *et al.* (2009) Bio-effects of Nano-TiO_2 on DNA and cellular ultrastructure with different polymorph and size. *Materials Science and Engineering C - Biomimetic and Supramolecular Systems*, **29** (3), 691–6.

183 Seo, J.W., Chung, H. *et al.* (2007) Development of water-soluble single-crystalline TiO_2 nanoparticles for photocatalytic cancer-cell treatment. *Small*, **3** (5), 850–3.