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NANOPARTICLES AND THE INTESTINE:

in vitro and *in vivo* investigations on genotoxic and inflammatory effects

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Der Beginn aller Wissenschaften
ist das Erstaunen,
dass die Dinge sind, wie sie sind

Aristoteles
384 - 322 v.Chr.

Für meine Eltern

Table of contents

CHAPTER 1

1 Introduction

The human intestinal tract is challenged daily by a broad diversity of solid or liquid substances, containing essential nutrients such as carbohydrates, proteins, fats, minerals or vitamins, but also pathogens that easily find their way into this open, tubular system. Throughout a total surface area of 200-300 m^2 (Wells *et al*., 1995) the healthy gut is well prepared to protect the body against possibly harmful substances, viruses, microorganisms or antigens by a tight mucosal lining, a well developed innate immune system and a variety of immune tissue and resident immune cells (Taniguchi *et al*., 2009; Garrett *et al*., 2010).

The adverse effects of nanoparticles (NP) in the lung are a long-standing field of research and the underlying mechanisms are nowadays well understood (Oberdörster *et al*., 2005, Borm *et al*., 2006, Nel *et al*., 2006). In contrast, the use of NP in food and food related products is recently gaining increasing interest. Several types of microparticles are well established as food additives, known to be inert and therefore harmless to the human body. However, the number of nanoparticle-containing food products increases immensely (homepage nanoproject.org), but yet little is known about the cytotoxic or inflammatory potential of these novel materials and their impact on the human intestine. Patients suffering from intestinal diseases that are accompanied by disturbance of the epithelial layer, such as inflammatory bowel diseases, might be even more susceptible to possible harmful NP effects.

1.1 The gastrointestinal tract

1.1.1 The Anatomy of the gastrointestinal tract

The gastrointestinal (GI) tract is a complex system, designed to ensure optimal nutrition and water absorption from ingested food. The human diet consists of a wide range of soluble to macromolecular structures, and the GI tract is challenged to handle not only this broad variety of substances that needs to be prepared for

optimal uptake of the containing nutrients. It also needs to deal with potentially harmful substances that can invade this long, tubular system via our daily food. A first barrier for the invasion of undesirable organisms, such as bacteria, is sterilisation by the acidic milieu of the stomach. The pH ranges from 1.5-2.0 in the fasting state and might rise up to 7.0 after ingestion of a meal. However, re-acidification takes place rapidly via increased secretion of gastric acid, mainly consisting of hydrochloric acid, sodium chloride and pepsin, by the gastric glands (Powell *et al*., 1994; Lindahl *et al*., 1997; Hörter and Dressman, 2001; Kwiecien and Konturek, 2003). Upon increasing motility of the stomach, the meal is transported to the small intestine. Here, the duodenum is the first segment and the regulator of digestion, as it is involved in inhibiting gastric secretion. Furthermore, pancreatic bicarbonate secretion leads to neutralisation of the chyme to a pH ranging from 6.4 to 7.5, depending on the intestinal section. This influences the solubility of certain nutrients and protects the intestinal mucosa (Evans *et al*., 1988; Powell *et al*., 1994; Hörter and Dressman, 2001; Kwiecien and Konturek, 2003). The small intestine further consists of the jejunum and, the most distal section, the ileum. Together with the duodenum, the jejunum is the major site of nutrient absorption. It is responsible for secretion of mucins, lactoferrin, albumin and other factors that are capable of mineral chelation to prevent nutrients, solubilised at the low gastric pH, from polymerising (Powell *et al*., 1994, 1999). Its surface area is amplified immensely due to the presence of crypts, named the crypts of Lieberkühn, and projections called villi. Upon differentiation, the epithelial cells develop microvilli on the apical side, around 3000-7000 per cell in the small intestine (DeSesso and Jacobson, 2001). These villi further enhance the surface area to provide optimal nutrient absorption (Sancho *et al*., 2004). Altogether, the intestine features a surface area of 200-300 m^2 (Wells *et al*., 1995). The anatomy of the small intestine, including the villi and a schematic epithelial cell with microvilli, is illustrated in Figure 1.1.

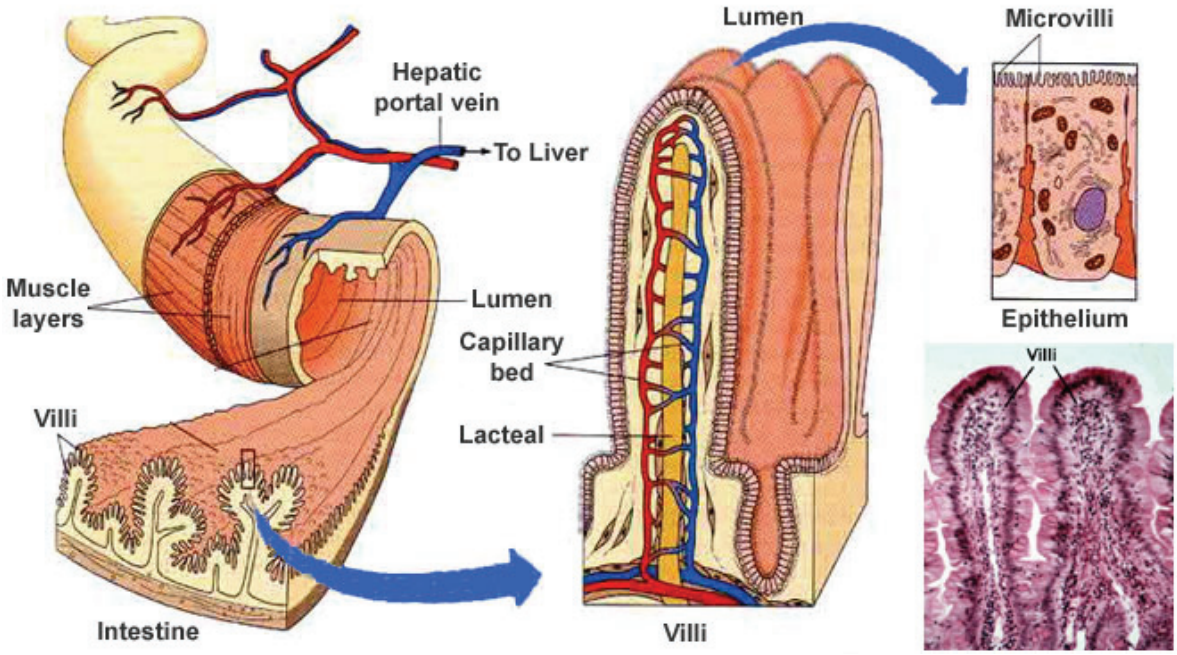

Figure 1.1 Illustration of a cross section through the small intestine with villi and crypts and an exemplary epithelial cell, featuring microvilli.
Taken from the homepage of the University of Colorado

The length of the villi decreases within the ileum. Finally, the colon, or the large intestine, marked by a considerably larger diameter, mainly consists of a smooth surface interspersed with crypts. The colon is divided into several segments, namely the ascending, the transverse, the descending and the sigmoid colon, terminating in the rectum. It is responsible for the storage of indigestible materials and waste products, the maintenance of the water balance as well as Vitamin K absorption. Three types of differentiated cells are present in the colon: enterocytes for nutrient absorption and secretion of hydrolases, goblet cells for mucus secretion, and the rare enteroendocrine cells that secrete several hormones, such as secretin or serotonin (Hollander *et al*., 1977; Christensen, 1985; Powell *et al*., 1994; Sancho *et al*., 2004). Overall, the transit time of ingested food or beverages through the intestine varies between 3 to 8 hours and is driven by movement of the haustra, which are pouches caused by sacculation. Not only propulsive, but also non-propulsive haustra-activity was reported, and ensures homogenisation of the chyme and intensifies the contact with the mucosal surface for optimal nutrient absorption (Ritchie, 1968; Powell *et al*., 1994).

The intestinal epithelial cells underlie constant renewal. Around four to six multipotent Leucine-rich repeat-containing G-protein coupled receptor 5 (Lgr5)-

expressing stem cells are located near (small intestine), or in (large intestine), each crypt bottom and constantly produce proliferating cells. These cells migrate either to the crypt bottom to differentiate to Paneth cells, exclusively found in the small intestine, or along the crypt-villus axis to proliferate several times and finally differentiate to enterocytes, enteroendocrine cells or goblet cells. The life span of a normal epithelial cell amounts to around five days, before it undergoes apoptosis, followed by cell shedding, at the tip of the villi or the surface epithelium in the colon, as depicted in Figure 1.4 (Ramachandran *et al*., 2000; Sancho *et al*., 2004; Sato *et al*., 2009). The epithelium directly overlies a thin layer of lose connective tissue, the lamina propria (LP), which contains blood and lymph capillaries. Underneath the LP lies the submucosa followed by a circular and a longitudinal smooth muscle layer for optimal intestinal motility (DeSesso and Jacobson, 2001). The various layers are shown in Figure 1.2 by a cross-section of a healthy murine colon.

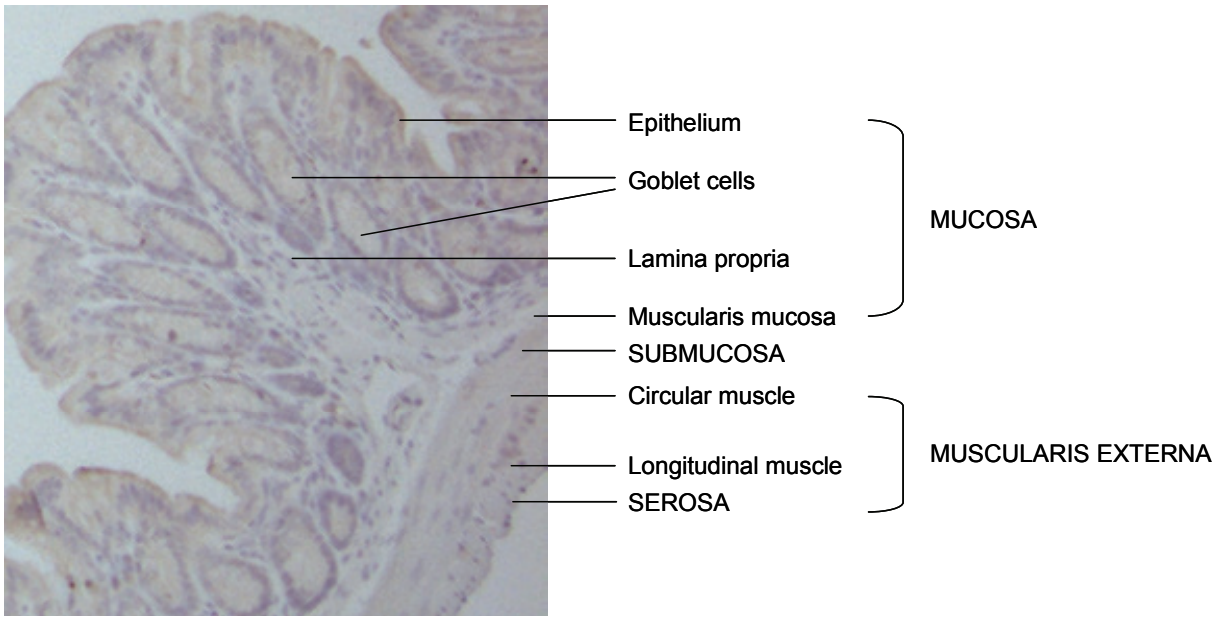

Figure 1.2 Cross section through a healthy murine colon, the cell nuclei were stained using haematoxylin (blue) (The picture is from a 9 weeks old C57BL/6 mouse as used in the study described in Chapter 5)

1.1.2 Caco-2 cells

As an *in vitro* model of the human intestine, Caco-2 cells are widely used in the laboratory. In 1974, they were established from the primary colon tumor, an adenocarcinoma, of a 72-year-old Caucasian man. Upon cell-cell contact, Caco-2 cells accumulate in the G_0/G_1 phase of the cell cycle and then undergo spontaneous enterocytic differentiation at standard culture conditions after reaching confluency (Mariadason *et al*., 2002; Chopra *et al*., 2010). Although isolated from colonic tissue, upon differentiation a loss of the colonic phenotype is detected, and expression of several markers of small intestine-like enterocytes increase, such as expression of villin and various brush-border associated hydrolases (e.g. sucrase, lactase, dipeptidylpeptidase 4 (DPP4), aminopeptidase N, and alkaline phosphatase), displaying their unique property of developing an apical brush border (Chantret *et al*., 1988; Engle *et al*., 1998; Mariadason *et al*., 2002). Caco-2 cells, as shown in Figure 1.3, are also useful tools for studying differentiation processes occurring physiologically upon migration along the crypt–villus axis (Chopra *et al*., 2010). Furthermore, undifferentiated cells might represent an inflamed intestinal epithelium, as hyperproliferative epithelial cells are known to occur during ulcerative colitis e.g. (Huang *et al*., 1997). They also resemble human colon cancer cells in a wide range of their gene expression patterns (Sääf *et al*., 2007). However, next to their various characteristics of small intestinal cells, gene expression patterns of differentiated Caco-2 cells appear similar to normal human colonic epithelial cells (Sääf *et al*., 2007).

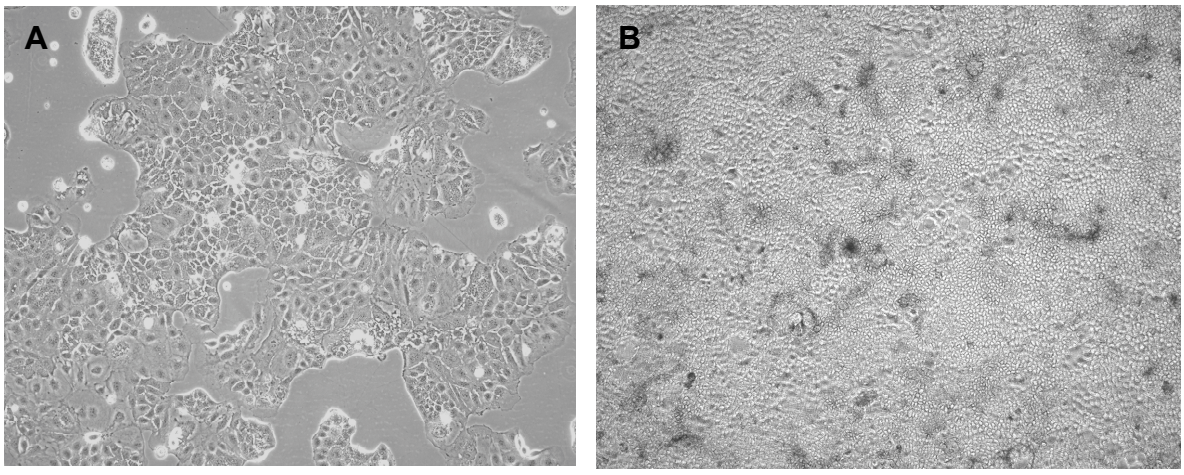

Figure 1.3 Undifferentiated (A, 2 days after seeding) and differentiated (B, 11 days after seeding) Caco-2 cells (100 x magnification)

1.1.3 The immune system in the gastrointestinal tract

The intestinal tract constitutes the largest immune system of the body. While the small intestine is only sparsely populated, the large intestine hosts around 10^{14} microorganisms. The gastrointestinal microbiota is composed of 500-1000 species, including eukarya, archea and bacteria (Taniguchi *et al*., 2009; Wells *et al*., 2010). The human body highly benefits from most of these, as many live in a homeostatic symbiosis with the host. The bacteria are capable of providing nutrient and energy metabolism, influence tissue development and repair, protect against harmful microbiota and are responsible for infant immune development (Leser and Mølbak, 2009; Barbosa and Rescigno, 2010). Upon ingestion however, potential pathogens constantly enter the intestinal lumen. Therefore, the epithelium is permanently challenged with the discrimination between friend and foe. The mucus layer acts as the first barrier of undesired invasion of substances and organisms, and the gut epithelium consists of several immune tissues: the Peyer's patches (PP), which are gut-associated lymphoid tissue (GALT), the lamina propria, mesenteric lymph nodes (MLN) or intraepithelial lymphocytes (IEL) to maintain homeostasis. PP are covered with an epithelial layer rich of microfold (M)-cells. These M-cells are highly specialised for uptake of pathogens or other substances that need to be transported towards the underlying phagocytes (Powell *et al*., 2010), and are shown to be upregulated in the presence of pathogens (Borghesi *et al*., 1999).

The innate immune system of the intestine is well orchestrated by various pathogen-recognising receptors and immune cells. Thus, Toll-like receptors (TLR) play a key role in the innate gut immunity. They recognise so-called pathogen-associated molecular patterns (PAMP), which are common structures on microorganisms. TLR are not only present on the epithelial cells of the intestine, but also on dendritic cells, macrophages or Paneth cells (Taniguchi *et al*., 2009; Wells *et al*., 2010; Garrett *et al*., 2010). Binding of PAMP to TLR can activate, amongst others, nuclear factor-κB (NF-κB), leading to an upregulation of chemokines and inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukins or proteases (Medzhitov *et al*., 1997). Upon cytokine secretion, polymorphonuclear neutrophils (PMN) that amongst others, circulate in the capillaries of the LP, can become activated and migrate towards the centre of inflammation. PMN are potent phagocytes, but they also lead to pathogen destruction upon oxidative bursting and

are for their part capable of pro-inflammatory cytokine production as well. The mechanisms underlying neutrophilic host defence are described in more detail in paragraph 1.1.3.1. Alongside neutrophils, dendritic cells (DC) and macrophages (m^{ϕ}) are the other important team players in intestinal host defence, and both are resident in PP, MLN and the lamina propria, as depicted in Figure 1.4. DC have a key role in antigen-processing and -presenting and are characterised by a high migratory capacity. Upon stimulation, they are able to induce proliferation and differentiation of both naïve and memory T cells, which is known to be uniquely for DC (Ng *et al*., 2010; Garrett *et al*., 2010). Next to DC, m^{ϕ} are able to internalise partially degraded luminal products from absorptive enterocytes as well as apoptotic epithelial cell fragments even without prior activation. Similar to DC, they can directly induce the differentiation of regulatory T cells. m^{ϕ} are widely unreactive upon repeated stimulation with the bacterial compound lipopolysaccharide (LPS) or endotoxins both *in vitro* and *in vivo* when pre-exposed with low doses of the respective substances. Thus, this tolerance development is considered a key role in maintaining homeostasis (West and Heagey, 2002; Ng *et al*., 2010).

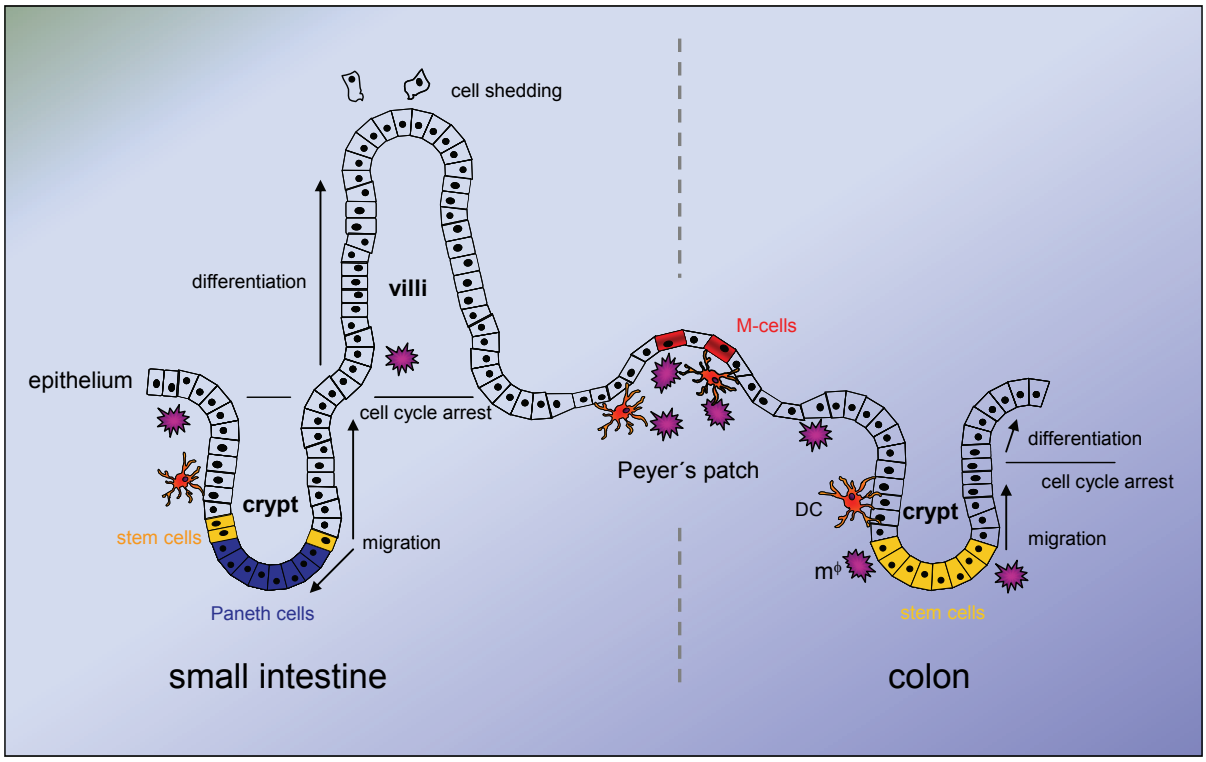

Figure 1.4 Schematic intestinal construction with crypts and villi representing the small intestine, a Peyer´s patch with M-cells, and the colon, solely exhibiting crypts. DC and macrophages are resident in the PP and LP.

1.1.3.1 Neutrophilic granulocytes

Neutrophilic granulocytes, or polymorphonuclear neutrophils (PMN), are counted among the most important phagocytes, and are in the first line of host defence. They circulate in the blood and have a life span of only 7-12 hours. Therefore, around 1-2 x 10^{11} PMN are produced daily in the human body. Neutrophils account for about 50-70 % of all blood leukocytes in the human body (Freitas *et al*., 2009; Wessels *et al*., 2010). Upon an inflammatory event, neutrophil production is upregulated, and its lifetime increases as a response to platelet activating factor (PAF), granulocyte-colony stimulating factor (G-CSF) or various pro-inflammatory cytokines, such as interleukin-1β (IL-1β) (Wessels *et al*., 2010). The crucial role of PMN in the human immune system is long known. In 1968, Baehner and Karnovsky described a link between a reduced PMN activity and the development of chronic granulomatous disease (CGD) (Baehner and Karnovsky, 1968). The important peroxidase-mediated bactericidal role of PMN and the formation of superoxide radicals as one of the main bactericidal mechanisms was already described more than 30 years ago (Klebanoff, 1967; Babior et al., 1973; Klebanoff and Rosen, 1978). A strong negative correlation between the chemotactic ability of PMN and patients with increased bacterial sepsis was demonstrated (Christou and Meakins, 1979), and clinical morbidity from infections is clearly increased with a reduced number of circulating PMN in the blood (Nauseef, 2007).

Various endothelial adhesion molecules, such as the intercellular adhesion molecule 1 (ICAM-1), mediate neutrophil adhesion to endothelial cells. ICAM-1 expression on the luminal surface of the capillary is increased during inflammation, and interacts with β2 integrin, which is expressed on the surface of PMN. Subsequent to adhesion, neutrophils begin to migrate across the endothelium and towards the centre of inflammation (Drost and MacNee, 2002; Wang *et al*., 2004). Interleukin-8 (IL-8) is known to be one of the most potent chemoattractants for the recruitment and activation of neutrophils into various organs (e.g. lung, intestine), and binds to the human CXC chemokine receptor 1 (CXCR1) and CXC chemokine receptor 2 (CXCR2) on the surface of the PMN (Kunkel *et al*., 1991; Mitsuyama *et al*., 1994; Buanne *et al*., 2007). IL-8 is mainly regulated by the NF-κB signalling pathway (Wang *et al*., 2009). Upon incubation with IL-8, increased PMN deformability and upregulation of the adhesion receptor CD11b was observed (Drost and MacNee,

2002). The neutrophilic cytosol contains granules that are filled with a variety of proteins, such as defensins, bactericidal-permeability-increasing protein, proteases (e.g. elastase, cathepsins), and myeloperoxidase (MPO) that consumes hydrogen peroxide (H_2O_2) and generates hypochlorous acid (HOCl), the most bactericidal oxidant that is produced by PMN (Hampton *et al*., 1998; Nauseef, 2007; Freitas *et al*., 2009). Activated neutrophils are capable of producing a variety of pro-inflammatory cytokines, e.g. IL-1β, IL-6, IL-12 and IL-23, and transport internalised pathogens to lymph nodes to support macrophages and DC in antigen presentation (Silva, 2010). Also, contact with pathogens results not only in phagocytosis, but also in the so-called oxidative burst, marked by an increased consumption of molecular oxygen and resulting production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Babior, 2000). ROS generation by PMN is catalysed by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, a membrane-bound enzyme complex that can be stimulated *ex vivo* by phorbol myristate acetate (PMA) (Suzuki and Lehrer, 1980). The NADPH oxidase complex consists of various subunits that are located in the cytosol and in the membranes of intracellular vesicles and organelles. The cytosolic subunits migrate towards the membrane bound subunits upon activation, to bind and configure the active enzyme complex (Knaapen *et al*., 2006).

In PMN, the catalytic subunit of the predominantly expressed NADPH oxidase is NOX2 (gp91phox), and the active enzyme complex is completed by the regulatory subunit p22phox, the cytosolic subunits p40phox, p47phox, and p67phox and the guanosine triphosphate hydrolase (GTPase) Rac. Moreover, several human homologs of NOX2 are present in nonphagocytic cells, such as NOX1, NOX3, NOX4, NOX5, and the dual oxidases Duox1 and Duox2 (Frey *et al*., 2009).

The crucial role of the NADPH oxidase *in vivo* is shown in mice deficient in specific subunits of this enzyme complex as well as in chronic granulomatous disease (CGD) patients; animals lacking a functional NOX2 have been shown to rapidly develop spontaneous multi-organ bacterial and fungal infections early in life and their life-expectancy has been reduced to a few months only, even when raised under specific pathogen-free (SPF) conditions (Ostanin *et al*., 2007). p47phox deficient mice develop CGD, a genetic childhood disease that is marked by non-functional phagocytes, thus leading to severe infections and early death (Jackson *et al*., 1995), and a lack of either NOX2, p22phox, p47phox or p67phox is linked to CGD development in humans (Krause and Bedard, 2008; Matute *et al*., 2009).

1.1.4 Oxidative stress, free radicals and the formation of reactive oxygen or nitrogen species

The concept of oxidative stress describes the imbalance between oxidants and antioxidants in favour of the oxidants, which might result in cell or tissue damage (Sies, 1997). In healthy conditions, formation of oxidants is balanced by the presence of a variety of antioxidants. Oxidants can be generated by the reduction of molecular oxygen to water, leading to the formation of the intermediates superoxide anion radical ($O_2^{\cdot -}$), H_2O_2 or the hydroxyl radical ($HO^{\cdot}$). Furthermore, free radicals in general are considered oxidants. Free radicals are molecules or molecular fragments that possess an unpaired electron, which is conventionally indicated by a superscript bold dot and responsible for the high reactivity and the paramagnetism of the radical. Free radicals can be charged positively or negatively or be electrically neutral. They are formed from a normal molecule by either homolytic cleavage of a covalent bond, requiring high energy input (e.g. ultrasound, UV-radiation), or addition or loss of a single electron. In biological processes, the electron transfer plays a major role in the generation of free radicals, as it can be initiated by enzymatic reactions (Slater, 1984; Cheeseman and Slater, 1993).

In healthy conditions, oxidants formation is scavenged by various antioxidant mechanisms. Some of the main antioxidant enzymes are superoxide dismutase (SOD) or catalase. Moreover, an important intracellular antioxidant is glutathione (GSH; L-γ-glutamyl-L-cysteinyl-glycine), a tripeptide containing a thiol group. Upon reaction with an oxidant such as H_2O_2 GSH is oxidised to glutathione disulfide (GSSG), and H_2O_2 is reduced to water:

$$H_2O_2 + 2\ GSH \rightarrow 2\ H_2O + GSSG$$

Subsequently, GSH is recovered by the GSSG reductase (Sies, 1997, 1999). Determination of the changes in intracellular GSH content and the GSH/GSSG ratio is a widely used marker for cellular response to oxidative stress in particle toxicity (Li *et al*., 2008).

ROS but also reactive nitrogen species (RNS) formation may occur via a multitude of chemical reactions (Barthel and Klotz, 2005; Knaapen *et al*., 2006; Freitas *et al*., 2009):

$O_2^{\cdot -}$ is a short-living radical and directly generated upon reaction of oxygen with NADPH oxidase:

$$NADPH + 2O_2 \rightarrow NADP^+ + 2O_2^{\cdot -} + 2H^+$$

Either spontaneously or through catalysis by SOD $O_2^{\cdot -}$ is dismutated into H_2O_2, which is relatively stable and capable of intracellular diffusion:

$$2\ O_2^{\cdot -} + 2H^+ \rightarrow O_2 + H_2O_2$$

In PMN, the highly abundant enzyme MPO catalyses the formation of hypochlorous acid (HOCl), a highly bactericidal oxidant, and directly consumes up to 70 % of the formed H_2O_2:

$$Cl^- + H_2O_2 \rightarrow HOCl + OH^-$$

The hydroxyl radical ($HO^{\cdot}$) is a highly reactive and very short living radical. It is formed according to the Haber Weiss-reaction, which includes the Fenton reaction, and is catalysed by iron:

$$O_2^{\cdot -} + Fe(III) \rightarrow Fe(II) + O_2$$

$$H_2O_2 + Fe(II) \rightarrow Fe(III) + HO^{\cdot} + HO^- \qquad \text{(Fenton reaction)}$$

Furthermore, neutrophils and, more importantly macrophages, are capable of producing reactive nitrogen species (RNS), especially nitric oxide ($NO^{\cdot}$), via the inducible nitric oxide synthase (iNOS) by consumption of NADPH and conversion of L-arginine to L-citrulline:

$$L\text{-arginine} + O_2 + NADPH \rightarrow NO^{\cdot} + L\text{-citrulline} + NADP^+$$

The resulting $NO^{\cdot}$ can further react with $O_2^{\cdot -}$ to generate the highly mutagenic and cytotoxic peroxynitrite anion ($ONOO^-$):

$$NO^{\cdot} + O_2^{\cdot -} \rightarrow ONOO^-$$

1.1.5 Ulcerative colitis

A disturbance of the well balanced immune system of the intestine can lead to the development of inflammatory bowel diseases (IBD), such as ulcerative colitis (UC) or Crohn´s disease (CD). CD may affect parts of the whole gastro-intestinal system, including the oral cavity, whereas UC is reduced to the large intestine, starting from the rectum and continuing up the colon. Both diseases are characterised by periodic exacerbation and remission of the symptoms. Patients suffering from CD or UC experience chronic diarrhoea, sometimes with blood, weight loss and abdominal pain (Fatahzadeh, 2009)

UC mainly occurs in early adulthood and again between the ages of 50-70 years. Environmental, hereditary or immunological factors play a role in the outbreak and progression of the disease. Therapeutic approaches nowadays involve treatment with steroids, immunosuppressive drugs or heparin and, in severe cases, a colorectomy. UC is marked by crypt abscesses, ulceration of the colonic epithelium and epithelial barrier dysfunction, accompanied by neutrophilic infiltration. A pronounced and long-lasting UC can also contribute to an increased risk for colorectal cancer (Itzkowitz and Yio, 2004; Wirtz and Neurath, 2007; Fatahzadeh, 2009; Wang *et al*., 2009). The main cause for exacerbation of UC is discussed to be the imbalance of the intestinal immune system, marked by continuous migration of activated lymphocytes, granulocytes and macrophages into the mucosa. Increased levels of inflammatory cytokines and chemokines are found in the tissue of UC patients. IL-8 levels are found significantly increased in colitic tissue. Further cytokines that are upregulated in UC are IL-1β, IL-6 or TNF-α e.g. T-cell response was found to be mainly type 2 helper cell (Th2) dominant in UC, marked by an increase in IL-5 and IL-13 secretion, in contrast to CD, where T-cell response appeared to be mainly Th1 dominant (Mazzucchelli *et al*., 1994; Mitsuyama *et al*., 1994; Rogler and Andus, 1998; MacDermott, 1999; Shih and Targan, 2008).

Various animal models are nowadays used to induce experimental colitis. For example, intestinal inflammation can occur by disturbance of the epithelial barrier. Feeding with the widely used dextrane sulphate sodium (DSS), a substance that is directly toxic to epithelial cells, leads to induction of colitic symptoms, such as bloody diarrhoea, ulcerations and neutrophil infiltration. Multi drug resistant 1 (MDR1) gene deficient mice are known to develop spontaneous bowel inflammation triggered by

the bacterial flora. In addition to this, animals with a disturbed immune response, such as signal transducer and activator of transcription 3 or 4 (STAT3 / STAT4) gene deficient mice (Gaudio *et al*., 1999; Wirtz and Neurath, 2007), develop spontaneous enterocolitis.

1.1.5.1 The role of neutrophils in ulcerative colitis

Neutrophils play a major role in the exacerbation of ulcerative colitis. The number of neutrophils present in colitic tissue correlates directly to the amount of secreted IL-8 and the grade of local inflammation (Mazzucchelli *et al*., 1994; Mitsuyama *et al*., 1994). Neutrophilic bursting is known to directly leading to an increased production of ROS (Knaapen *et al*., 2006), which in turn is closely related to the amplification of the inflammatory response in UC (Babbs, 1992). Polymorphisms in the ICAM-1 encoding gene as well as elevated levels of ICAM-1 were found in tissues of UC patients (Dippold *et al*., 1993; Vainer, 2005; Naito *et al*., 2007). Neutrophil infiltration is also known to play a crucial role in DSS induced experimental colitis. DSS-treated $CXCR2^{-/-}$ mice did not exhibit PMN infiltration into the mucosa, and the clinical colitis conditions were noticeably improved in comparison to wild type (WT) mice (Buanne *et al*., 2007). Specific depletion of neutrophils in a DSS-colitic rat model, using the monoclonal antibody RP-3, reduced the colitic symptoms significantly (Natsui *et al*., 1997), whereas this attenuating effect seems to be mainly dependent on the depletion of iNOS and not on a defective NADPH oxidase (Krieglstein *et al*., 2001).

1.1.6 Genomic instability and carcinogenesis

Colorectal cancer (CRC) mainly arises from genomic instability such as chromosomal instability (CIN) or microsatellite instability (MSI). Aneuploidy, i.e. an abnormal number of chromosomes, as well as loss of heterozygosity arise from CIN. This can lead to large-scale chromosomal losses and therefore also loss or mutation of essential tumour suppressor genes such as p53 or adenomatous polyposis coli (APC), a key suppressor in the human colon. CRC in the colitic colon is evident by the presence of multifocal areas, unlike sporadic colorectal carcinoma (SCC), where dysplastic lesions usually develop in one or two focal areas. Additionally, these areas

are found to arise within tissue areas that are already affected by colonic inflammation (Boland *et al*., 2005; Itzkowitz *et al*., 2004).

The epithelial cells in active UC are known to be hyperproliferative, marked by increased apoptosis and an accelerated cell turnover that might contribute to carcinogenesis (Arai *et al*., 1999). Further, the ongoing inflammation, accompanied by constant influx of activated phagocytes and permanent ROS/RNS generation, is an important risk factor for cancer development. Thus, DNA damage can be induced by oxidation, nitration, depurination, methylation and deamination (Knaapen *et al*., 2006). The hydroxyl radical is the most reactive radical that directly interacts with the DNA by H-atom abstraction, to generate manifold products with all four bases (Pryor, 1988). One of the most important and well-studied oxidative lesions generated by $HO^{\cdot}$ is 8-hydroxydeoxyguanosine (8-OHdG), generated by oxidation of guanine in DNA at C8, as shown in Figure 1.5.

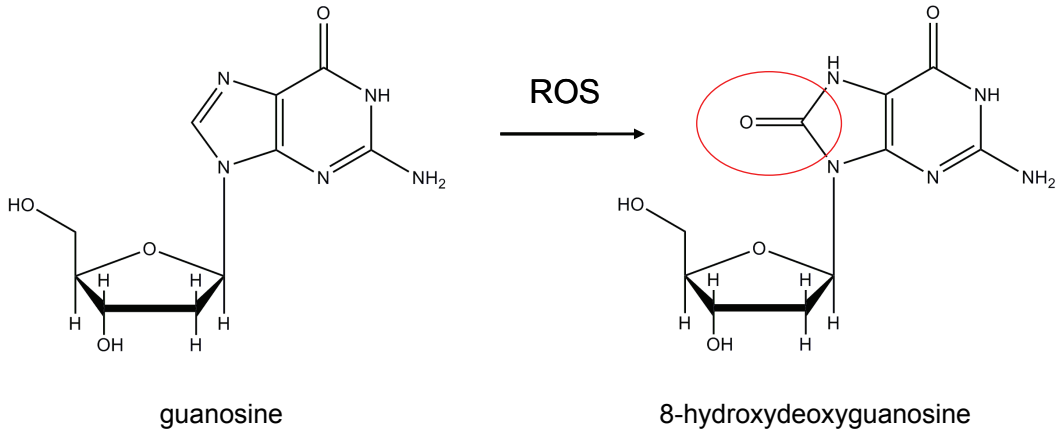

Figure 1.5 ROS-induced formation of 8-hydroxydeoxyguanosine (8-OHdG) from guanosine

It is found to be elevated in colonic tissues of patients with long-lasting UC (Itzkowitz *et al*., 2004) and well known for it's mutagenic potential, as dAMP, instead of dCMP, might be incorporated wrongly opposite 8-OHdG (Shibutani *et al*., 1991). The analysis of alterations in 8-OHdG sites is discussed to find use as a marker of oxidative stress in carcinogenesis (Kasai, 1997). Important enzymes for repair of oxidative base damage are 8-oxoguanine DNA glycosylase (OGG1) and its bacterial homologue formamidopyrimidine glycosylase (Fpg) that has found use in the laboratory to specifically detect and quantify oxidative lesions (Pflaum *et al*., 1997; Burrows and Muller, 1998).

1.2 Nanoparticles

The use of engineered nanoparticles (ENP) is a rapidly growing economic field. These particularly small materials are characterised by a size range between 1 and 100 nm, with one or more external dimensions in the nanoscale (Stone *et al*., 2009). Thus, the classification as a "nano" structure does not only include spherical particles, but also nanofibres, single- and multiwalled nanotubes, nanowires, nanorings, nanostructured surfaces and composite materials (Oberdörster *et al*., 2005; Borm *et al*., 2006). Not only size is a main feature of ENP, but also their large specific surface area (SSA), that increases exponentially with decreasing primary particle size (Oberdörster *et al*., 2005). It is well known that NP tend to form aggregates by fusion of primary particles, and agglomerates, defined as undispersed clusters of aggregates during their production or generation as well as in suspension (Donaldson *et al*., 2005; Zhang *et al*., 2008). Particles are commonly discriminated between particles with inherent toxic activity, such as welding fume, quartz dust or hard-metal dusts, and poorly soluble low toxicity particles (PSP) such as titanium dioxide (TiO_2) or carbon black (CB) (Borm *et al*., 2004).

Nanotechnology will gain significant importance in the near future, in particular in the fields of optics, fabrics, displays and pigments, as well as in industries such as space, automotive, and microelectronics. In recent years, increasing research is focused on using ENP in medicine and pharmacology, for cosmetics, food or food related products (Oberdörster *et al*., 2005; Borm *et al*., 2006; Chaudhry *et al*., 2008; Geiser and Kreyling, 2010). For example, nano-enabled products worth $147 billion were produced in 2007, whereas the sales volume of nano-containing products is expected to be around $3.1 trillion in 2015 (luxresearchinc.com).

Humans can be exposed to ENP intentionally by use of new drugs to deliver medical agents to specific target organs or by the use of quantum dots for *in vivo* targeting of tumour cells e.g. (Gao *et al*., 2004; Mühlfeld *et al*., 2008; Matsusaki and Akashi, 2009). Furthermore, various functional foods are launched regularly that promise enhanced nutrient absorption, or general increased well-being, by consuming nanoparticle-enriched products (homepage nanotechproject.org).

Unintentional uptake of ENP often occurs at the workplace simply by inhalation, and potentially through the skin by use of novel cosmetic products. Food and toothpaste can contain ENP, thus ingestion is another important route of

nanoparticle exposure, and the impact on human health is still under discussion. NP exposure of the brain is another very recent field of interest. There is evidence that NP might be taken up by the olfactory bulb upon inhalation and nasal deposition, and subsequently be transported directly to the brain (Hautot *et al*., 2003; Oberdörster *et al*., 2005). Also, systemic NP uptake can be an issue, either upon inhalation, ingestion or uptake by the skin, and high doses of ingested NP are already found to cause DNA damage in blood and bone marrow cells, suggesting that there may be significant translocation (Trouiller *et al*., 2009). Possible routes of NP uptake are illustrated in Figure 1.6.

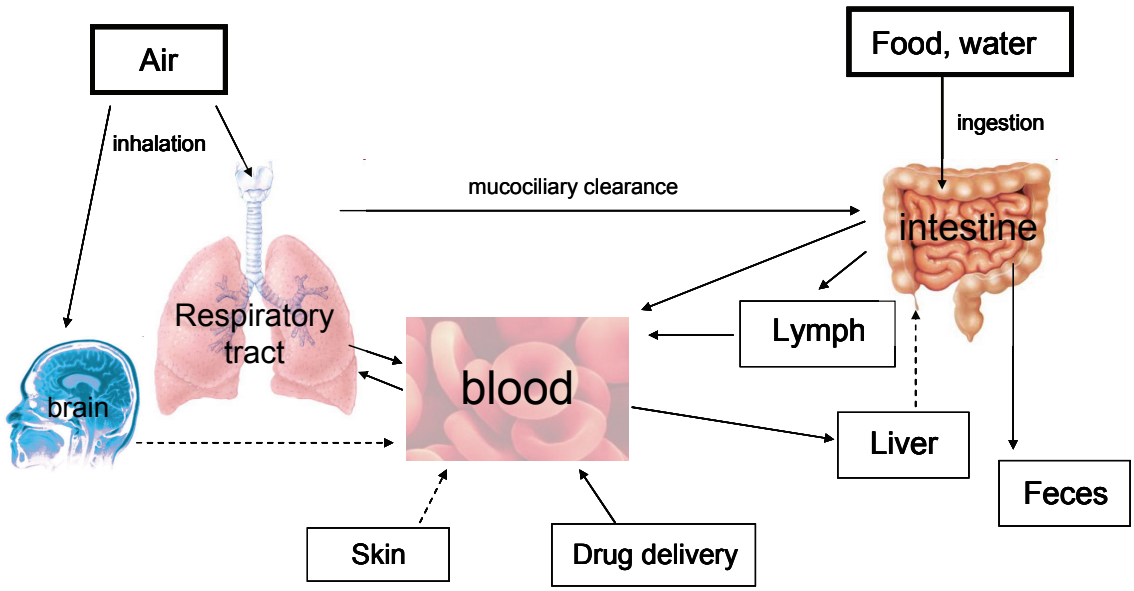

Figure 1.6 Possible routes of human NP-uptake, allocation and excretion. Modified from Oberdörster *et al*., 2005

1.2.1 Ambient particulate matter

Not only intentionally produced nanoparticles are a source for human exposure, but also a wide range of particulate matter (PM) challenge human health and the lung tissue is the first organ to get in contact with these materials. Forest fires and volcanoes, but also viruses and pollen are considered natural PM sources to which humans where constantly exposed throughout their evolution. In contrast, combustion or engine exhausts, power plants or welding applications are examples for anthropogenic sources for PM. Ambient particulate matter is divided into different categories in accordance to their aerodynamic diameters, namely the diameter of a

spherical particle with a density of 1 g/cm^3. PM_{10} is defined as having coarse, fine and ultrafine particles with aerodynamic diameters between 2.5-10 µm, hence $PM_{2.5}$ are particles < 2.5 µm, and $PM_{0.1}$ are NP < 0.1 µm. (Oberdörster *et al*., 2005; Mühlfeld *et al*., 2008).

1.2.2 Nanoparticles and the lung

Occupational exposure to inhaled nanoparticles and the discovery of its link to fibrosis originated the first nanotoxicological inquiries (Borm, 2002). Epidemiological studies with coal miners and silica-exposed workers further revealed a correlation between fibrosis and lung cancer (Borm *et al*., 2004). Nowadays, inhaled particles are known to play an important role not only in fibrosis or cancer development, but also in chronic obstructive pulmonary disease (COPD) and asthma. PM_{10} are further discussed to exacerbate cardiovascular effects in predisposed individuals (Donaldson *et al*., 2000; 2009). The mechanisms underlying toxicological NP effects and their impact on carcinogenicity and cancer development in the lung are partly understood. Nevertheless, particle hazard is often hard to predict, as their numerous properties (e.g. size, specific surface area (SSA), chemical composition, solubility) each might influence their toxic potential. However, studies on the lung and lung epithelial cells have shown that carbon black and TiO_2 particles of the same composition and crystalline structure display elevated toxicity and pro-inflammatory potential with an increasing SSA (Oberdörster *et al*., 2005; Monteiller *et al*., 2007; Johnston *et al*., 2009).

According to their size and mass, inhaled ENP or PM tend to invade and deposit differently into the three major regions of the respiratory tract. Upon nose breathing and during rest, a mathematical model predicts deposition of particles > 1000 nm or < 1 nm mainly in the nasal, pharyngeal and laryngeal region. Particles of a size between 1-100 nm tend to deposit in the tracheobronchial region but also in the alveoli (Oberdörster *et al*., 2005). Clearance of particles in the lung can occur either by chemical processes, if the material is biodegradable, or by physical translocation, such as mucociliary clearance, whereupon after swallowing these particles finally reach the gastrointestinal tract (Oberdörster *et al*., 2005).

Different mechanisms of cellular NP uptake are currently discussed. Amongst others, phagocytosis, macropinocytosis, clathrin- and nonclathrin-mediated endocytosis or diffusion can play a role. However, cellular uptake is dependent on particle size or chemical composition as well as the exposed cell type (Unfried *et al*., 2007).

1.2.2.1 Mechanisms of nanoparticle toxicity

The main contributor to NP toxicity is considered to be their ability to induce ROS (Donaldson *et al*., 2005; Oberdörster *et al*., 2005). Molecular oxygen can be converted into ROS directly on the surface of metal-containing particles or by particle contaminants such as iron, via the Fenton-like reaction (paragraph 1.1.4). Indirectly, and upon single electron transfer, this may be catalysed by the NADPH oxidase, or it may occur as a side reaction of electron transport along the mitochondrial respiratory chain. Oxygen can react to $O_2^{\cdot-}$ resulting in formation of H_2O_2 (paragraph 1.1.4) (Unfried *et al*., 2007). Likewise, photoactivation leads to the formation of singlet oxygen ($^1O_2$) followed by transformation into $O_2^{\cdot-}$, and is discussed to play a role for NP-containing cosmetics as sun creams and thus NP toxicity to the skin. But also in the laboratory, photoactivation can play a role in inducing ROS by particles such as TiO_2, which might lead to positive outcomes in *in vitro* toxicology tests. Obviously however, it is not expected to play a role *in vivo* when exposed to inner organs such as lung or intestine (Nakagawa *et al*., 1997; Sayes *et al*., 2006; Johnston *et al*., 2009).

Low amounts of ROS can induce activation of various signalling cascades within the cell, such as the phosphoinositide 3'-kinase (PI 3-kinase)/protein kinase B (PKB) pathway (Barthel and Klotz, 2005). Excessive ROS formation however can induce genotoxicity, leading to DNA strand breakage, oxidative lesions, micronucleus formation or sister chromatid exchanges, and thus induce carcinogenicity (Schins and Knaapen, 2007). DNA damage and oxidative stress was found *in vitro* and *in vivo* after exposure to both PM (Risom *et al*., 2003; Gallagher *et al*., 2003; Albrecht *et al*., 2005) and ENP (Johnston *et al*., 2000; Gurr *et al*., 2005; Falck *et al*., 2009).

Next to the primary particle toxicity described above, inflammation plays an important role in particle induced lung injury and is referred to as secondary particle

toxicity when the inflammatory processes overwhelm the antioxidant and DNA damage repair capacities in the lung (Schins and Knaapen, 2007; Figure 1.7). Resident alveolar macrophages, that usually phagocyte microorganisms or particles will be activated when overwhelmed by the amount of invading pathogens and release inflammatory cytokines and chemokines. Consequently, neutrophils are recruited and activated, leading to enhanced generation of reactive oxygen and nitrogen species by mechanisms as described above, thus featuring a high mutagenic potential by induction of genotoxicity (Driscoll *et al*., 1997; Knaapen *et al*., 1999; 2006).

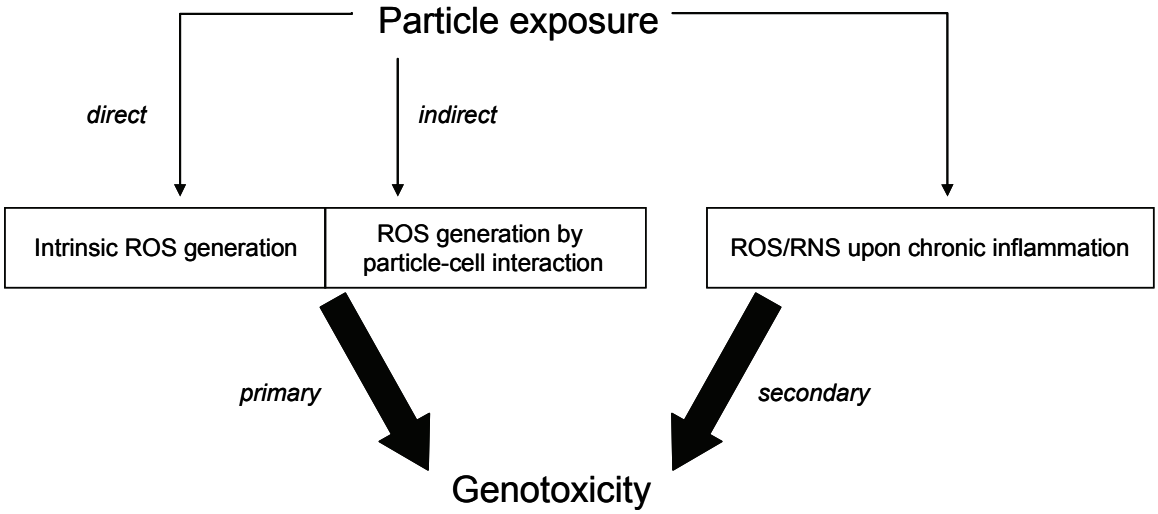

Figure 1.7 Primary versus secondary particle induced genotoxicity mechanisms

1.2.3 Nanoparticles and the gut

Inorganic microparticles have a long-standing use in the food industry. They are appreciated for being inert within the human body and are approved as food additives, finding use in both conventional and functional food (homepage food.gov.uk). Several materials, their E numbers and applications are listed in Table 1.1.

E Number	Material	Application
171	Titanium dioxide	Colouring
172	Iron oxides / hydroxides	Colouring
173	Aluminium	Colouring
174	Silver	Colouring
175	Gold	Colouring
529	Calcium oxide	Acidity regulator
530	Magnesium oxide	Acidity regulator / Release agent
551	Silicon dioxide	Anticaking agent

Table 1.1 Metals and metal oxides used as food additives (homepage food.gov.uk)

Nowadays, the use of ENP for food and food related products (food packaging/wraps, food storage containers, refrigerators, kitchenware e.g.) gains increasing attention (homepage nanotechproject.org). The reduction of particle size involves changing of the physico-chemical properties. Consequently, ENP often possess different colours, solubility, or thermal behaviour compared to their microparticulate equivalents (Tiede *et al*., 2008). Titanium dioxide (TiO_2) for example is popular as a whitener when utilised in the micro-size range and was already approved as a food additive by the FDA in 1966. Food products may contain up to 1 % of TiO_2 by weight (homepage cfsan.fda.gov). Nano-sized TiO_2 however has a different refractive index leading to loss of its white colour and therefore allowing it to be applied as transparent films. This can either prevent confectionary products from melting, improve shelf life when applied to food directly (Chaudhry *et al*., 2008; Mars Inc.'s US Patent US 5741505), or act as an oxygen barrier or UV absorber in (plastics) food packaging (Chaudhry *et al*., 2008; Arora and Padua, 2010). Taking into account that the first official approval already took place more than 40 years ago, and that nanotechnology was then a relatively young science, it is not surprising that the size of food additives was originally not regulated. However, international efforts have been made recently to address this issue and if deemed necessary, regulations were put in place to make up for previous omissions (homepage fda.gov; EFSA scientific opinion 2009).

Besides TiO_2, various other metal or metal oxide nanoparticles are already used in food packaging. Silver (Ag), zinc oxide (ZnO) or magnesium oxide (MgO)

nanoparticles are potent antimicrobial agents, whereas silicon dioxide (SiO_2), alumina (Al_2O_3), iron oxides (Fe_3O_4, Fe_2O_3) and, again, ZnO, are appreciated as UV absorbers in plastics that prevent UV-degradation of the food (Chaudhry *et al*., 2008; Bouwmeester *et al*., 2009). Several of these materials however can also be directly applied to functional or conventional food, such as TiO_2 or MgO in confectionary products, SiO_2 in slim shakes, as fluxing agents or for coatings, or selenium in a so-called nano-tea (Chaudhry *et al*., 2008; Schmid and Riediker, 2008; homepage nanotechproject.org). In this case, one needs to distinguish between products that might contain ENP for improvement of the product properties, and that nowadays do not need to be specifically labelled as “nano-containing food”. This may lead to unintentional ingestion. Functional food in turn is promoted especially for its nano-contents. Therefore, the containing nanomaterials are ingested intentionally.

Besides, great effort is made to develop organic and biodegradable nanoparticles that can be used in medical approaches, for example for targeted drug delivery (Lamprecht *et al*., 2001; Bouwmeester *et al*., 2009). Furthermore, self-assembling nanotubes can be made of milk proteins, and liposomal vesicles are used as nutritional carriers (Das *et al*., 2009). Upon their biodegradability, these nanomaterials are generally considered as safe to use in food and are regarded to be less contrary in comparison to metal and metal oxide particles that are often biopersistent.

Taken together the several sources of ENP, a daily ingestion of 10^{12}-10^{14} particles per day is suggested, with an increasing tendency (Lomer *et al*., 2001; 2004). Currently there are major research initiatives including EU projects aiming to improve detection of NP in food and in the GI tract in order to further improve exposure assessment aspects (e.g. Nanolyse project, Inlivetox project; homepage European commission).

1.2.3.1 Intestinal uptake of engineered nanoparticles

At present, the actual amount of nanoparticulate uptake within the intestine is still unclear and dependent on the applied material as well as their unique properties (size, surface structure, chemical composition e.g.). Generally, intestinal particle-absorption appears to be below 10% (Jani *et al*., 1994; Hillery et al., 1994), whereas

the uptake of small particles (~110-120 nm) within the small intestine *in situ* is shown to be 15-250 fold higher in comparison to larger particles (~510-550 nm, Desai *et al*., 1996). The M-cells of the intestinal PP are specialised for absorption of macromolecules and particles (Powell *et al*., 2010). Nonetheless, after ingestion of 60 nm polystyrene particles, only 60 % of the absorbed materials could be recovered in the PP, whereas 40 % were found in non-lymphoid tissue of Sprague-Dawley rats after 5 days of gavaging (Hillery *et al*., 1994). This suggests that next to absorption via the M-cells additional uptake mechanisms play a role in the intestinal epithelium. Epithelial particle uptake can occur via manifold mechanisms such as phagocytosis, macropinocytosis, clathrin- and nonclathrin-mediated endocytosis or diffusion (Unfried *et al*., 2007), depending on both the type of material and the exposed cell type. Also, persorption was described as a mechanism for particle translocation and that it can occur upon cell shedding, which might be associated with formation of epithelial “gaps”. However, this mechanism seems to be of minor importance (Volkheimer, 1974; Powell *et al*., 2010). Nonetheless, nanoparticles tend to accumulate at the site of inflammation in IBD and can be efficiently taken up by macrophages within the affected tissue (Ashgar and Chandran, 2006).

1.2.3.2 Toxicity of ingested nanoparticles

Even though the uptake of nanoparticles into the gastrointestinal tract appears low, the health risks of ingested ENP are still unclear. Not much data is currently available that face the toxic or inflammatory potential of these novel materials, and very few studies deal with their impact on existing diseases or their outbreak, such as IBD. Powell *et al*. analysed the effect of a microparticulate (200 nm) TiO_2-LPS conjugate to stimulate peripheral blood mononuclear cells from healthy controls and CD patients (Powell *et al*., 2000). They found the strongest increase of IL-1 secretion after incubation with the conjugate, compared to TiO_2 or LPS alone, with a slightly higher response in cells from CD patients. Contrasting however, stimulation of colonic explants of both healthy controls and CD patients resulted in comparable IL-1 levels and the response in general appeared low. Ambiguous outcomes were also found in patients studies when IBD patients received a low particle diet. A pilot study pointed towards a positive effect as promotion of disease remission by avoiding

particle-containing food, whereas these findings could not be confirmed in a follow-up study (Lomer *et al*., 2001; 2004).

Only very few *in vitro* approaches are nowadays published that determine metal or metal oxide particle toxicity in intestinal cell lines. Nano-vanadium oxide was found cytotoxic to Caco-2 cells (Rhoads *et al*., 2010). Furthermore, the cytotoxic effects of nano-ZnO and its potential to induce oxidative stress in human colon carcinoma LoVo cells (De Berardis *et al*., 2010) and human colon-derived RKO cells (Moos *et al*., 2010) were recently confirmed.

To date, only few *in vivo* studies have addressed possible DNA damage and toxicity of NP in mice and rats. Dybdahl and co-workers exposed rats orally to diesel exhaust particles for 21 days. DNA strand breaks and oxidative stress were found both locally in colon epithelial cells and systemically in the liver (Dybdahl *et al*., 2003). Furthermore, oral administration of single high doses (5 g Zn/kg) of nano-ZnO to mice were found to induced death in two cases as well as growth retardation in the first days after treatment. Various systemic effects were reported, such as renal damage and anaemia (Wang *et al*., 2006). By gavaging of a single high dose (5 g/kg) of different sized nano-TiO_2 hepatic injury as well as Ti distribution into various tissues such as liver or kidneys was further observed (Wang *et al*., 2007). Moreover, the study by Trouiller *et al*. revealed systemic DNA damage after ingestion of TiO_2. After 5 days oral exposure to high particle concentrations, dose dependent double strand breaks could be detected in bone marrow by measure of γ-H2AX foci. By using the alkaline comet assay for detection of double and single strand breaks and/or strand breaks induced by alkali-labile sites, increasing damage could be found in white blood cells from peripheral blood after incubation with the highest dose (500 mg/kg). Moreover, blood-derived erythrocytes revealed an increased number of micronuclei at high dose treatment, and increased levels of 8-OHdG, a marker for oxidative DNA damage, were detected in the liver (Trouiller *et al*., 2009).

Overall, knowledge of possible adverse effects of ingested ENP and their impact on intestinal health is very low in the research community, and more research is urgently needed to obtain a better understanding of possible adverse effects and the underlying mechanisms.

1.3 Aim of the thesis

The toxicological mechanisms of nanoparticles (NP) in the respiratory tract are reasonably well understood. Many metal oxide nanoparticles (e.g. TiO_2) as well as carbonaceous nanoparticles derived from combustion processes, such as diesel engine exhaust particles and carbon black are known to be capable of generating reactive oxygen species that can induce intracellular oxidative stress (Oberdörster *et al*., 2005; Donaldson *et al*., 2005).

This in turn may lead to cytotoxicity or induction of pro-inflammatory cytokines. Oxidative stress and inflammation in the lung are implicated in various lung diseases including COPD and asthma. Particle-induced DNA damage may occur either directly, referred to as primary genotoxicity, or in an indirect way by activation of phagocytes such as neutrophils or macrophages, hence inducing inflammatory effects that further lead to DNA damage (secondary genotoxicity) (Schins and Knaapen, 2007). Elevated DNA strand breakage and oxidative DNA damage, also resulting from oxidative stress, is further known to contribute to carcinogenicity. An increased lung cancer risk is linked to inhalation of ambient particulate matter and diesel exhaust particles (Knol *et al*., 2009; HEI research report, 1995), as well as chronic inhalation of nano-sized metal oxide particles (Donaldson *et al*., 2005).
Industrial application of nano-sized materials in food and food products have increased dramatically. However, possible adverse effects of particles in the human intestine are poorly investigated to date and these might play a role in the development and progression of chronic inflammatory bowel diseases or bowel cancer.

The present thesis aimed to investigate whether primary and/or secondary genotoxic mechanisms were induced by ingested NP, and their relevance for the gastrointestinal tract was to be evaluated. Therefore, the induction of oxidative stress as well as the cytotoxic and DNA damaging potential of food relevant nanomaterials on the intestinal epithelium was determined *in vitro* and *in vivo*. Further, evaluations were conducted on the pro-inflammatory effects and the arising relevance of inflammatory processes in (nano)particle toxicology.

The particles used in these studies and their potential applications were

TiO_2	potentially present in food
SiO_2 (amorphous)	
ZnO	potentially present in food / food packaging
MgO	
carbon black (CB)	model of an environmental NP

In **Chapter 2**, a subset of four food relevant (nano)particles, as well as carbon black as a model of an environmental combustion-derived nanomaterial, were analysed for their cytotoxic potential on the intestinal epithelial cell line Caco-2. The induction of oxidative stress and the resulting level of oxidative DNA damage were evaluated. Selected materials were further analysed for the influence of size on their DNA damaging and cytotoxic properties.

To follow up on the previous studies, and to gain deeper insight in size and specific surface area dependent adverse effects of TiO_2, five different samples were characterised in detail in **Chapter 3**. The materials were compared for their cytotoxic and DNA damaging potential in Caco-2 cells with regard to their major physico-chemical properties (e.g. size, crystalline structures, aggregation).

The influence of gastric and intestinal pH conditions that impact on ingested nanoparticles was evaluated in **Chapter 4**. Two food relevant materials, namely SiO_2 and ZnO were analysed for solubility and size distribution within suspension. Uptake into Caco-2 cells was determined, and the influence of cell differentiation and simulated particle digestion was evaluated with regard to cytotoxicity and pro-inflammatory cytokine induction.

Activated primary human neutrophils (PMN) were co-cultured with Caco-2 cells, and in **Chapter 5** the generation of ROS within this model as well as the impact of PMN on the DNA integrity of the intestinal cells were evaluated. The influence of SiO_2 on PMN induced ROS generation and on DNA damage in the co-culture model was determined. An acute and chronic mouse model of colitis was used for the evaluation of DNA integrity and oxidative stress upon ingestion of SiO_2 in the intestinal epithelium *in vivo*.

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CHAPTER 2

Cytotoxicity and oxidative DNA damage by nanoparticles in human intestinal Caco-2 cells

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Abstract

The use of engineered nanoparticles in the food sector is anticipated to increase dramatically, whereas their potential hazards for the gastrointestinal tract are still largely unknown. We investigated the cytotoxic and DNA damaging effects of several types of nanoparticles and fine particles relevant as food additives (TiO_2 and SiO_2) or for food packaging (ZnO and MgO) as well as carbon black on human intestinal Caco-2 cells.

All particles, except for MgO, were cytotoxic (LDH and WST-1 assay). ZnO, and to lesser extent SiO_2, induced significant DNA damage (Fpg-comet), while SiO_2 and carbon black were the most potent in causing glutathione depletion. DNA damage by TiO_2 was found to depend on sample processing conditions. Interestingly, application of different TiO_2 and ZnO particles revealed no relation between particle surface area and DNA damage. Our results indicate a potential hazard of several food-related nanoparticles which necessitate investigations on the actual exposure in humans.

2.1 Introduction

Engineered Nanoparticles (ENP) have gained strong interest in various fields of research and industry, since their small size offers new features and enhanced reactivity in comparison to larger particles of the same chemical composition. Their use can be found in a broad field, for instance as pigments and resins, as UV-filters in cosmetics, in strategies to improve drug delivery, or for applications in medical diagnostics (Borm *et al*., 2006). The food industry is starting to use various nanoparticles as food additives or to improve food packaging in an attempt to optimise their products. While microparticles, such as Titanium dioxide or Silicon dioxide have had a long-standing use as food additives, for example as whiteners (e.g. TiO_2) or enhancers of viscosity and fluxing agents (e.g. SiO_2) (Schmid and Riediker, 2008; Chaudhry *et al*., 2008), in the future nanoparticles of this composition will be finding their way into foods, i.e. as coatings of confectionary products (e.g. SiO_2, MgO and TiO_2; Chaudhry *et al*., 2008) or as additives for functional food (e.g. amorphous SiO_2; Nanotechproject homepage). Specific ENP are also considered as promising components of novel food packaging materials in view of their ability to enhance gas exchange and their antimicrobial properties (e.g. ZnO and MgO). More advanced approaches in the food industry include nanoparticle-based sensors to monitor food edibility or nano-encapsulation applications to improve nutrient stability and delivery (Taylor *et al*., 2005; Asuri *et al*., 2007; Kaittanis *et al*., 2007). Little is known, however, about the potential risks of ENP application to the human body. Especially the effects of ENP within the gastrointestinal tract are poorly investigated.

In the field of nanotoxicology research, inhalation and ingestion are considered as two major uptake routes of nanoparticles to the human body (Oberdörster *et al*., 2005a, 2007). Depending on their physicochemical properties, nanoparticles can be taken up by different types of cells via active or passive uptake mechanisms (reviewed in Unfried *et al*., 2007). Within biological systems, they are able to generate reactive oxygen species (ROS) like superoxide ($O_2^{\cdot -}$), hydroxyl radicals ($OH^{\cdot}$) or hydrogen peroxide (H_2O_2) as well as reactive nitrogen species (RNS) like nitric oxide ($NO^{\cdot}$) and peroxynitrite ($ONOO^-$) in a direct or indirect manner (Unfried *et al*., 2007). The surface chemistry of particles can lead to direct ROS formation (Brown *et al*., 2001; Stoeger *et al*., 2009), whereas cellular targets such as Nicotinamide adenine dinucleotide phosphate (NADPH)-like enzymes, mitochondria, or intracellular calcium stores can be activated to produce ROS in an indirect way (Brown *et al*., 2004; Singh *et al*., 2007). Alternatively, particles can lead to

indirect ROS generation by inducing inflammation characterised by the recruitment and activation of ROS producing phagocytic cells such as monocytes and neutrophils (Schins and Knaapen, 2007).

The ability of particles to generate ROS and to induce oxidative stress has been proposed as a unifying mechanism of their toxicity (Oberdörster *et al*., 2005a, 2007; Xia *et al*., 2006). Such properties have been associated not only with the activation of inflammatory mediators, but also with the induction of oxidative DNA damage and associated mutagenesis (Oberdörster *et al*., 2007; Schins and Knaapen, 2007). In the respiratory tract, the potential adverse effects of inhaled particles are well recognised and relevant toxicity mechanisms have been identified. Previous studies have for instance shown that combustion derived nanoparticles (including carbon black and diesel exhaust) elicit inflammation in the respiratory tract and may result in chronic inflammatory diseases such as fibrosis or chronic obstructive pulmonary disease (Donaldson *et al*.,2005).

Possible harmful effects of (engineered) nanoparticles in the gastrointestinal tract are still largely unknown. Lomer and co-workers have estimated that around 10^{12}-10^{14} microparticles are ingested daily per person (Lomer *et al.*, 2004). However, nanofood applications are expected to increase dramatically in the near future and this strengthens the need for research to increase knowledge on the potential toxicological effects of nanoparticles to the gut.

The aim of our present study was to investigate the cytotoxic and DNA damaging properties of several types of ENP and fine particles to the human colon epithelial cell-line Caco-2, recognised as an *in vitro* model of the human small intestinal epithelium. TiO_2 and SiO_2 particles were chosen as examples of food additives, while ZnO and MgO particles were selected as promising constituents of food packaging. Finally, carbon black was evaluated as a model of ambient air nanoparticles, since these can reach the gastrointestinal tract upon pulmonary clearance. For the determination of cytotoxicity, the LDH assay as well as the WST-1 assay was used. The reduction of total glutathione (GSH) content was determined as a marker for oxidative stress. To investigate oxidative DNA-damage, the formamidopyrimidine glycosylase (Fpg)-modified comet assay was used, which specifically detects oxidative lesions including 8-hydroxy-2-deoxyguanosine (8-OHdG).

2.2 Methods

Materials. The characteristics of all (nano)particles used in this study are listed in Table 1. Trypsin, Dulbecco's Ca^{2+}/Mg^{2+}-free phosphate buffered saline (PBS), agarose, low melting point (LMP) agarose, Triton X-100, Dimethyl sulfoxide (DMSO), ethidium bromide, Glutathione-reductase, reduced L-Glutathione, fetal calf serum (FCS), KH_2PO_4, K_2HPO_4 and BCA-assay kit (bicinchoninic acid- and copper(II) sulphate solution) were all purchased from Sigma (Germany). Minimum essential Medium (MEM) with Earle´s salts and non essential amino acids were obtained from invitrogen (Germany). Lactate dehydrogenase (LDH) Cytotoxicity Detection Kit and Cell Proliferation Reagent WST-1 were obtained from Roche (Switzerland). Formamidopyrimidine-glycosylase (Fpg)-enzyme was kindly provided by Dr. Andrew Collins, Institute for Nutrition Research, University of Oslo, Norway. All other chemicals were from Merck (Germany).

Code	**Material specification**	**BET** (m^2/g) *	**primary particle size range** (nm) ***	**Source**
TiO_2-F	TiO_2 fine (pure anatase)	10	40-300	Aldrich
TiO_2	TiO_2 ultrafine (80% anatase, 20% rutile), p25	50	20-80	Degussa
TiO_2-HSA	TiO_2 (anatase modified), Hombikat	> 300	< 10	Sigma
SiO_2	Amorphous SiO_2 fumed	200	14	Sigma
CB	Carbon black, Printex 90	300	14	Degussa
MgO	MgO nanoactive**	600	8	Nanoscale Materials Inc., USA
ZnO	ZnO nanoactive**	70	10	Nanoscale Materials Inc., USA
ZnO-Ns	ZnO nanoscale	50	20	Nanostructured and Amorphous Materials Inc.
ZnO-F	ZnO fine	< 10	n.d.	Sigma

Table 2.1 Characteristics of (nano)particles used in the present study
* Surface area according to Brunauer, Emmett and Teller, as provided by company
** Specified as nanoactive particles by company (i.e. composed of aggregates with a geometric size above nano-size, but with specific properties of nanoscale particles)
*** As provided by company
n.d. no information provided

Culture and treatment of the cells. The human colon adenocarcinoma cell line Caco-2 was obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH**,** Germany) and grown in MEM with Earle´s salts and Non Essential Amino Acids, supplemented with 20 % FCS, 1 % L-glutamine and 30 IU/ml penicillin–streptomycin. For experiments, cells were trypsinised at near confluency and $4x10^4$ cells per cm^2 were seeded into 60 mm culture plates (Fpg comet assay), 6-well tissue culture plates (glutathione assay) or 96-well tissue culture plates (LDH- and WST-1 assay) and grown overnight to 70-80% confluency. Cells were then starved for 20 h in serum free medium. Experiments were always performed between cell passages 5 to 30. For treatment, all particles were suspended in starvation culture medium. The suspension was sonicated (Sonorex TK52 water-bath; 60 Watt, 35 kHz) for 7 min and then directly added to the cells at the indicated concentrations. For the Fpg comet assay and glutathione assay, cell monolayers were rinsed repeatedly with PBS after the end of the treatments. This was done to remove excess of extracellular particles that might interfere with the various assays, as well as to remove detached (dead) cells and their debris.

Cytotoxicity. Cytotoxicity was determined by the lactate dehydrogenase (LDH) assay as a marker of cell membrane integrity as well as the water-soluble tetrazolium salt (WST-1) as a measure of the metabolical activity of the cells. For both the LDH and the WST-1 assay 96-well tissue culture plates were used and cells were treated at the indicated concentrations and time intervals with the particles as described above. LDH was determined using a commercial diagnostic kit (Roche, Switzerland). The cleavage of the tetrazolium salt WST-1 to formazan dye via mitochondrial dehydrogenases was measured using a commercial WST-1 diagnostic kit (Roche). Cells were incubated for additional 2 h with WST-1 and analysed directly.

Detection of oxidative DNA damage by Fpg-modified comet assay. The Fpg-modified comet assay was used to measure DNA strand breaks and specifically oxidative DNA damage in the cells, based on the method as described by Speit *et al*. (2004). As a positive control, the photosensitiser Ro-19 8022 (Roche, Switzerland) was used, which induces specific oxidative damage after 2 min light exposure. On the basis of the kinetics of DNA damage induction and its repair in Caco-2 cells, a treatment of 4 h was chosen to reveal possible DNA damage by the particles with avoidance of effective DNA repair. After incubation, cells were rinsed twice with PBS, detached by

trypsinisation and immediately suspended in 1 ml culture medium. Cells were centrifuged for 10 min at 1300 rpm and 4 °C and resuspended in starvation medium at a concentration of 1.5×10^6 cells/ml. A mixture of 10 µl cell suspension with 70 µl 0.5 % LMP agarose was added onto 1.5 % agarose precoated slides in duplicates. Following 10 min of solidification on ice, slides were lyzed overnight at 4 °C in lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris-base, pH 10, containing 10% DMSO and 1% Triton X-100). The slides were washed three times for 5 min in Fpg-enzyme buffer (40 mM HEPES, 100 mM KCl, 0.5 mM EDTA, 0.2 mg/ml BSA, pH 8), covered with 100 µl of either buffer or Fpg in buffer, sealed with a coverslip and incubated for 30 min at 37 °C. All slides were then transferred into the electrophoresis tank. After alkaline unwinding (pH 13) for 20 min, electrophoresis was performed subsequently for 10 min at 270 mA, 26 V. Slides were neutralised three times for 5 min using neutralisation buffer (0.4 M Tris, pH 7.5). Before analysis, slides were dried on air for 10 min and stained with ethidium bromide (10 µg/ml, 40 µl per slide). Comet appearances were analyzed using an Olympus BX60 fluorescence microscope at 400× magnification. A comet image analysis software program (Comet Assay II, Perceptive Instruments, Haverhill, UK) was used for quantification of DNA damage by analysis of the % tail DNA. A total of 50 cells were analyzed per slide per experiment.

Evaluation of comet assay sample processing conditions. Since TiO_2 is suspected to cause oxidative ROS production by photosensitising mechanisms, Caco-2 cells treated with TiO_2 were applied to the comet assay slides in duplicates. For each individual experiment, two slides were processed as usual, with all washing and incubation steps in the dark, while two further slides were handled under normal laboratory light conditions from lysing until Fpg-incubation (daylight + additional illumination by standard neon tubes 18 W, Philips).

Total glutathione content. For measurement of total glutathione content of the Caco-2 cells, cells were treated for 4 hours after which they were rinsed twice with PBS and scraped in 1 ml KPE-buffer (1:6.25 phosphate buffer A [0.1 M KH_2PO_4] and phosphate buffer B [0.1 MK_2HPO_4*$3H_2O$], 10 mM EDTA , pH 7.5) plus 0.1% Triton-X-100 and 0.6% sulfosalicylic acid, sonicated and vortexed for 3 times (30 sec each). After centrifugation (6200 rpm, 5 min at 4 °C) supernatants were frozen at -20 °C until analysis. A mixture of 0.8 mM NADPH and 0.6 mM DTNB (1:1) was added to 50 µl samples or GSH standard

in a 96 well-plate, followed by the immediate addition of 50 µl GSH reductase (4 units/ml). The absorbance was measured at 412 nm for 5 times during 2 min and the values were adjusted to the total protein content as determined by BCA-assay.

Statistics. All means were calculated from three independent experiments, and are expressed in the graphs as mean and standard deviation (SD). Analysis of statistical significance was done by Student's t-test with *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ as levels of significance.

2.3 Results

The cytotoxic potential of ZnO, TiO_2, MgO, SiO_2 and CB was evaluated in dose- and time-dependent manner by measuring the LDH leakage as marker of the cell membrane integrity and the reduction of WST-1 as marker of changes in the metabolic activity. To this extent, cells were treated 4 and 24 hours with 20 and 80 µg/cm^2 particles. After 4 h incubation, 80 µg/cm^2 SiO_2 significantly increased LDH release in comparison to the controls (Figure 2.1 A). After 24 h incubation, SiO_2, TiO_2 and ZnO induced a significant LDH release and thus loss of membrane integrity at both treatment concentrations (Figure 2.1 B). Notably, with CB significant LDH release could only be measured after 24 hours at the lower concentration. The absence of significant LDH increase at the higher treatment concentration may be an artefact due to the absorption of the LDH onto the large surface of the carbon black particles. MgO did not show any membrane-damaging effects at the concentrations and time points tested.

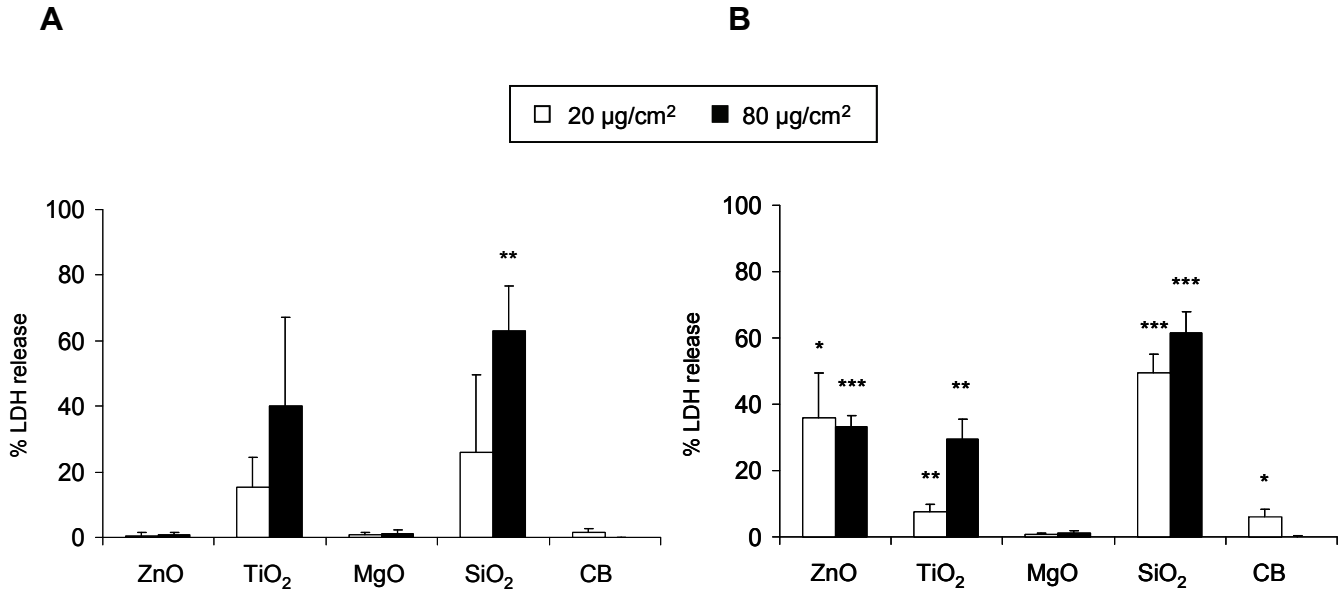

Figure 2.1 **Cytotoxic effects of several particles on Caco-2 cells.** Cells were incubated with 20 and 80 µg/cm^2 particles for 4 (A) and 24 (B) hours. LDH leakage from the cells was measured as marker of cell toxicity and is presented as percentage of the whole LDH content of the cells in the control incubation (ctr). Values are expressed as mean and standard deviation.
* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ versus control

With the WST-1 assay, all particles with the exception of MgO, were found to reduce metabolic activity and thus induce toxicity. After 4 h incubation, the high dose of

all particles significantly decreased the metabolic activity of the Caco-2 cells (Figure 2.2 A), whereas after 24 h significant toxicity occurred at both concentrations tested (Figure 2.2 B). Interestingly, the 4 h incubation with 80 µg/cm^2 MgO, caused a significantly increased formazan dye formation compared to the control cells.

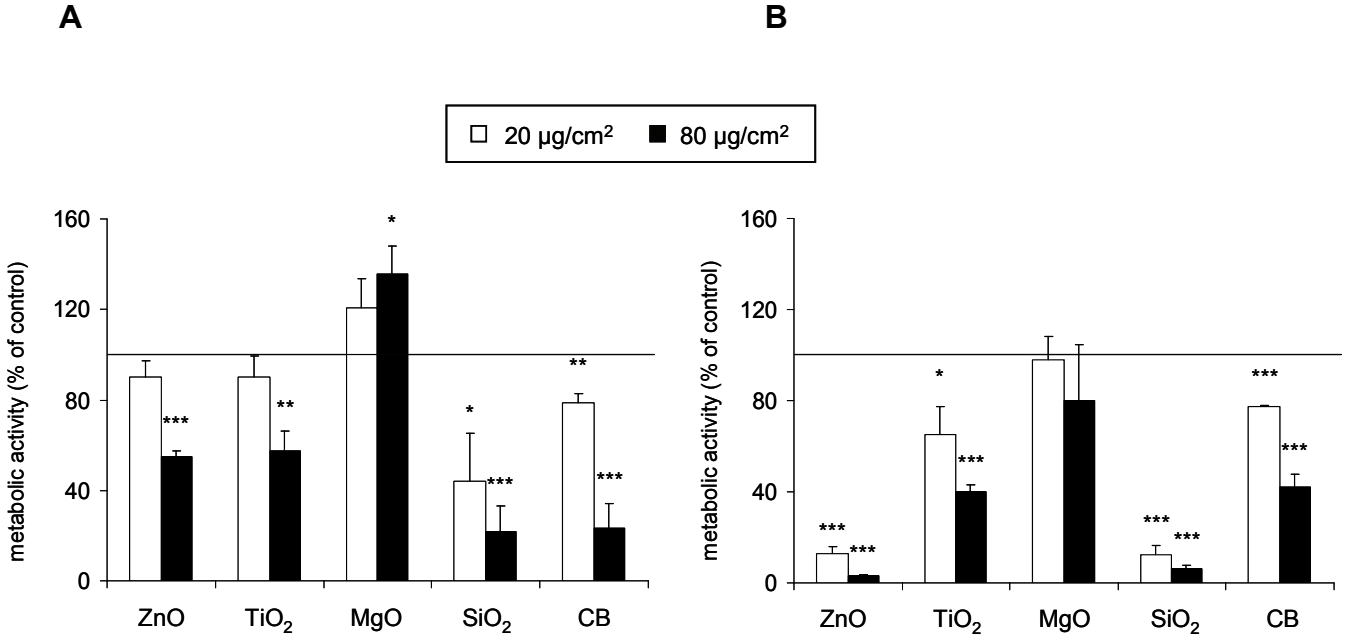

Figure 2.2 **Influences of several particles on the viability of Caco-2 cells.** Cells were incubated with 20 and 80 µg/cm^2 particles for 4 (A) and 24 (B) hours. Mitochondrial enzyme activity was measured via conversion of WST-1 as a marker of cell viability. Values are expressed as mean and standard deviation. The control level is indicated by the horizontal line for clarification.
* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ versus control

To investigate their dose-dependent cytotoxicity in more detail, the effects of additional concentrations of two of the strongly cytotoxic particles, i.e. TiO_2 and ZnO, were examined. Both the LDH and WST-1 assay revealed a clear dose dependent cytotoxic effect of both particle types after 24 hours incubation with an even more pronounced toxicity caused by ZnO in comparison to TiO_2 (Figure 2.3).

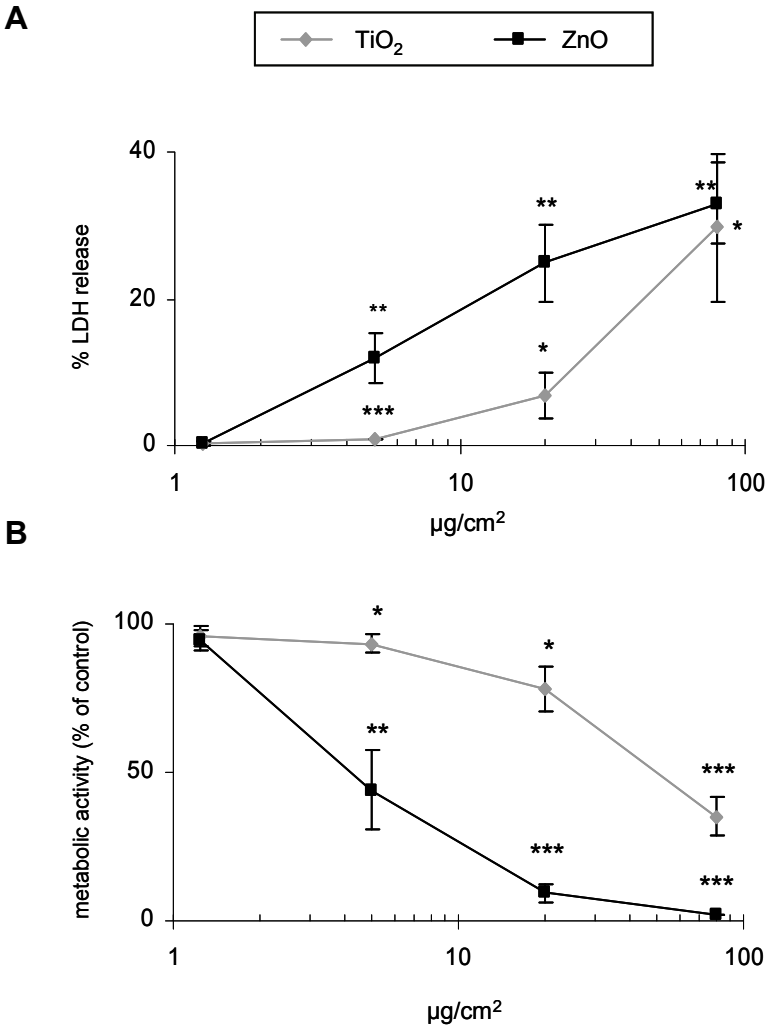

Figure 2.3 **Dose dependent cytotoxic effects of TiO_2 and ZnO on Caco-2 cells.** Cells were incubated with 1.25, 5, 20 and 80 µg/cm^2 particles for 24 hours. LDH leakage (A) and mitochondrial activity (B) were measured as markers of cell toxicity. Values are expressed as mean and standard deviation.
* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ versus control

To determine the potential DNA damaging properties of the particles to colon cells, the Fpg modified comet assay was used. In this assay, after electrophoresis DNA damage becomes visible as a “tail” of DNA fragments behind the cell core and is scored by determination of the percentage of DNA in tail. The specific induction of oxidative DNA damage is determined by the incorporation in the assay of the Fpg-enzyme, which specifically cleaves oxidative DNA lesions, particularly 8-OHdG sites. The activity of the Fpg was verified by the treatment of the cells with Ro-19 8022, a photosensitiser known to induce oxidative DNA damage via generation of singlet oxygen (data not shown).

Already after 4 h incubation, ZnO caused a significant increase of both DNA strand breaks and oxidative DNA damage in the Caco-2 cells (Figure 2.4). Additionally, treatment with SiO_2 caused significant oxidative DNA damage to the cells. None of the

other particles were found to cause DNA strand breakage or oxidative DNA damage in the Caco-2 cells. However, TiO_2 treated Caco-2 cells that were processed in the presence of light (see methods section for details) showed increased DNA damage as well as significant oxidative damage in contrast to the dark-processed TiO_2 treated cells (see Figure 2.4).

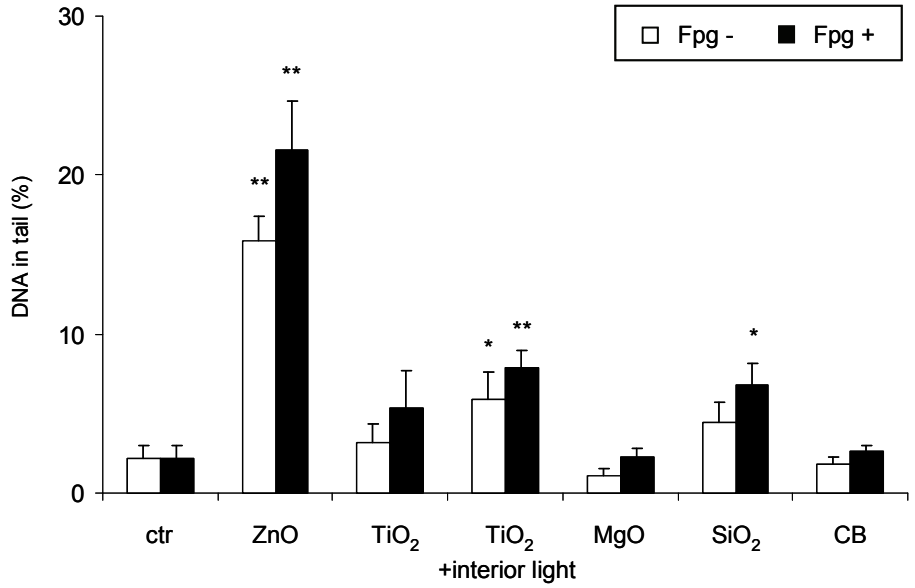

Figure 2.4 Particle-induced DNA strand breakage and oxidative DNA damage in Caco-2 cells using Fpg comet assay. DNA strand breakage and oxidative DNA damage was determined in Caco-2 cells by Fpg-modified comet assay following 4 h treatment with several particles at 20 µg/cm^2. After treatment all samples were processed for comet assay in the dark, while additional samples of TiO_2-treated cells were processed under normal interior light (see method section for details). Values are expressed as mean and standard deviation.
* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ versus control

Since it is known cells with diminished intracellular glutathione (GSH) levels may be more sensitive to oxidative DNA damage induction, the total GSH content of the Caco-2 cells was measured after 4 h particle incubation. Treatment of the Caco-2 cells with all particles, except for TiO_2, led to significant GSH depletion with SiO_2 causing the strongest effect of 40 % depletion (Figure 2.5).

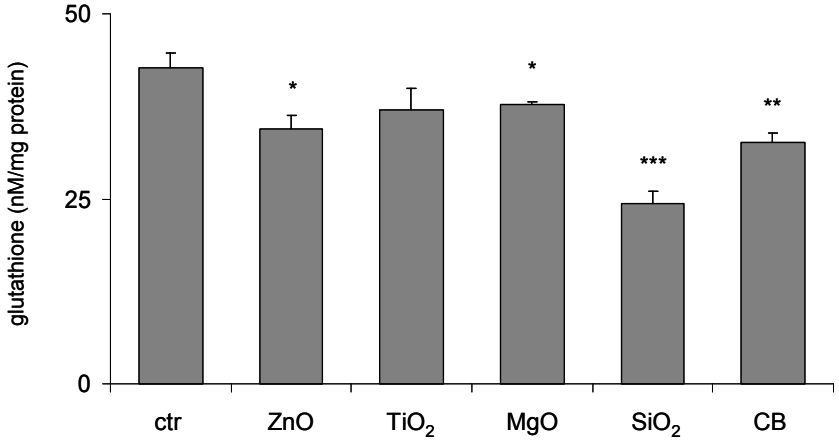

Figure 2.5 **Effects of several particles on total glutathione content of Caco-2 cells.** Cells were incubated with 20 µg/cm^2 particles for 4 hours. Total glutathione content of the Caco-2 cells is expressed as nM/mg protein. Values are expressed as mean and standard deviation.
* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ versus control

To investigate whether the cytotoxic and DNA damaging effects of the particles occurred in proportion to their surface area, multiple types of TiO_2 and ZnO particles were simultaneously tested for their cytotoxicity as well as their capability to induce DNA damage. To this extent, Caco-2 cells were incubated for 4 hours with the TiO_2 and ZnO samples used so far as well as with two further samples of both TiO_2 and ZnO with different surface area (see sample specifications in Table 2.1).

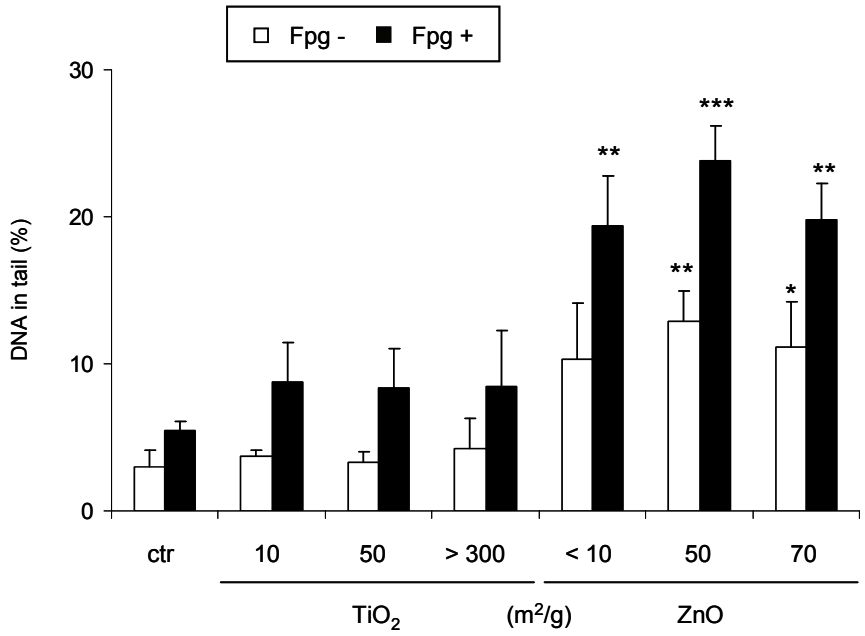

Figure 2.6 **Oxidative DNA damage in Caco-2 cells using Fpg comet assay induced by different-sized particles.** Damage was determined in Caco-2 cells using the Fpg-modified comet assay following 4 h treatment with TiO_2 and ZnO particles with BET-values of 10, 50, >300 m^2/g (TiO_2) and <10, 50, 70 m^2/g (ZnO) at 20 µg/cm^2. Values are expressed as mean and standard deviation.
* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ versus control

For TiO_2, no significant DNA strand breaks or oxidative DNA lesions could be observed for any of the samples (Figure 2.6). In contrast to these findings, cytotoxicity was significantly induced by the highest doses of the two nano-TiO_2 samples (TiO_2 and TiO_2-HSA) but not by fine TiO_2 (TiO_2-F) (Figure 2.7 A). Additionally, fine TiO_2 only reduced metabolic activity of the cells in high doses, whereas both samples of nano-TiO_2 already showed effects at the treatment concentration 20 µg/cm^2 (Figure 2.7 B).

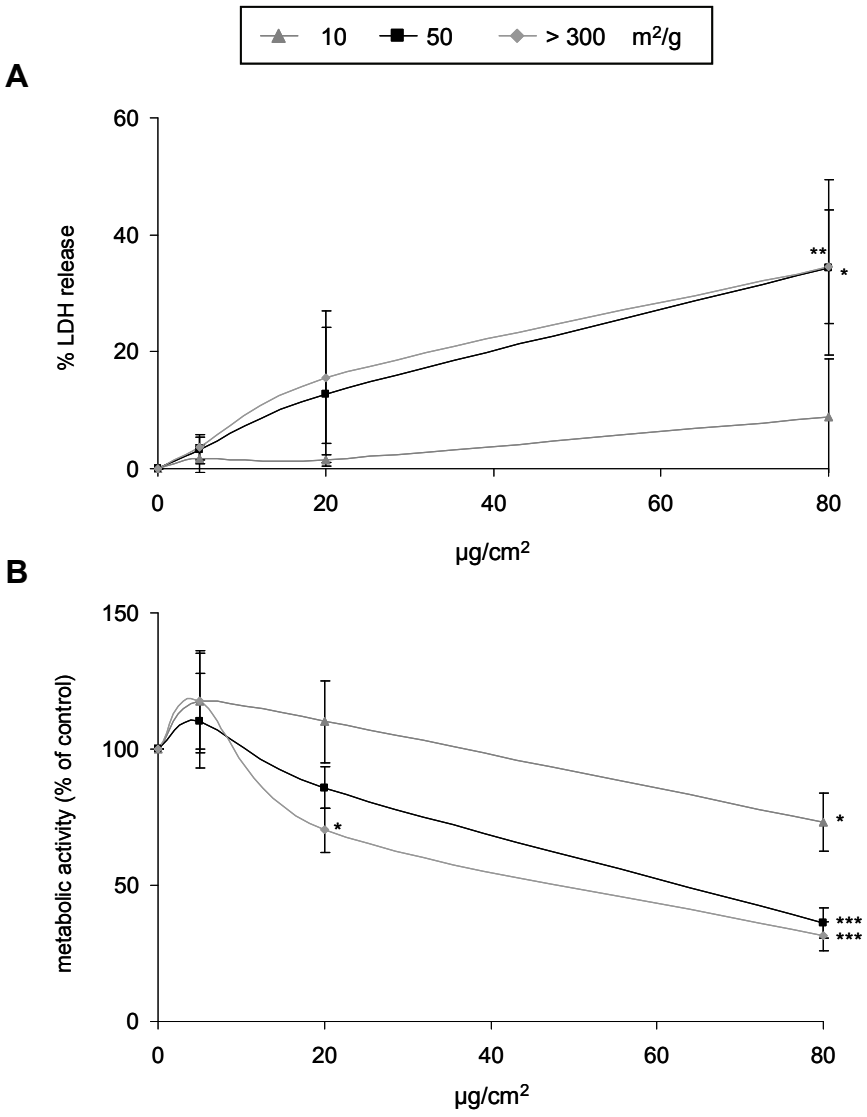

Figure 2.7 Cytotoxicity of TiO_2 particles of with different surface area to Caco-2 cells. Cells were incubated for 4 hours with 5, 20 and 80 µg/cm^2 of TiO_2 particles with BET-values of 10, 50 and > 300 m^2/g. LDH leakage (A) and mitochondrial activity (B) were measured as markers of cell toxicity. Values are expressed as mean and standard deviation.
$^{+}$ $p < 0.05$ versus control for the 10 m^2/g particles
* $p < 0.05$ and *** $p < 0.001$ versus control for the 50 m^2/g particles
$^{\#\#}$ $p < 0.01$ and $^{\#\#\#}$ $p < 0.001$ versus control for the > 300 m^2/g particles

For ZnO, all three samples caused a significant increase of DNA strand breaks as well as enhanced oxidative damage, with no significant differences in potency between the individual ZnO particles (Figure 2.6). In agreement with our earlier observations (see Figure 2.1 A), the ZnO sample with the surface area of 70 m^2/g did not induce increased LDH leakage after 4 h. This also appeared true now for the fine ZnO sample (ZnO-F) as well as the ZnO sample with the BET surface area of 50 m^2/g (ZnO-Ns) (see Figure 2.8 A).

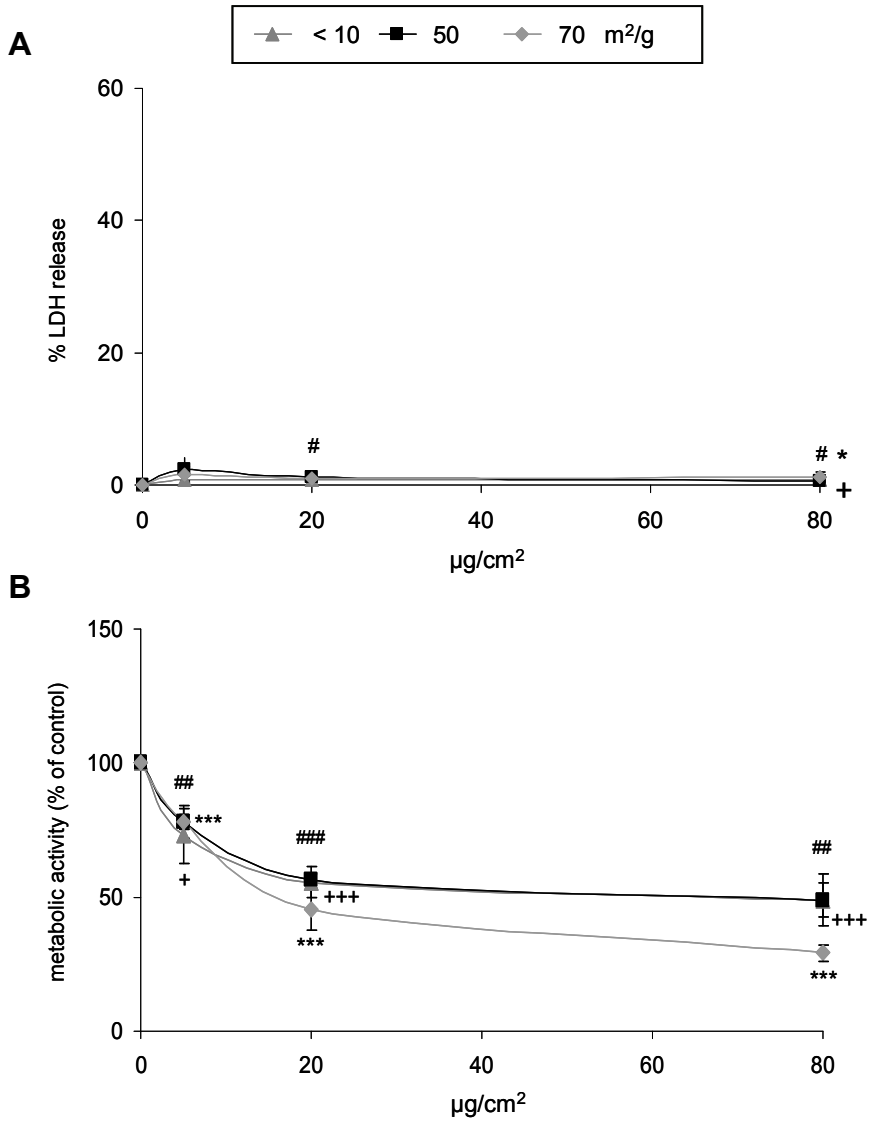

Figure 2.8 **Cytotoxicity of ZnO particles of different surface area to Caco-2 cells.** Cells were incubated for 4 hours with 5, 20 and 80 µg/cm^2 of ZnO particles with BET-values of <10, 50 or 70 m^2/g. LDH leakage (A) and mitochondrial activity (B) were measured as markers of cell toxicity. Values are expressed as mean and standard deviation.
$^{+}$ $p < 0.05$ and $^{+++}$ $p < 0.001$ versus control for the <10 m^2/g particles
* $p < 0.05$ and *** $p < 0.001$ versus control for the 50 m^2/g particles
$^{\#}$ $p < 0.05$, $^{\#\#}$ $p < 0.01$ and $^{\#\#\#}$ $p < 0.001$ versus control for the 70 m^2/g particles

A slight, but significant LDH release could be shown for the ZnO-Ns at 20 µg/cm^2 as well as the highest dose (80 µg/cm^2) for all ZnO particles. Finally, irrespective of their surface area, all ZnO particles were found to reduce metabolic activity in a clear dose-dependent manner, already being significant at 5 µg/cm^2. (Figure 2.8 B). The ZnO sample with the highest surface area tended to be more toxic than both other samples, but this difference was not statistically significant.

2.4 Discussion

In the present study, the toxic effects of a panel of fine and nano-size particles to the human colon epithelial cell line Caco-2 were evaluated. At first, we investigated the cytotoxic effects of the particles by measuring their influence on membrane integrity and metabolic activity of the cells. We found that both TiO_2 and SiO_2 already display significant dose-dependent membrane damage after 4 hours. ZnO and CB only revealed their membrane damaging properties after 24 hours. Analysis of changes in metabolic activity indicated cytotoxicity towards the Caco-2 cells for all samples, with the exception of MgO. Over all, the WST-1 assay tended to be more sensitive to detect particle toxicity when compared to the LDH assay, in concordance with previous findings by us in lung epithelial cells (Schins *et al*., 2002) and macrophages (Albrecht *et al*., 2009). Our data also highlight the importance of using more than one cytotoxicity assay; this is most notably shown by the contrasting effects with the CB nanoparticles, whereby the lacking -or even seemingly decreasing effect in the LDH in high concentrations- is considered to result from LDH absorption artefacts (Monteiro-Riviere *et al.* 2008; Stone *et al*., 2009). In this regard it also remains to be investigated whether the enhanced formation of formazan dye in the WST-1 assay as observed with the MgO is due to increased cell proliferation, or an artefact.

Within the panel of tested particles, ZnO induced a significant amount of DNA strand breaks and oxidative damage in the Caco-2 cells, while SiO_2 only induced oxidative DNA damage in the absence of a significant increase in DNA strand breakage. The lack of significance in the observations upon SiO_2 treatment is in line with the recent study of Barnes *et al.* (2008) that could not find significant strand breaks in the conventional comet assays performed in two separate laboratories. It is also important to notice that the DNA strand breakage induction of SiO_2 was measured at a concentration that also causes significant cytotoxicity (WST-1 assay). In contrast, the DNA damaging effects of ZnO were observed to occur in the absence of cytotoxicity (both LDH and WST-1 assay). Notably, to determine the true genotoxic potential of these and other particles, more detailed dose-response investigations will be required, i.e. according to testing guidelines and by employing independent genotoxicity assays (Stone *et al*., 2009). However, such an approach was beyond the scope of our current study, which was aimed to provide first clues for the toxic properties of various nanoparticles in human colon epithelial cells.

In the present study, both SiO_2 and ZnO treatment also caused a clear depletion of total glutathione (GSH) within the Caco-2 cells, in association with the observed oxidative DNA damage induced by these particles. This indicates that, alongside GSH measurements (Oberdörster *et al*., 2005b; Hussain *et al*., 2005), oxidative DNA damage analysis by Fpg-comet assay can also be considered a highly sensitive marker of oxidative stress in the toxicity screening of nanoparticles. However, one should also be cautious about potential artefacts with this assay. For instance, we could observe that incubation with TiO_2 caused an obvious but not statistically significant increase of strand breaks and oxidative DNA damage when the comet assay was processed as usual in the dark. After handling of the already lysed slides by normal interior light, these damages became statistically significant. This reveals the necessity of careful handling when performing the comet assay to avoid false positive results, especially when photosensitising compounds such as TiO_2 are used.

The relevance of the photosensitising action of TiO_2 is under ongoing discussion, most importantly in relation to its application in skin formulations. Nakagawa et *al.* could demonstrate enhanced strand breaks detected by using the comet assay, but also increased cytotoxicity, in mouse lymphoma cells when treated with different TiO_2 particles and irradiated with UV light (Nakagawa *et al*., 1997). On the other hand, Theogaraj and co-workers could not find increased chromosome aberrations after treatment of Chinese hamster ovary cells with several ultrafine TiO_2 particles and UV light irradiation (Theogaraj *et al*., 2007).

Since toxic effects of nanoparticles are considered to be at least in part due to their increased surface area (Oberdörster *et al*., 2005a), we also investigated cytotoxicity and DNA damaging properties in Caco-2 cells of TiO_2 and ZnO samples that differed in their surface area. Remarkably however, we could not find any surface area-dependent effects concerning DNA strand breaks or oxidative damage, neither by the three different TiO_2 particles nor by the various ZnO particles used. Only slightly increased cytotoxic effects of both nano-sized TiO_2 particles in comparison to fine TiO_2 could be observed. As such, our data are in contrast to observations on the DNA damaging effects of fine versus ultrafine TiO_2 in bronchial epithelial cells (Gurr *et al*., 2005) and also differ from the hallmark observations in pulmonary toxicology where ultrafine TiO_2 causes increased inflammatory responses in comparison to fine TiO_2 at equal mass dose or lung burden (reviewed in Oberdörster *et al*. 2005a). Our findings therefore suggest that particle surface area may not necessarily be the best dose-metric to predict the

genotoxic properties of poorly soluble low toxicity particles and/or the over all toxic potency of particles to the gastrointestinal tract, unlike e.g. their inflammogenic potency as clearly demonstrated for the respiratory tract (Oberdörster *et al*., 2005a; Donaldson *et al*., 2008). Our data reveal marked differences in the responding of each single particle tested. Table 2 summarises the various outcomes of the toxic properties of the different types of particles that were investigated. The findings of our study demonstrate that the various properties are not predictable and points out the need of differentiated investigation of each single property for every particle.

	Membrane damage (LDH) 4 h		Membrane damage (LDH) 24 h		Impaired metabolic activity (WST-1) 4 h		Impaired metabolic activity (WST-1) 24 h		DNA strand breakage	Oxidative DNA damage	Glutathione depletion
µg/cm²	20	80	20	80	20	80	20	80	20	20	20
TiO_2	-	-	++	++	-	++	+	+++	-	-	-
SiO_2	-	++	++	++	+	+++	+++	+++	-	+	+++
CB	-	-	+	- (a)	++	+++	+++	+++	-	-	++
MgO	-	-	-	-	-	+ (↑)	-	-	-	-	+
ZnO	-	-	+	++	-	+++	+++	+++	+++	+++	+

Table 2.2 Overview of particle effects on different endpoints
- : no significant effects found
+, ++ and +++ : significant effects found (number of symbols indicates the strength of significance)
(a) potential artefact
(↑) opposite, i.e. possible proliferative effect

An estimated daily ingestion of 10^{12}-10^{14} particles per day has been reported (Lomer *et al*., 2001, 2004), with an increasing tendency, while still very little is known about the possible cytotoxic or genotoxic effects of nanoparticles reaching the colon (Oberdörster *et al*., 2007; Borm *et al*., 2006; Chaudhry *et al*.,2008). Nowadays, the actual daily ingestion of particles and its burden to the colon seems to be still below risk for the consumer. However, it is well anticipated that the occurrence of nanoparticles, i.e. novel materials as well as modified formulations of existing particles will increase in food products or contaminants in the near future (Chaudhry *et al*., 2008; Tiede *et al*., 2008; Bouwmeester *et al*., 2009). Presently, it is still largely unclear to which extent ingested nanoparticles are actually taken up by the epithelial cells in relation to their physicochemical properties and to which extent they can translocate into the bloodstream. Earlier studies suggest an estimated particle-absorption of less than 10 % (Jani *et al*., 1994). However, the uptake of small particles (~110-120 nm) by the small intestine *in situ* is shown to be 15-250 fold higher in comparison to larger particles

(~510-550 nm, Desai *et al*., 1996). Studies with lung epithelial cells indicate that specific nanoparticles, depending on their primary size and aggregation status may be taken up by cells via various mechanisms including phagocytosis, pinocytosis and passive diffusion and thereby occasionally may reach important intracellular targets including mitochondria and the nucleus (Geiser *et al*., 2005; Unfried *et al*., 2007; Mühlfeld *et al*., 2008).

In relation to our current observations it should also be kept in mind that many factors influence and possibly enhance the toxicity of ingested nanoparticles. For example, the acidic gastric fluid may modify the chemical composition or have an impact on solubility of the particles. Additionally, the recent observation by Ashwood *et al.* (2007), showing that interactions with the microflora of the colon can increase the toxicity of particles, suggests that the toxicity observed in our *in vitro* model might even be an underestimation of that occurring *in vivo* upon exposure to these compounds. Possible interactions between different ingested particles should also be kept in mind. Exemplary, MgO, which showed no cytotoxic effects in our experiments, might nevertheless render cells more susceptible to the effects of other particles due to its ability to decrease cellular GSH levels.

Finally, it should be kept in mind that our current *in vitro* study represents a model of healthy intestine, and that our current observations do not elaborate on the potential role of intestinal inflammation in the toxicity of ingested particles. On the one hand, it should be addressed to which extent specific particles can trigger pro-inflammatory processes in the gastrointestinal tract as this may increase their toxicity. For the respiratory tract it has been established that low-toxicity nanoparticles cause inflammation in proportion to their surface area, and this inflammatory response in turn, has been associated with tissue damage, remodelling and mutagenesis (Duffin *et al*., 2007; Schins and Knaapen, 2007). On the other hand, it should also be investigated whether the toxic potency of ingested nanoparticles differs between healthy individuals and those with (chronic) inflammatory bowel diseases.

In conclusion, our results show that a variety of nanoparticles, which may occur in food or food packaging, can exert cytotoxic as well as DNA damaging effects in target cells of the human intestine. Presently, we cannot entirely rule out any harmful effect of such particles upon ingestion. Obviously, the results of our study should be interpreted in relation to the applied particle concentrations. However, the mere lack of information about in which products they can actually be found, to which extent they are used in

processed food and in which dose they will actually reach the intestine upon ingestion (Tiede *et al*., 2008; Bouwmeester *et al*., 2009) complicates an appropriate evaluation of the risks of nanoparticle ingestion.

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CHAPTER 3

Specific surface area independent effects of titanium dioxide particles in human intestinal Caco-2 cells

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Abstract

Titanium dioxide (TiO_2) has a long-standing use as a food additive. Micrometric powders are e.g. applied as whiteners in confectionary or dairy products. Conversely, possible hazards of nanometric TiO_2-particles for humans and the potential influence of a varying specific surface area (SSA) are currently under discussion. To follow-up on our previous study (Gerloff *et al*., 2009; *Nanotoxicology*) we have analysed five different samples of TiO_2 for crystallinity, primary particle size, SSA and aggregation/agglomeration. Their cytotoxic and DNA damaging potential was evaluated in human intestinal Caco-2 cells by LDH assay and Fpg-modified comet assay. Two anatase-rutile containing TiO_2 samples, in contrast to three pure anatase powders, caused mild membrane damage and DNA oxidation under exclusion of illumination. The effects could not be explained by the different SSA of the samples, and indicated that cytotoxic and DNA damaging properties of TiO_2 in this system are more likely driven by their chemical composition.

3.1 Introduction

The use of particles as food additives has been well established throughout the last decades. Titanium dioxide (TiO_2) for example is well appreciated for its inert capacities and as such widely used as a white food coloring. Indeed, it can be found in many foods, as for example dairy products (GSFA online) and is accepted by the EU with the E-number 171. Currently, a lot of research is in progress to expand the application areas of this additive using nano TiO_2-particles, for example as a coating in confectionary products to prevent melting or improve shelf life (Chaudhry *et al*., 2008). Bulk TiO_2 is regarded as a highly biocompatible material. However, TiO_2 nanoparticles have been found to elicit toxic responses in various *in vivo* and *in vitro* systems (Oberdörster *et al*. 2005; Oberdörster *et al*. 2007; Johnston *et al*. 2009). The observed adverse effects in these studies have been contributed to the small particle size, the high specific surface area (SSA) and the reactivity of nanometric TiO_2 powders. Consequently, investigations on possible adverse effects of ingested TiO_2-nanoparticles are necessary to rule out any health risks to humans consuming particle-containing products.

Previously, we have investigated the potential of different nano-sized materials to induce cytotoxicity and DNA damage in Caco-2 cells (Gerloff *et al*., 2009). Amongst others, we have also aimed to address the influence of an increasing particle specific surface area (SSA evaluated by means of Brunauer, Emmett and Teller method) on both endpoints mentioned by comparative testing of three different types of TiO_2-particles. In this study we did not observe a distinction with respect to DNA damage. Recently however, we have found out that the SSA value listed by the supplier in the catalogue as well as on the label of the specific batch was incorrect. Contrary to the provided SSA value of > 300 m^2/g, this TiO_2 sample appeared to have a SSA value of about 50 m^2/g, closely resembling that of one of the two other previously tested samples (Gerloff *et al*., 2009). Accordingly, the statement on the apparent lack of a surface area dependent DNA damaging effect for TiO_2 on our previous paper could not be truly made.

To clarify on this matter, we have now further screened a broader range of TiO_2-particles, by including two additional types of particles of high SSA values. In the present study, for all samples the SSA values were newly determined. The mean primary particle size as well as their organisation in aggregates was evaluated by

using transmission electron microscopy (TEM) and dynamic light scattering (DLS). Using both these novel and the earlier investigated materials, we re-evaluated the potential role of surface area of TiO_2-particles on cytotoxicity and oxidative DNA damage in Caco-2 cells.

3.2 Methods

Materials. Trypsin, Dulbecco's Ca^{2+}/Mg^{2+}-free phosphate buffered saline (PBS), agarose, low melting point (LMP) agarose, Triton X-100, Dimethyl sulfoxide (DMSO), ethidium bromide and fetal calf serum (FCS) were all purchased from Sigma (Germany). Lactate dehydrogenase (LDH) Cytotoxicity Detection Kit was obtained from Hoffmann-LaRoche (Switzerland). Minimum essential Medium (MEM) with Earle´s salts and non essential amino acids was purchased from invitrogen (Germany). [R]-1-[(10-Chloro-4-oxo-3-phenyl-4H-benzo[*a*]quinolizin-1-yl)-carbonyl]-2-pyrrolidinemethanol (Ro19-8022) was a gift from Hoffmann-LaRoche (Switzerland). Formamidopyrimidine-glycosylase (Fpg)-enzyme was kindly provided by Dr. Andrew Collins, Institute for Nutrition Research, University of Oslo, Norway. All other chemicals were from Merck (Germany).

TiO_2 samples. A set of five different TiO_2 samples was used. Three of the samples were already used in our previous study (Gerloff *et al*., 2009). For the present investigations, two further samples were included, i.e. JRC 12, supplied by the Japan Catalysis Society, and Hombikat UV100, obtained from Sachtleben Chemie GmbH, Germany.

XRD spectroscopy. XRD spectra were collected on a diffractometer (PW1830, Philips) using CoKα radiation, in the (20 – 90) 2θ range, with step width 2θ = 0.05 and diffraction peaks have been indexed according to ICDD database (International Centre for Diffraction Data).

Surface area measurements. The surface area of the particles has been measured by means of the Brunauer, Emmett and Teller (BET) methods based on N_2 adsorption at 77K (Micrometrics ASAP 2010).

Morphological characterisation. The mean size of primary particles as well as their organisation in aggregates/agglomerates has been evaluated by electron transmission microscopy TEM (JEOL 3010-UHR instrument operating at 300 kV, equipped with a 2k × 2k pixels Gatan US1000 CCD camera).

Average hydrodynamic size. TiO_2 particles were suspended in water at pH 9 to maximise the electrostatic repulsion between particles. The suspensions were sonicated for 2 min with a probe sonicator (100 W, 20 kHz, Sonoplus, Bandelin, Berlin, Germany). The average hydrodynamic size of particles or aggregates has been evaluated by means of dynamic light scattering (DLS) (Zetasizer Nano–ZS, Malvern Instruments, Worcestershire, UK). The detection limit for this technique is of 6 µm.

Culture and treatment of the cells. The human colon adenocarcinoma cell line Caco-2 was obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH**,** Germany) and grown in MEM with Earle´s salts and Non Essential Amino Acids, supplemented with 20 % FCS, 1 % L-glutamine and 30 IU/ml penicillin–streptomycin. For LDH assay and Fpg-modified comet assay, cells were prepared and treated as described in Gerloff *et al*., 2009.

Cytotoxicity. Cytotoxicity was determined by the lactate dehydrogenase (LDH) assay as a marker of cell membrane integrity. 96-well tissue culture plates were used and cells were treated at the indicated concentrations for 4 hours with the particles as described above. LDH was determined using a commercial diagnostic kit according to the manufacturer's instructions (Roche, Switzerland).

Detection of oxidative DNA damage by Fpg-modified comet assay. The Fpg-modified comet assay was used to measure DNA strand breaks and specifically oxidative DNA damage in the cells, based on the method described by Speit *et al*. (2004) with minor modifications described earlier (Gerloff *et al*., 2009). As positive control, the photosensitiser Ro-19 8022 was used, which induces specific oxidative lesions after 2 min light exposure (Angelis *et al*., 1999).

Statistics. All means were calculated from three independent experiments, and are expressed in the graphs as mean and standard deviation (SD). Analysis of statistical significance was done by unpaired Student's t-test with *$p<0.05$ and **$p<0.01$ as levels of significance.

3.3 Results

The characteristics of the samples used in this study are listed in Table 3.1.

Sample	Crystalline phases [a]	Particle specific surface area (m^2/g) as reported in Gerloff *et al.*, 2009	Particle specific surface area (m^2/g) as determined in current study	Primary particles mean diameter (nm)	Aggregates/ agglomerates size range (nm)
TiO_2-F	100 % anatase	10	10	> 100 [b]	70 – 1000 [d]
TiO_2(p25)	77 % anatase 23 % rutile	50	52.6 ± 0.1	25.2 ± 0.2 [c]	40 - 500 [d]
TiO_2-HSA	90 % anatase 10 % rutile	> 300	52.8 ± 0.4	21.9 ± 0.3 [c]	20 - 2000 [d]
JRC 12	100 % anatase	-	282.3 ± 1.9	7 [b]	30 - 1000 [d]
UV100	100 % anatase	-	342.4 ± 1.3	3.94 ± 0.05 [c]	50 - 2000 [d]

Table 3.1 Characterisation of different-sized titanium dioxide particles.
[a] evaluated by means of XRD
[b] theoretical, calculated from the surface area value
[c] evaluated by TEM
[d] evaluated by Dynamic Light Scattering (DLS). This technique has an upper detection limit of 6000 nm, therefore the presence of larger aggregates may not be excluded.

The samples were found to differ to great extent in terms of their crystalline phases, SSA values and nanostructure. Two samples, i.e. TiO_2(p25) and TiO_2-HSA are composed of anatase and rutile phases in different proportions. The other three samples are pure anatase. TiO_2-F is a micrometric powder as suggested by the low SSA which corresponds to a theoretical particle size larger than 100 nm. Although a fraction of single monodisperse particles is present, the powder is mainly composed by aggregates or agglomerates, as suggested by DLS evaluation. TiO_2(p25) and TiO_2-HSA exhibited similar SSA values. As already mentioned, TiO_2-HSA, which was used in our previous study (Gerloff *et al*., 2009) turned out to have a much lower BET value upon analysis than the value originally provided by the supplier. The TEM images of these two different samples show the presence of primary particles having a mean diameter of around 20 nm, mainly organised in nanometric and micrometric agglomerates (Figure 3.1 A, B). Single nanoparticles were also detected by TEM. The presence of agglomerates was confirmed by DLS measurements. Well defined crystallographic planes are visible in the HRTEM images (see inserts in Figure 3.1 A and B) suggesting a high degree of crystallinity of the materials. The XRD patterns (Figure 3.2) reveal the presence of very sharp peaks corresponding to anatase and rutile phases, which further confirms the crystallinity of both samples.

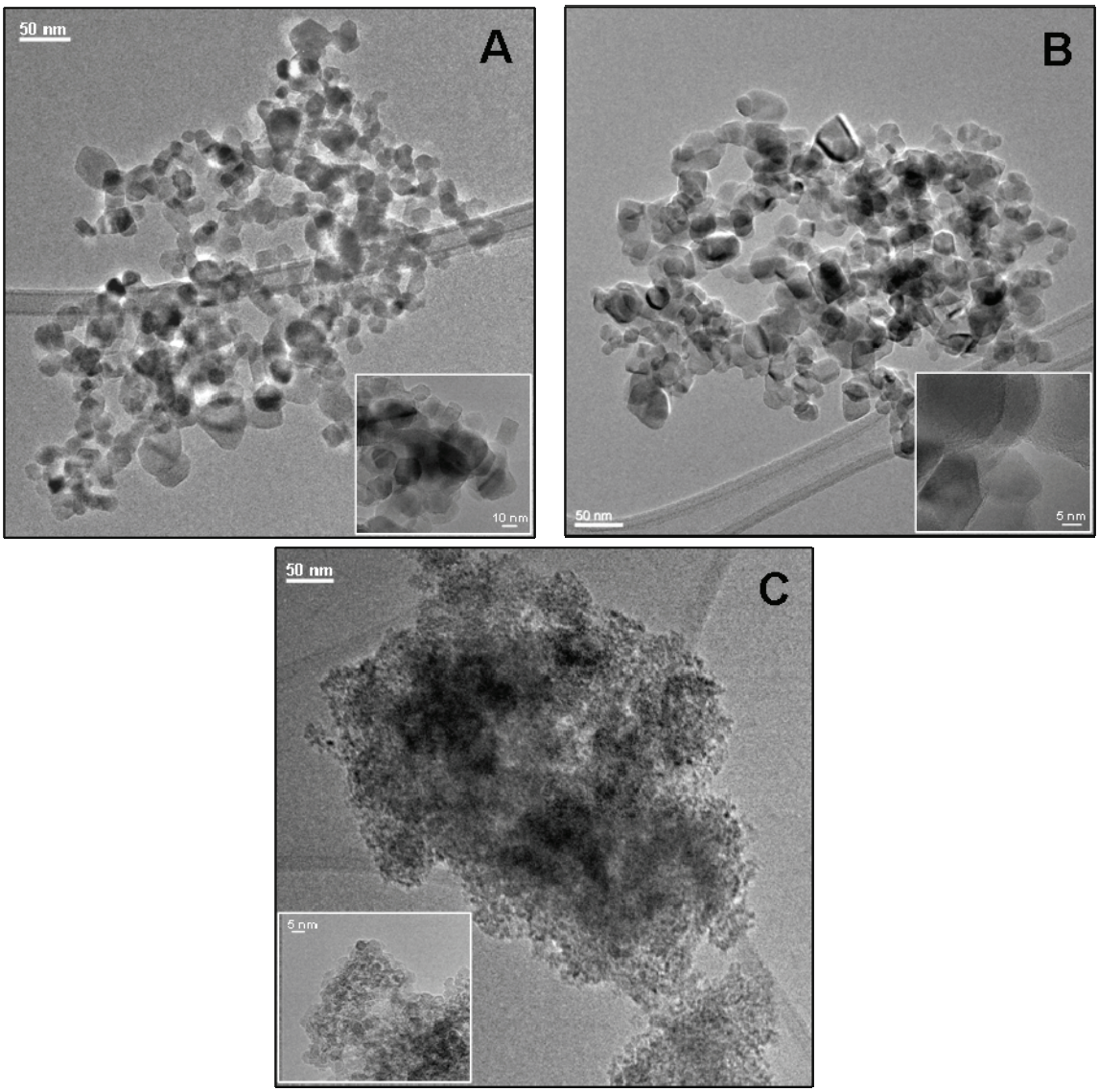

Figure 3.1 **Morphological characterisation of titanium dioxide particles.** TEM images of (A) TiO_2-HSA, 30kX; (B) TiO_2(p25), 40kX; (C) UV100, 40kX. In the insets the HRTEM images (200kX, 500kX and 300kX respectively) are shown.

The high surface area sample UV100 exhibits a very different structure: in this case nanometric primary particles are strongly bonded together to form nanometric and micrometric aggregates (Figure 3.1 C). No single monodisperse nanoparticles were detected in both TEM and DLS analysis. The broadening of the diffraction peaks corresponding to the anatase phase in the XRD pattern (Figure 3.2) reveals a highly disordered structure which is a direct consequence of the small size of primary particles.

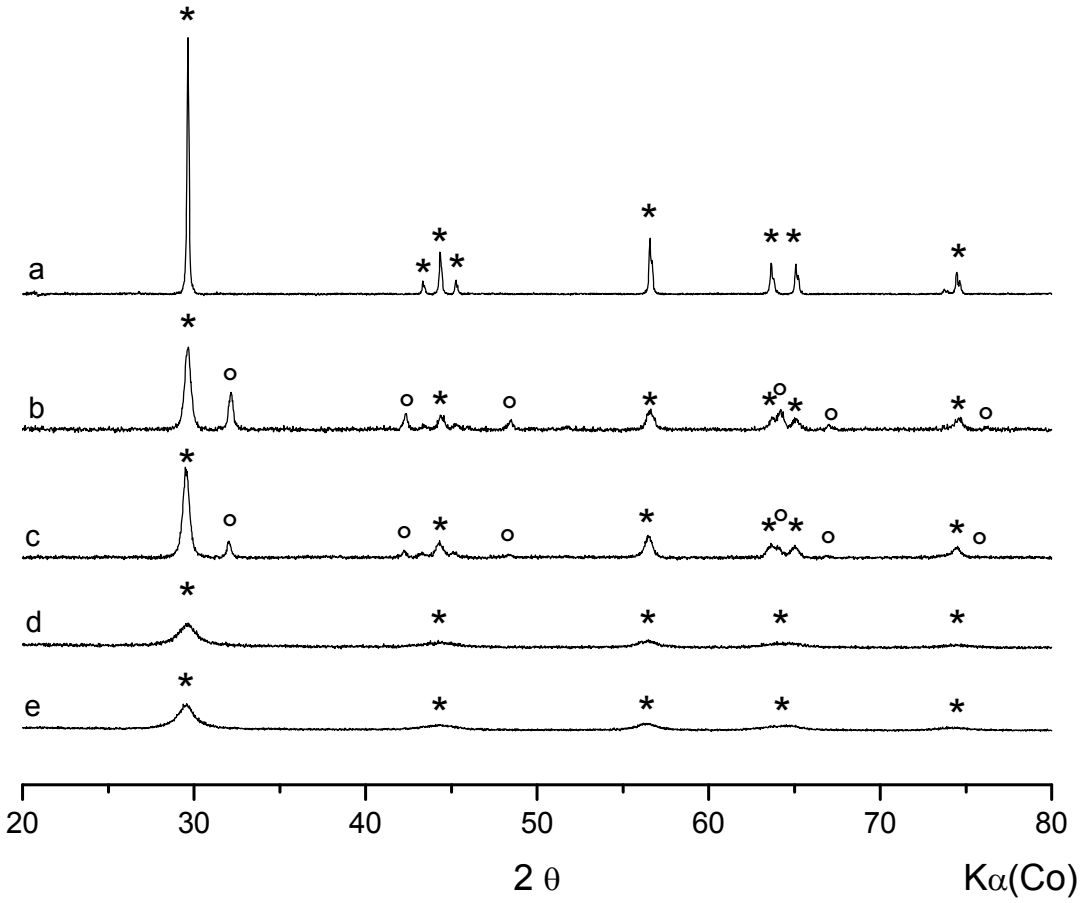

Figure 3.2 **Crystalline phases in titanium dioxide particles.** XRD patterns of (a) TiO_2-F; (b) TiO_2(p25); (c) TiO_2-HAS; (d) UV100; (e) JRC 12; in the 20-80 = 2θ range. The diffraction peaks of anatase (*) and rutile (°) are indicated above the patterns.

The presence of amorphous TiO_2 is confirmed by the low abundance of crystallographic planes in the HRTEM images (see insert of Figure 3.1 C). A similar structure is likely for the other high surface area sample JRC 12: the broad diffraction peaks in the XRD patterns are indicative of a disordered anatase structure while DLS analysis revealed the presence of nanometric and micrometric aggregates/agglomerates.

To determine whether the differences in primary particle size, surface area or crystalline phase of the TiO_2 samples result in an altered cytotoxic or DNA damaging potential to Caco-2 cells, all five particle types were simultaneously tested in the LDH assay and the Fpg-modified comet assay. Cell membrane integrity was measured after incubation for 4 h with 20 and 80 µg/cm^2 particles respectively as the degree of LDH leakage in comparison to a positive (100 %) and negative control (0 %) (Figure 3.3). No cytotoxic effect was found after incubation with TiO_2-F. Moreover,

both particles of the highest surface area and the lowest primary particle size (JRC 12 and UV100) did also not induce any LDH leakage. In contrast, TiO_2(p25) as well as TiO_2–HSA, both rutile-containing particle types, induced significant cytotoxicity after incubation with 80 µg/cm^2. Additionally, the incubation with 20 µg/cm^2 TiO_2(p25) induced mild but statistically significant effects.

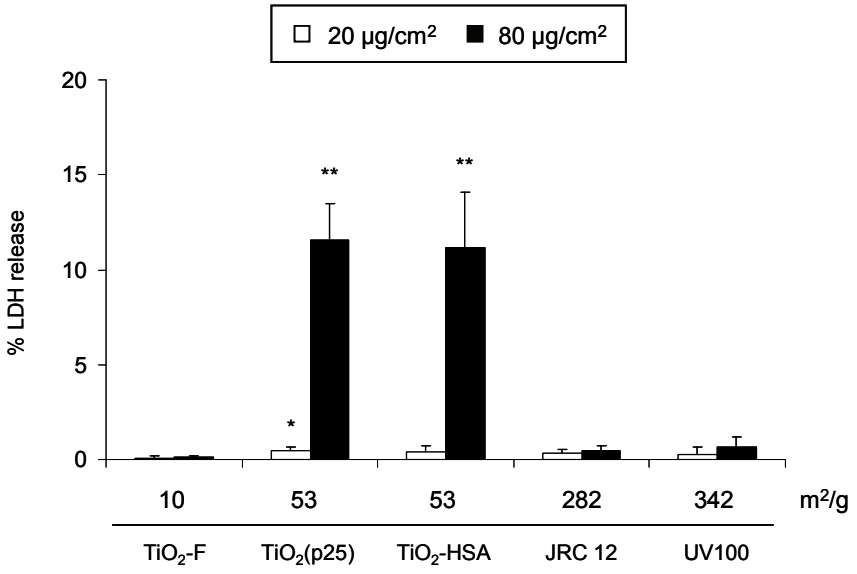

Figure 3.3 Cytotoxic effects of different titanium dioxide particles on Caco-2 cells. Cells were incubated with 20 and 80 µg/cm^2 of different TiO_2 particles for 4 hours. Specific surface area is indicated in m^2/g (BET). Membrane integrity was measured as marker of cell toxicity using the LDH (lactate dehydrogenase) assay. Values are expressed as mean +/- standard deviation, n=3.
* $p < 0.05$ and ** $p < 0.01$ versus control

DNA strand breaks and alkali labile sites as well as specific oxidative DNA damage were analyzed by the Fpg-modified comet assay (Figure 3.4). In this assay, DNA damage becomes visible as a “tail” of DNA fragments behind the cell core after electrophoresis and is scored by determination of the percentage of DNA in tail. The Fpg-enzyme is used to specifically identify oxidative DNA damage by cleavage of oxidative DNA lesions, particularly 8-OHdG sites. As a positive control, the photosensitiser Ro-19 8022, known to induce oxidative DNA damage (Will *et al.*, 1999) was applied to verify the activity of the Fpg. Incubating the Caco-2 cells with 20 µg/cm^2 particles for 4 hours resulted in a damage for the different samples with tendencies similar to that observed for the cell integrity (Figure 3.3). Both particles of the highest surface area and the lowest primary particle size (JRC 12 and UV100) did not induce any significant DNA damage. Fine TiO_2 was able to slightly, but significantly induce DNA strand breaks but no oxidative lesions, whereas both

TiO_2(p25) and TiO_2-HSA led to significant oxidative DNA damage. Moreover, TiO_2(p25) also caused significant DNA strand breaks. Compared to the positive control, the damage induced by all TiO_2 samples appeared very low.

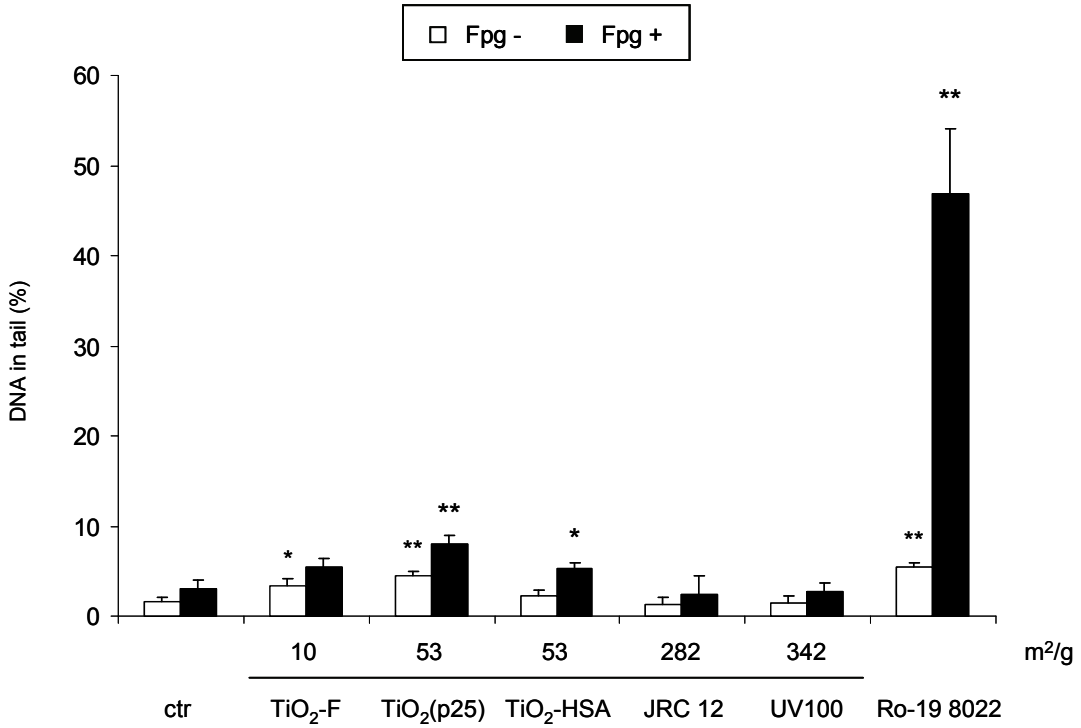

Figure 3.4 Oxidative DNA damage in Caco-2 cells using Fpg-modified comet assay induced by different titanium dioxide particles. DNA strand breakage and oxidative DNA damage were determined in Caco-2 cells using the Fpg-modified comet assay following 4 hours treatment with 20 µg/cm^2 of different TiO_2 particles. Specific surface area is indicated in m^2/g (BET). ctr=control. Values are expressed as mean +/- standard deviation, n=3.
* $p < 0.05$ and ** $p < 0.01$ versus control

3.4 Discussion

In our previous study (Gerloff *et al*., 2009) we have investigated the possible hazards of food-related nanoparticles with respect to cytotoxicity and genome integrity in human intestinal cells. Additionally, in the previous study, we compared the potential cytotoxic and DNA-damaging effects of zinc oxide (ZnO) and titanium dioxide (TiO_2) powders of different particle sizes and surface areas in more detail. To this extent, we included a specific TiO_2 sample with a very high SSA (TiO_2-HSA), chosen especially to compare with our sample of interest, i.e. TiO_2(p25). Since this specific sample was needed only for comparative analyses, we did not fully characterise it ourselves but instead relied on the SSA value provided by the supplier (> 300 m^2/g).

After publication of our previous study, by chance we found new and contradictory information regarding the surface area of TiO_2-HSA, which was confirmed upon our further inquiries by the supplier. To rule out any possible false conclusions about the absence of an influence of a high SSA on cytotoxicity and DNA damage, we therefore decided to characterise TiO_2-HSA in detail. To our surprise, we found a SSA of only 52.8 ± 0.4 m^2/g. Moreover, the sample was found to be composed of 90 % anatase and 10 % rutile (Table 3.1). Both characteristics thus turned out to be quite similar to those of TiO_2(p25). Consequently, it is not surprising at all that we have shown similar levels of toxicity for both particles in our previous study (Gerloff *et al.*, 2009).

A thorough evaluation of the physicochemical properties and composition of the materials used is considered as a prerequisite for an appropriate interpretation of nanotoxicology studies (Warheit, 2008; Stone *et al*., 2009). Our previous and current study provides a clear example of this, and that one should not rely solely on characterisation data provided by suppliers.

In order to verify our previous conclusions stating that there is no dependency on the specific surface area in terms of cytotoxicity and DNA damage in Caco-2 cells, we included two additional TiO_2 powders both having a very high SSA (around 300 m^2/g) and a composition of 100 % anatase (Table 3.1) and repeated a set of experiments. The high SSA of the two new samples corresponded to a very low primary particle size (4 and 7 nm, respectively). However, the size distribution of particles was very similar to the other samples (Table 3.1), since primary particles appeared organised in nanometric and micrometric aggregates.

In accordance to our previous findings, only the rutile-containing TiO_2-particles that also possess an intermediate specific surface area (i.e. TiO_2(p25) and TiO_2-HSA) induced cytotoxicity after 4 h treatment with the highest concentration. None of the other particles (TiO_2-F, JRC 12 and UV100) showed cytotoxicity at the tested concentrations. A similar tendency was observed for the endpoints evaluated by the Fpg-modified comet assay, i.e. DNA strand breakage and oxidative DNA damage. In concordance with the cytotoxicity findings, no DNA damaging effect was found after incubation for 4 hours with JRC 12 and UV100. Surprisingly however, in our present study we detected a low, but nonetheless significant induction of DNA damage by TiO_2-F. Likewise, TiO_2-HSA was shown to induce a mild but significant oxidative DNA damage. For TiO_2(p25) both oxidative damage and strand breakage were significantly induced. None of these current findings were observed in our previous study (Gerloff *et al*., 2009). The most likely explanation for this phenomenon lies within the highly sensitive comet assay itself. The level of DNA damage induced by the TiO_2 samples was found to be relatively close to background in contrast to our positive control, and variations in comet assay measurements are well known to occur in relation to cell culture conditions (e.g. cell passage number) or gel to gel variations (Liao *et al*., 2009). Obviously, our current observations also have to be interpreted in terms of statistical versus biological significance, in relation to the dose applied.

It has been shown that crystallinity influences the toxic potential of TiO_2-particles with anatase usually showing a greater tendency to induce adverse effects such as cyto- or genotoxicity (reviewed in Johnston *et al*., 2009). This may be related to the higher photocatalytic activity of the anatase phase in biological tests performed under UV irradiation, while no effect should be expected in tests performed in the dark. Some studies reported that free radical species may be generated at the surface of TiO_2 also in the dark or under ambient illumination (Sayes *et al*., 2006; *Fenoglio et al.* 2009) but there are no evidences of a higher reactivity of anatase with respect to rutile or mixed phases in these conditions. Besides, rutile or TiO_2 powders composed by the two crystalline phases were reported to be more active than anatase in several studies. Gurr and co-workers compared the DNA-damaging effects of rutile- and anatase-TiO_2 particles of 200 nm primary particle size on human bronchial epithelial cells, BEAS-2B. They demonstrated a significant increase in the release of hydrogen peroxide in cells treated with rutile TiO_2, but not with anatase

TiO_2 if incubated in the dark. Additionally, using the comet assay they found that a mixture of anatase and rutile particles showed a more pronounced DNA damaging effect in the absence of light, than pure anatase or pure rutile TiO_2 (Gurr *et al*., 2005). More recently, Grassian and colleagues studied the possible influence of primary particle size on the inflammatory potential of nano-TiO_2 in mice. Two samples were considered, having different particle size and, consequently, different SSA. The sample having smaller primary particles consisted of pure anatase, while those having the larger particles contained both anatase and rutile crystalline phases. Stronger effects were expected after inhalation or instillation of the higher SSA TiO_2. Interestingly however, exactly the opposite was observed: after a 4 hours low dose inhalation of the anatase/rutile-containing TiO_2 a significantly increased number of total cells in bronchoalveolar lavage was observed, whereas anatase-TiO_2 could cause this effect only at high exposure concentrations. A similar effect was found after instillation of mice with a medium and high concentration of anatase/rutile-containing TiO_2 but not by pure anatase-TiO_2. Additionally, a significant release of LDH and IL-1β was induced by the highest, and an increased release of TNF-α by the medium and high concentration of anatase/rutile-TiO_2 (Grassian *et al*., 2007). Toxic effects towards keratinocytes have been reported for both rutile and anatase (Braydic-Stolle *et al*. 2009), while micrometric rutile appeared more active in inducing IL-1β release in macrophage-like THP-1 cells than anatase TiO_2 (Morishige *et al*., 2010).

In our study, a higher reactivity of the anatase-rutile containing TiO_2 in comparison to the anatase-TiO_2 was found while working under dark conditions. The significant induction of DNA strand breaks and oxidative DNA damage by TiO_2(p25) and TiO_2-HSA was surprising. However, it confirms our previous conclusion (Gerloff *et al*., 2009) that the TiO_2-reactivity in Caco-2 cells is independent of the specific surface area. In contrast, a hazardous potential of TiO_2 seems to be mainly associated with the crystalline phase of the particles. Moreover, JRC 12 and UV100 were characterised by a low degree of crystallinity, which may account for the observed absence of cytotoxicity and DNA damage induction of these specific samples despite their high SSA.

In conclusion, we were able to verify our previous observations on the absence of an association between the specific surface area of TiO_2 particles and their cytotoxic and DNA damaging effects in Caco-2 cells (Gerloff *et al*., 2009).

Furthermore, our present study also highlights, once again, the need for a proper physico-chemical characterisation of the nanoparticles used in toxicity tests.

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Declaration

The manuscript is submitted to a peer-review journal.
The cytotoxicity measurements and detection of DNA strand breakage and oxidative lesions were performed by Kirsten Gerloff. The impact on authoring this paper can be estimated in total with 90%.

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CHAPTER 4

Influence of simulated gastrointestinal digestion on particulate mineral oxide-induced cytotoxicity and interleukin-8 regulation in differentiated and undifferentiated Caco-2 cells

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Abstract

Novel aspects of engineered nanoparticles may offer many advantages for optimising food products and food packaging over bulk materials. However, their potential hazards in the gastrointestinal tract require further investigation. Here, we evaluated the uptake of two types of particles relevant to the food industry, namely silicon dioxide (SiO_2) and zinc oxide (ZnO) in human intestinal Caco-2 cells. Furthermore, we have determined the ability of these particles to generate reactive oxygen species (ROS), induce cytotoxicity and affect interleukin-8 (IL-8) both mRNA expression and protein secretion by Caco-2 cells. The role of gastrointestinal digestion was assessed by pre-incubating the materials in simulated gastric and intestinal pH-conditions. We evaluated the solubility and agglomeration properties of both the native and the pre-incubated particles, and both particle suspensions were used for the incubation of undifferentiated and differentiated Caco-2 cells. Pre-treatment under gastrointestinal conditions reduced ROS formation but did not influence cytotoxicity (based on the WST-1 assay) or IL-8 expression for either material. In contrast, cell differentiation markedly determined the cytotoxic potential of the SiO_2 and ZnO particles, reflected as significantly lower TD_{50} values in the undifferentiated Caco-2 cells. Interestingly,

the pro-inflammatory effects of SiO_2 on the cells appeared to be higher in the undifferentiated cells, whereas ZnO only induced IL-8 secretion in the differentiated Caco-2 cells. Further research is needed to determine the *in vivo* relevance of the state of differentiation of intestinal epithelial cells for the hazards of ingested particles.

4.1 Introduction

The production of functional foods is a fast growing field and the use of inorganic food additives herein as well as in conventional foods is widely accepted. Several microparticulate metal oxides have been used as food colouring agents (e.g. E171, titanium dioxide; E172, iron oxides and hydroxides), acidity regulators (e.g. E530, magnesium oxide) or anti-caking agents (e.g. 551, silicon dioxide) for many years (homepage food.gov.uk). However the use of similar mineral oxides, in smaller nanoparticulate sizes, is also receiving much interest as "nano-sizing" alters the properties of the materials and offers new applications for the food industry. Indeed, zinc oxide (ZnO) is already attracting attention in nanoparticulate form to develop novel food packaging products due to its antimicrobial and UV-absorbent properties (Chaudhry *et al*., 2007). An average Western diet provides a large mineral oxide intake of microparticles, typically 10^{12}-10^{14} particles per day (Lomer *et al.*, 2001 and 2004), so nanoparticle intakes could similarly become significant if food applications grow.

The altered properties of nano-sized materials is generating some concern as it is well recognised that their decreased size in conjunction with a highly increased specific surface area considerably influences their reactivity and may, therefore, increase aspects of their toxicity and inflammatory potential (reviewed in Oberdörster *et al*. 2005, Borm *et al*. 2006). Certain nanoparticles (especially those based on reactive metals e.g. Fe, Zn) proffer a unique capability to generate reactive oxygen species (ROS) such as superoxide ($O_2^{\cdot -}$), hydroxyl radicals ($HO^{\cdot}$) or hydrogen peroxide (H_2O_2) with the associated induction of cellular oxidative stress (Oberdörster *et al*., 2005, 2007; Xia *et al*., 2006; Unfried *et al*., 2007). This ROS formation can be triggered both directly by the specific surface chemistry of particles (Brown *et al*., 2001; Stoeger *et al*., 2009), or indirectly, for example by acting on ROS-producing cellular components such as membrane bound NADPH oxidase enzymes and mitochondria (Unfried *et al*., 2007). In turn, ROS formation and oxidative stress are implicated in the inflammatory effects of nanoparticles by activating pro-inflammatory signalling cascades. Particle-induced inflammation is typically characterised by the recruitment and activation of ROS producing phagocytic cells including monocytes and neutrophils, which exacerbates oxidative stress and tissue damage (Schins and Knaapen, 2007). One of the most prominent cytokines responsible for neutrophil-

recruitment is interleukin-8 (Kunkel *et al*., 1991; Mitsuyama *et al*., 1994; Kucharzik and Williams, 2002-2003). Toxic effects of particles are all described in the lung, whereas only few data are available concerning possible impacts of these compounds on the gastrointestinal tract. The uptake of nanoparticles can occur via different mechanisms (reviewed in Unfried *et al*., 2007) and varies with the nature of the material and also the cell type. The gastrointestinal tract features Peyer´s Patch lymphoid tissue, rich in 'M-cells' which are specialised and differentiated epithelial cells highly capable of taking up particles (reviewed in Powell *et al*., 2010). Other mechanisms may also be relevant as, for example, previous work has shown that 40 % of 60 nm polystyrene particles that were taken up could be recovered in non-lymphoid tissue of Sprague-Dawley rats after 5 days of gavaging (Hillery *et al*., 1994).

The human intestine undergoes constant regeneration, whereupon undifferentiated enterocytes generated from stem cells migrate from the crypts to the villus (small intestine) or the surface epithelium (colorectum) within 5 days. On their way, they begin to differentiate and become postmitotic before undergoing apoptosis followed by cell shedding (Ramachandran *et al*., 2000; Sancho *et al*., 2004). The Caco-2 cell line is derived from colonic epithelial adenocarcinoma cells. When grown under normal cell culture conditions, and after reaching confluence, they differentiate into small intestine enterocytes as the cells become polarised and express many of the characteristics of the enterocytes (e.g. tight junctions, microvilli, membrane transporters) (Chantret *et al*., 1988; Chopra *et al*., 2010). Therefore this cell line has been widely used as a model for *in vitro* studies looking at uptake of nutrients and pharmaceuticals. On the other hand, undifferentiated Caco-2 cells (i.e. prior to confluence in the exponential phase of growth) may resemble epithelial cells of the inflamed colon, as hyperproliferative epithelial cells have been shown to be present in ulcerative colitis (e.g.Huang *et al*., 1997; Arai *et al*., 1999), and they resemble human colonic cancer cells in a wide range of their gene expression patterns (Sääf *et al*., 2007). Thus, Caco-2 cells are a useful *in vitro* model to investigate both the pathological and the normal intestine.

Recently, using undifferentiated Caco-2 cells, we have demonstrated the DNA damaging and cytotoxic potential of various types of nanoparticles that might in the future occur in food or food-related products (Gerloff *et al*., 2009). Amongst other materials, we included amorphous silicon dioxide (SiO_2) particles which, as noted

above, is a relevant additive in foods and pharmaceutics (Schmidt and Riediker, 2008). Particulate zinc oxide (ZnO) was also investigated. In the undifferentiated Caco-2 cells, both SiO_2 and ZnO particles were found to induce cytotoxicity as well as oxidative DNA damage (Gerloff *et al*., 2009). The aim of the present study was to study the cytotoxic and pro-inflammatory effects of SiO_2 and ZnO particles in (i) undifferentiated compared to differentiated Caco-2 cells, and (ii) to examine the impact of gastrointestinal digestion of the particles on cellular response.

4.2 Methods

Materials. Two types of particle were used in the present study: amorphous SiO_2 (fumed; Sigma, Germany) with a mean primary diameter of 14 nm and specific surface area (SSA) of 200 m^2/g and ZnO (nanoactive: Nanoscale Materials Inc., US) with a reported crystallite size of ≤10 nm and SSA ≥ 70 m^2/g. The ZnO sample was kindly provided by Dr. Duffin (University of Edinburgh) who reported a SSA of 48.3 m^2/g by an independent analysis. Trypsin, Dulbecco's Ca^{2+}/Mg^{2+}-free phosphate buffered saline (PBS), ethylenediaminetetraacetic acid disodium salt dehydrate (EDTA), hydrogen peroxide (H_2O_2), nitric acid (HNO_3), deferoxamine (DFO), sucrose, fetal calf serum (FCS) and bovine serum albumin (BSA) were all purchased from Sigma (Germany). Minimum essential Medium (MEM) with Earle´s salts was obtained from Invitrogen (Germany). DC Protein Assay kit and iScript cDNA Synthesis Kit were purchased from BioRad Laboratories (USA). RNeasy Mini kit and QuantiFast SYBR Green PCR Kit were obtained from Qiagen (Germany). Calibration standard solutions (zinc or silicon solution 1000 ppm) were obtained from Fisher Scientific (UK), 1-hydroxy-3-carboxy-pyrrolidine (CPH) was purchased from Alexis Biochemicals (Germany). Cell Proliferation Reagent WST-1 and Lactate dehydrogenase (LDH) Cytotoxicity Detection Kit were purchased from Roche (Germany) and human IL-8 ELISA kit was obtained from Sanquin (The Netherlands). Spartan Syringe filters 0.2 µm were purchased from Whatman (Germany), ultrafiltration units (Vivaspin, 3kDa MWCO) were obtained from Sartorius Stedim Biotechnology (Germany). Interleukin-1β (IL-1β) was obtained from R&D Systems (USA). All other chemicals were from Merck (Germany). All assay kits were used according to the manufacturer's protocol.

Solubility and aggregation or agglomeration of the particles. The freshly-prepared particle suspensions were analysed using elemental mass analysis for both their soluble and insoluble (i.e. particulate) amounts. The latter was further subdivided into fractions < 200 nm, containing individual primary particles and/or smaller aggregates (generated by fusion of primary particles) and/or agglomerates (undispersed clusters of aggregates) (Donaldson *et al*., 2005), and fractions above 200 nm, consisting of large aggregates and/or agglomerates. Therefore, (i) the initial particle suspension (1 mg/ml serum free cell culture media), (ii) the suspension after

10 min of sonication as detailed below, and (iii) the final dilution as used in the cell culture experiments (i.e. 20 μg/cm^2), were filtered through a 0.2 μm syringe filter followed by ultrafiltration with a 3 kDa unit. This approach was applied to the original particles as well as to those that were subjected to the digestion simulation assay according to the procedure described below. Each fraction was analysed by inductively coupled plasma optical emission spectrometry (ICP-OES) for quantitative evaluation of elemental Si or Zn. The hydrodynamic size of the particle aggregates or agglomerates in suspension was also evaluated by means of laser diffraction using the Mastersizer 200 (Malvern Instruments). This measurement was performed only for the original (undigested) ZnO and SiO_2 suspensions immediately following sonication.

Inductively coupled plasma optical emission spectrometry (ICP-OES). The samples were digested in 5% HNO_3 and their elemental content was analysed using a JY2000-2 ICP Optical Emission Spectrometer (Jobin Yvon Horiba). External calibrations (0.1-20 ppm) were carried out for the determination of Si (251.611 nm) and Zn (202.551 nm).

Culture and treatment of the cells. The human colon adenocarcinoma cell line, Caco-2, was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) GmbH, Germany, and grown in MEM with Earle´s salts and Non Essential Amino Acids, supplemented with 20 % FCS, 1 % L-glutamine and 30 IU/ml penicillin–streptomycin. For the experiments, cells were trypsinised at near confluency and 4x10^4 cells per cm^2 were seeded into 6-well tissue culture plates (for particle cellular uptake, ELISA and qRT-PCR) or 96-well tissue culture plates (for the WST-1 assay) and grown overnight (undifferentiated cells) or for 10 days (differentiated cells). Prior to the experiments, the cells were incubated overnight (20 h) in serum free medium. All experiments were performed between cell passages 5 to 30. The SiO_2 and ZnO particles were suspended in serum-free culture medium. Two treatments were applied to the cells: (a) the suspension was sonicated (Sonorex TK52 water-bath; 60 Watt, 35 kHz) for 10 min and then directly added to the cells at the indicated concentrations or (b) particles were pre-incubated under simulated gastrointestinal pH-conditions (see below) and then applied to the cells.

Particle uptake. Cells were incubated in triplicate wells with 10, 20 or 40 µg/cm^2 particles at 37 °C for 1 h. The cell monolayer was washed three (SiO_2) or four (ZnO) times with DPBS containing 2 mmol EDTA to remove all loosely bound particles. To confirm removal of particles that were not taken up by the cells, the content of Si and Zn in the wash solutions was analysed by ICP-OES. For SiO_2, no remaining particles were detected in the wash solution from the second rinsing (data not shown). For ZnO, few particles (~6 %) could still be recovered within the 4th wash solution, but further washing repeats were not considered to prevent detachment of the cells. Then 2 ml HNO_3/H_2O_2 was added to the culture disks to lyse the cells overnight. After homogenisation by shaking, the Si or Zn content was determined by ICP-OES. To ensure that particles were not taken up by cells due to permeation of membrane damaged cells the LDH content of the media supernatants, after treatment, was determined using a commercial diagnostic kit according to the manufacturer's instructions (LDH leakage in each treatment < 5 %; data not shown). Additionally, triplicate wells were lysed with 2 ml 0.5 M NaOH for 15 min and homogenised by shaking for determination of total cell protein by the DC (detergent compatible) protein assay. Particle uptake was expressed in µg of Si or Zn per mg of cell protein.

Uptake inhibition. Two independent methods were used to inhibit particle uptake into the differentiated Caco-2 cells. For the inhibition of active energy-dependent particle uptake by destruction of microtubules, cells were incubated at 4 °C with the particles for 1 h on an ice slab in the incubator and washed with ice cold buffer. To block clathrin AP2-mediated endocytosis, cells were treated in hypertonic serum free cell culture medium with 0.45 M sucrose for 1 h. After the incubation period the particle uptake was determined as described above.

Simulation of gastrointestinal digestion. The SiO_2 and ZnO particles were used either directly after their suspension in serum-free medium and subsequent sonication (hereafter referred to as native particles, i.e. *SiO_2* and *ZnO*) or, alternatively, were pre-treated under gastro- and intestinal pH-conditions (referred to hereafter as "digestion simulated" particles, *SiO_2-DS* and *ZnO-DS*). The SiO_2 and ZnO particles were incubated at a concentration of 1 mg/ml for 30 min in 25 ml gastric solution (NaCl (34 mM) / HCl solution, pH 2.7). Following this acidic incubation, a 3.75 ml sample was taken for characterisation of dispersability of the

particles (solubility and aggregation/agglomeration) as described above, and the remaining suspension was neutralised to pH 7.5-7.7 with 2.5 ml intestinal solution, (a carbonate/bicarbonate buffer (50 mM), at pH 9.5 including 1.68 g of $NaCO_3$, 7.16 g $NaHCO_3$ and 4 g NaCl in 2 L H_2O) and incubated for a further 30 min. After the second incubation period the suspensions were characterised for dispersability as before. The simulated digestion was conducted at 37 °C under constant shaking. Finally, the samples were appropriately diluted in serum-free media to obtain the final particle suspension that Caco-2 cells were incubated with.

ROS measurements by Electron Paramagnetic Resonance (EPR). For detection of reactive oxygen species (ROS) formation, induced by the particles, 20 and 80 µg/cm^2 of SiO_2, ZnO, SiO_2-DS and ZnO-DS particles were preincubated for 30 or 60 minutes with the spin probe CPH (0.5 mM CPH (1-hydroxy-3-carboxy-pyrrolidine) in 10 µM DFO) for specific superoxide detection and analysed using a MiniScope MS200 Spectrometer (Magnettech, Berlin, Germany) at room temperature. The following instrumental settings were used: Magnetic field: 3360 G; sweep width: 97 G; scan time: 60 sec; number of scans: 1; modulation amplitude: 2000 mG. Data shown are calculated from the average peak amplitude of CPH characteristic triplet spectrum and expressed in arbitrary units (AU).

Cytotoxicity. Cytotoxicity was determined by the tetrazolium salt WST-1 for measurement of the metabolical activity of the cells. The cleavage of the tetrazolium salt WST-1 to formazan dye via mitochondrial dehydrogenases in the cells was measured using a commercial WST-1 diagnostic kit (Roche, Germany) in 96-well culture plates. Cells were incubated with various particle concentrations at different time points as described above. After the incubation period with the particles, the cells were incubated for a further 2 h (undifferentiated Caco-2 cells) or 30 min (differentiated cells) with WST-1 and analysed as per the manufacturer's protocol.

Enzyme-linked immunosorbent assay (ELISA). To measure IL-8 release, cells were treated with 20 µg/cm^2 particles or the positive control IL-1β (10 ng/ml) for 4 and 24 h. After the treatment period, cell monolayers were washed two times with PBS to remove loosely membrane-bound particles and detached (dead) cells and cell debris. Cell culture medium was collected, centrifuged (14000 rpm, 5 min at 4 °C) and

supernatants were frozen at -20 °C until analysis. IL-8 activity was determined by a commercial ELISA kit according to the recommendations of the supplier (Sanquin, The Netherlands).

RNA isolation and quantitative Real-Time reverse-transcription polymerase chain reaction (qRT-PCR). Cells were treated with 20 µg/cm^2 particles for 4 h as described above. Total RNA was isolated using a commercial RNeasy kit according to the manufacturer's instructions and 0.5 µg per sample was transcribed into complementary DNA using iScript cDNA Synthesis Kit according to the protocol. Total amount of mRNA was analysed in the Rotor-Gene Q (Qiagen) in 47 cycles, using 7.5 µl QuantiFast SYBR Green PCR Kit, 25 ng cDNA, and 0.5 µM primers. Human β-Actin was used as the internal reference gene. Primer sequences for IL-8 were 5'-ACT CCA AAC CTT TCC ACC C-3' (forward) and 5'-CCC TCT TCA AAA ACT TCT CCA C-3' (reverse), and for β-Actin 5'-CCC CAG GCA CCA GGG CGT GAT-3' (forward) and 5'-GGT CAT CTT CTC GCG GTT GGC CTT GGG GT -3' (reverse).

Statistics. All means were calculated from three independent cell experiments and each condition was investigated in triplicate wells in each experiment, with the error bars representing standard deviation (SD). Analysis of statistical significance was done by unpaired Student's t-test with *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ as levels of significance. Cytotoxicity data were analysed using ANOVA with Dunnett post-hoc comparison with *$p < 0.01$ and **$p < 0.001$. TD_{50} values were analysed by log-linear regression.

4.3 Results

Both particles used (i.e. amorphous SiO_2 and ZnO), were found to form large aggregates/agglomerates Figure 4.1 A shows the distribution of the amounts of Si or Zn quantified after fractionated filtration. For both types of material, the majority of particles were found to occur as large aggregates/agglomerates in each fraction (> 90 %). The amount of soluble material varied between 1 and 7 %. When considered on a mass basis, low to negligible amounts of Si or Zn were found to be in the < 200 nm fraction.

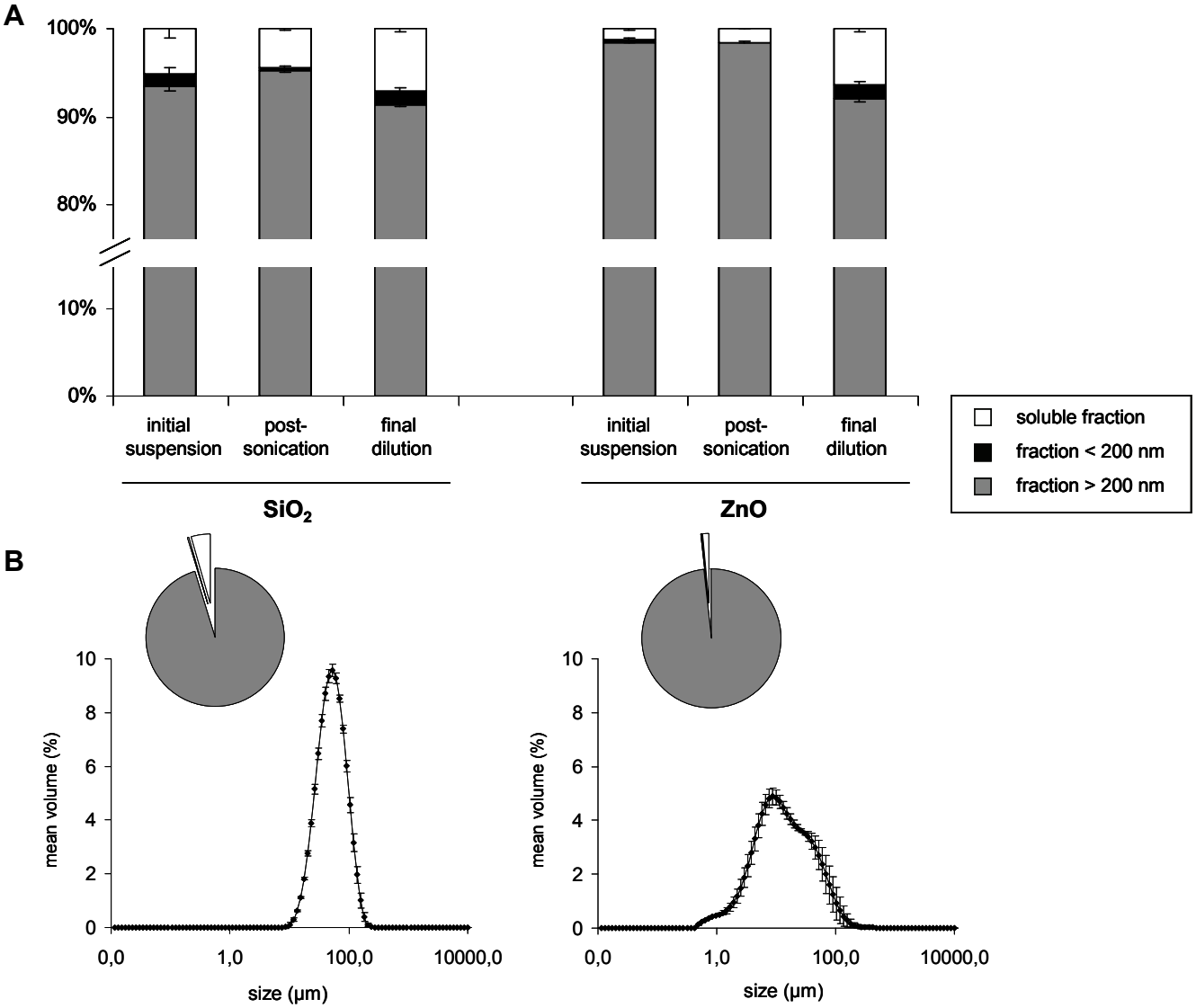

Figure 4.1 **Particle characterisation.** SiO_2 and ZnO particles were characterised for aggregate/agglomerate formation and solubility in serum free cell culture medium via fractionated filtration and quantitative element analysis (A). Final dilution = particle concentration used for cell incubation (20 µg/cm^2). Size distribution of post-sonicated SiO_2 and ZnO measured by laser diffraction (B). Values are expressed as mean and standard deviation (n=2-3 for A, and n=4 for B).

The presence of large aggregates/agglomerates in the post-sonicated fraction was confirmed by Mastersizer measurements as an independent method (Figure 4.1 B), revealing size ranges from 10-200 µm for SiO_2 and 1-100 µm for ZnO, respectively.

The ability of differentiated Caco-2 cells to take up the particles within one hour incubation is shown in Figure 4.2.

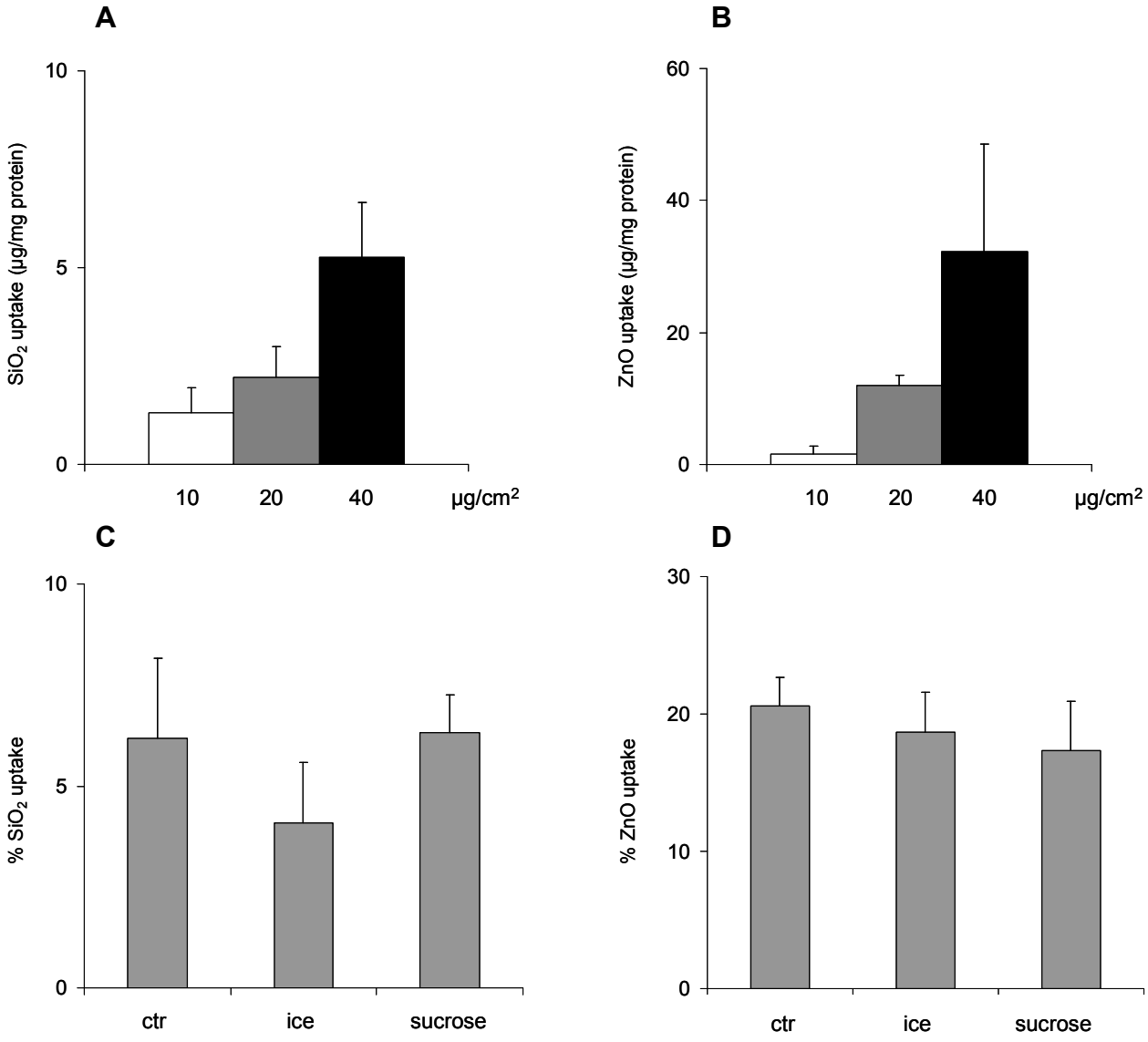

Figure 4.2 **Particle uptake and uptake inhibition in Caco-2 cells.** The uptake of SiO_2 (A) or ZnO (B) was determined in differentiated Caco-2 cells after treatment for 1 h at 10, 20 and 40 µg/cm^2. Inhibition of uptake of SiO_2 (C) or ZnO (D) particles was determined after 20 µg/cm^2 treatment by incubation at 4°C (ice) or introducing hypertonic conditions (sucrose, B). Values are expressed as mean and standard deviation, n=3.

Both SiO_2 and ZnO were taken up in a dose dependent manner, but ZnO uptake appeared to be much higher than uptake of SiO_2 (Figure 4.2 A, B). The

particle uptake was not apparently due to increased cell permeability (i.e. membrane damage) as after one hour incubation none of the doses tested induced significant leakage of LDH or, therefore, disruption of the cell membrane (we used a cytotoxicity cut-off of 5 %). To investigate possible uptake mechanisms, the particle-treated cells were either incubated on ice to reduce microtubule-dependent endocytosis or incubated with 0.45 M sucrose, since hypertonic conditions are known to inhibit clathrin AP2-mediated endocytosis (Figure 4.2 C, 4.2 D). The incubation on ice caused a slight reduction of the SiO_2 uptake, although this did not reach statistical significance. There was no reduction on the uptake of the ZnO particles when the cells were incubated at 4 °C. Similarly, the hypertonic conditions did not influence the uptake of either type of particle. Overall, therefore, uptake of particles onto the cells was likely to dominate over uptake into the cells.

To simulate gastrointestinal digestion processes, which could potentially alter the toxicity of particles after their oral intake, we developed a gastrointestinal assay. The amount of Si and Zn was determined in samples collected during all steps of this assay, namely, the treatment in gastric buffer, the additional treatment in neutralised intestinal buffer and the final diluted particle suspension used for the cellular experiment (Figure 4.3).

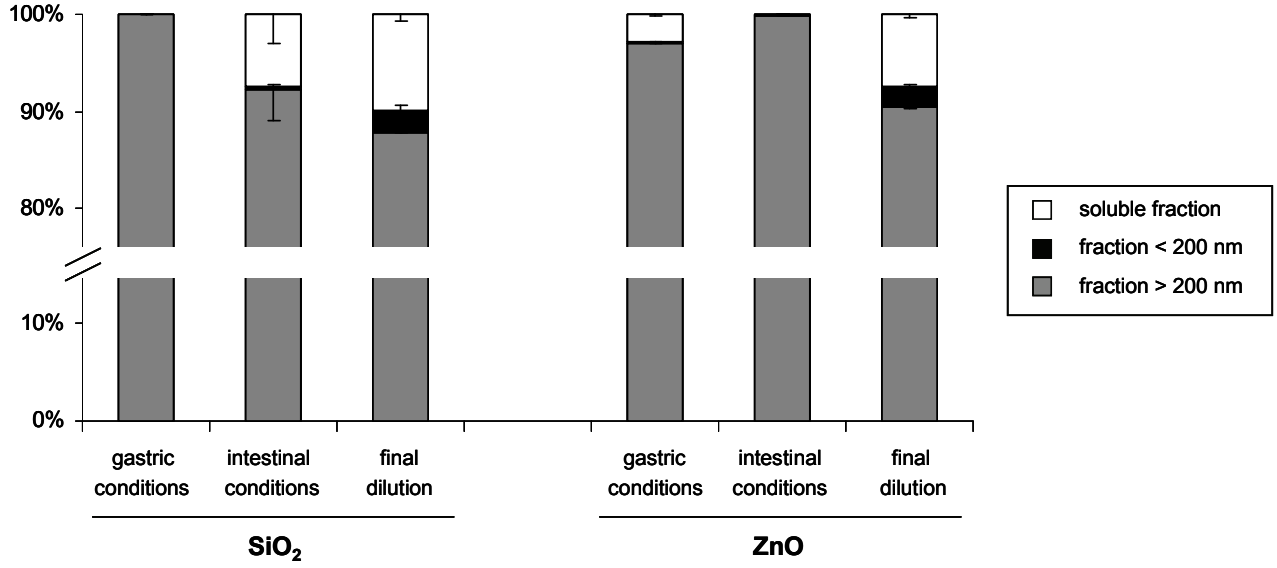

Figure 4.3 **Particle characterisation after gastrointestinal pH-treatment.** SiO_2 and ZnO particles were characterised for aggregate/agglomerate formation and solubility via fractionated filtration and quantitative element analysis after incubation in gastric pH-conditions followed by intestinal pH-conditions. Final dilution = particle concentration used for cell incubation (20 µg/cm^2) in serum free cell culture medium. Values are expressed as mean and standard deviation, n=2-3.

The agglomeration patterns of the final dilutions of the digested particles (SiO_2-DS and ZnO-DS; Figure 4.3) were similar to those of the un-digested (SiO_2, and ZnO), as can be seen in Figure 4.1 A. Suggesting particles were mainly present as large aggregates or agglomerates.

To determine the effect of the digestion simulation treatment on the surface reactivity of both types of particles, their ability to generate superoxide anion radicals ($O_2^{\cdot-}$) was determined by EPR spectroscopy using the spin probe CPH. Figure 4.4 A shows the time- and concentration dependent potential of both the native and the "digested" SiO_2 and ZnO particles to generate $O_2^{\cdot-}$. Interestingly, the formation of superoxide was significantly reduced after gastrointestinal simulation treatment of both particles followed by 60 minutes pre-incubation with CPH. Representative spectra of CPH control, digested and undigested SiO_2 (80 µg/cm^2, 60 min pre-incubation with CPH) are depicted in Figure 4.4 B.

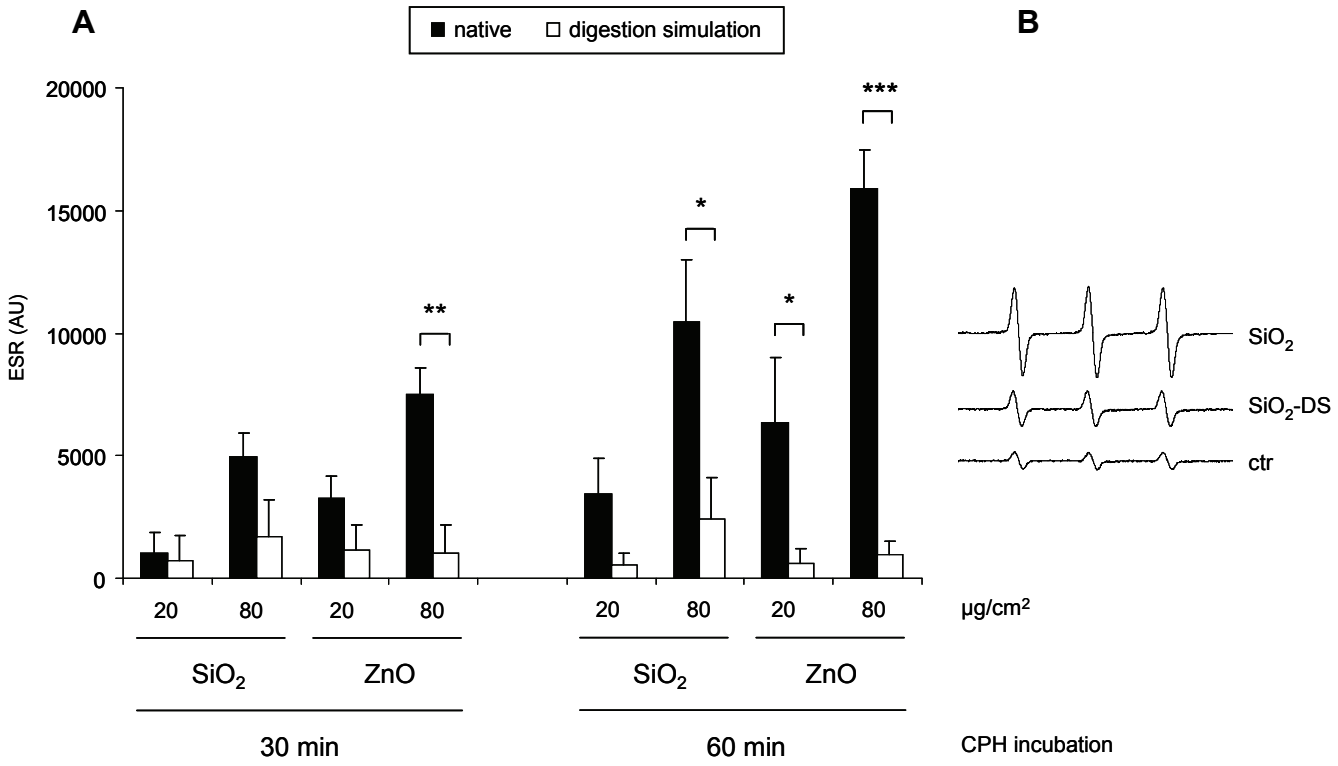

Figure 4.4 **Acellular ROS formation by SiO_2 and ZnO particles.** The formation of superoxide anion radicals was determined by EPR after 30 and 60 min pre-incubation with the spin probe CPH. 20 and 80 µg/cm^2 native or digestion buffers treated SiO_2 or ZnO were used (A). Representative spectra of CPH-control, 80 µg/cm^2 native or digested SiO_2 after 60 min pre-incubation (B). Values are expressed as mean and standard deviation, n=3.
* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$

To investigate whether the simulated gastric and intestinal digestion had an influence on cell viability, we performed the WST-1 assay after incubation of Caco-2 cells with the digested particle suspension for 4 (Figures 4.5, 4.6; A and C) or 24 h (Figures 4.5, 4.6; B and D). These experiments were performed both in undifferentiated and differentiated Caco-2 cells in order to address the possible impact of cell differentiation status on particle-induced toxicity. Results for the undifferentiated cells (i.e. at 70-80 % confluency) are shown in Panels A and B, while effects in the differentiated cells are depicted in Panels C and D of Figures 4.5 and 4.6.

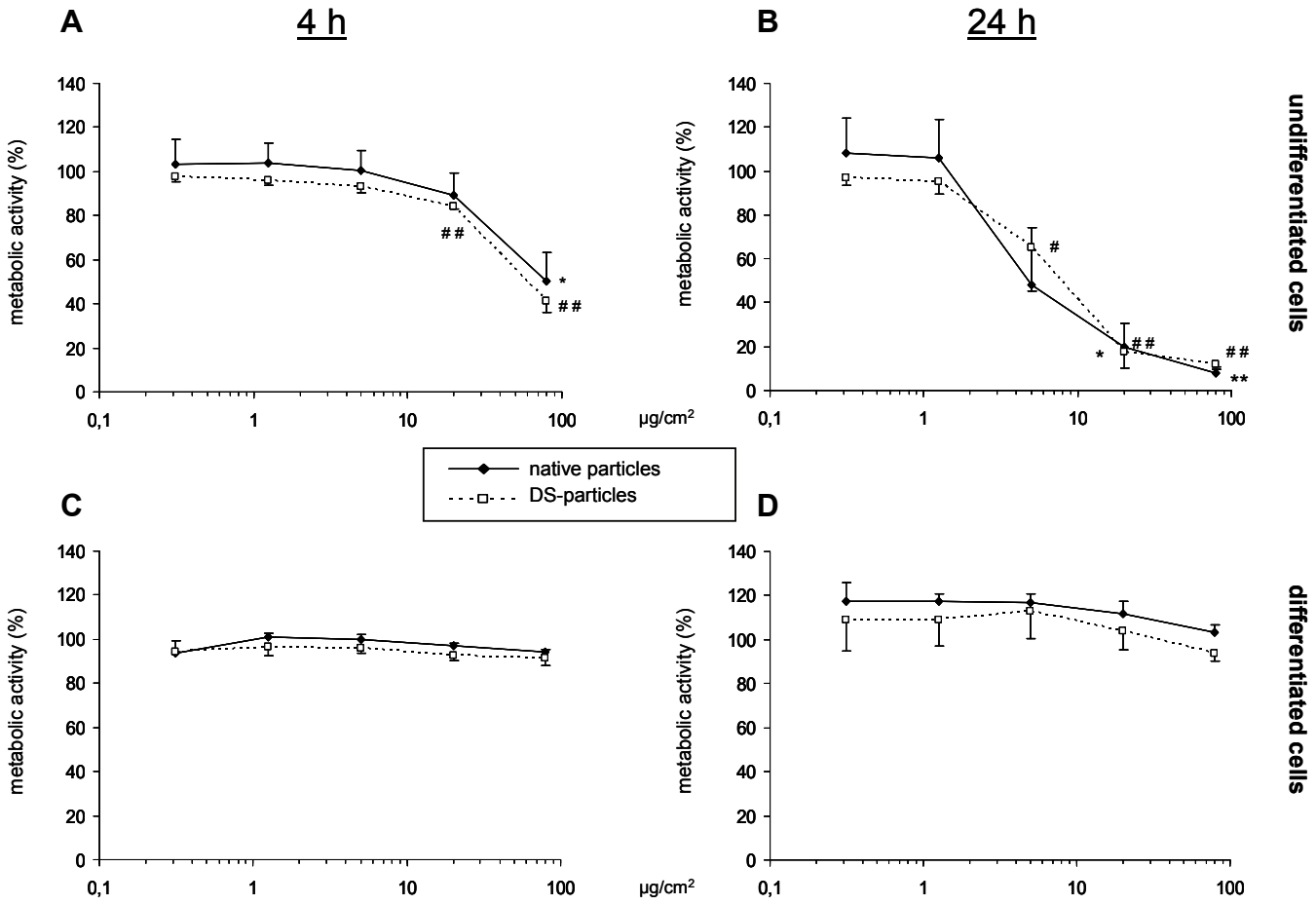

Figure 4.5 **Effects of SiO_2 on the viability of undifferentiated and differentiated Caco-2 cells.** Undifferentiated (A, B) and differentiated (C, D) Caco-2 cells were incubated with 0.3125, 1.25, 5, 20 and 80 µg/cm² native or pH-treated SiO_2 particles for 4 (A, C) and 24 (B, D) hours. Metabolic competence of the cells was determined via conversion of WST-1 as a marker of cell viability. Values are expressed as mean and standard deviation, n=3.
* $p < 0.01$ and ** $p < 0.001$ versus control for native particles
# $p < 0.01$ and ## $p < 0.001$ versus control for pH-treated particles

SiO_2 and SiO_2-DS were both found to reduce cell viability only in undifferentiated cells. For the 24h incubation, significant effects in the undifferentiated Caco-2 cells were even observed at low concentrations (5 µg/cm^2). In contrast, the differentiated cells were neither affected by SiO_2 nor by SiO_2-DS (Figure 4.5). Contrary to these observations, ZnO was found to be cytotoxic to both undifferentiated and differentiated cells after 24 h incubation, although considerably more so in the former (Figure 4.6).

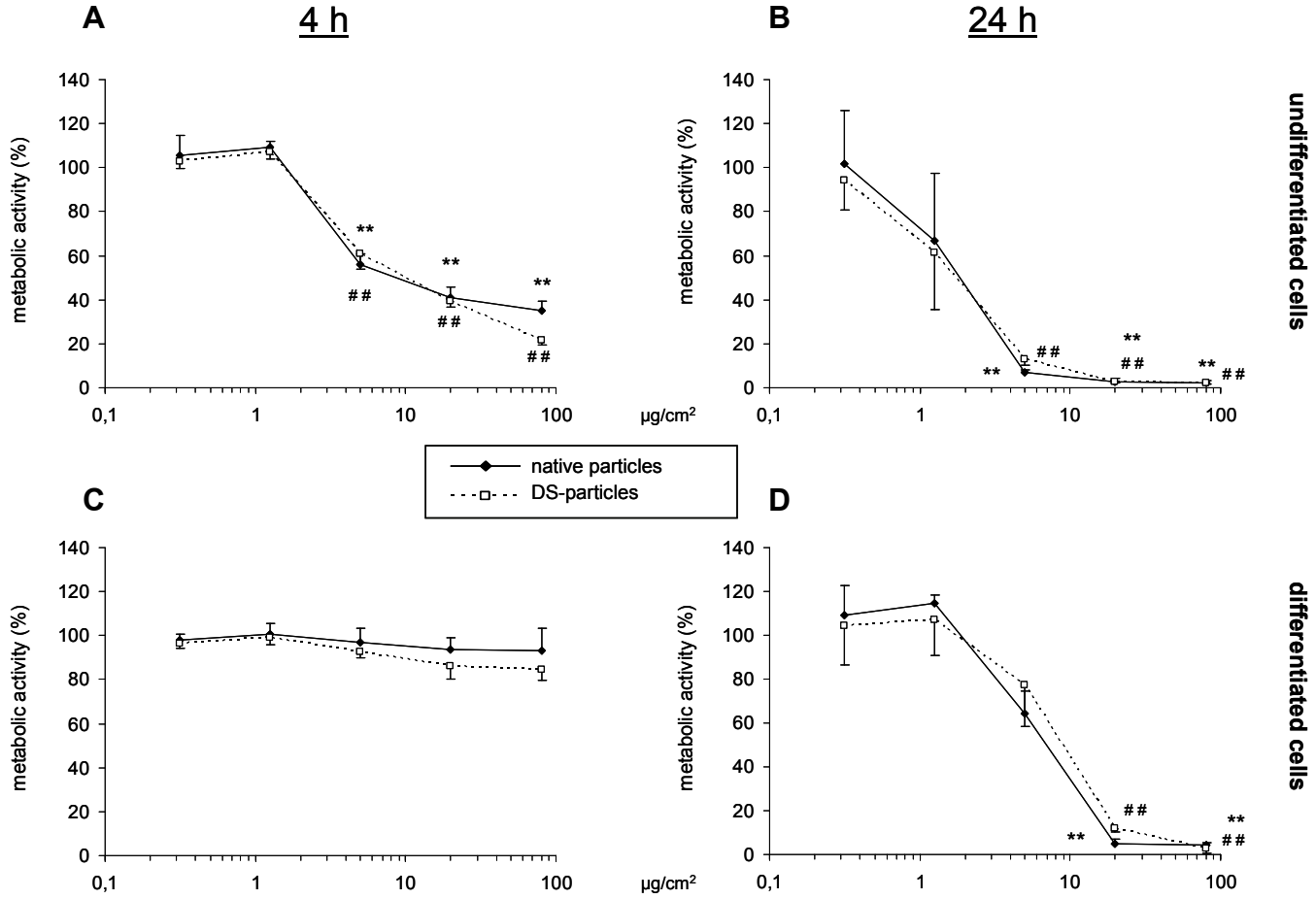

Figure 4.6 Influences of ZnO on the viability of undifferentiated and differentiated Caco-2 cells. Undifferentiated (A, B) and differentiated (C, D) Caco-2 cells were incubated with 0.3125, 1.25, 5, 20 and 80 µg/cm^2 native or pH-treated ZnO particles for 4 (A, C) and 24 h (B, D). Metabolic competence was measured as conversion of WST-1 as a marker of cell viability. Values are expressed as mean and standard deviation, n=3.
* $p < 0.01$ and ** $p < 0.001$ versus control for native particles
# $p < 0.01$ and ## $p < 0.001$ versus control for pH-treated particles

The TD_{50} values (24 h) in the undifferentiated cells were 1.74 ± 1.74 and 1.77 ± 1.6 for ZnO and ZnO-DS, respectively. For the differentiated cells, significantly higher TD_{50} values were obtained being, 8.99 ± 1.05 and 9.82 ± 2.98 for ZnO and ZnO-DS, respectively. The TD_{50} values did not differ between the native and the digested ZnO, indicating that the toxicity of these particles may be mostly dependent on the cell differentiation status. At 4 h, only the undifferentiated cells showed significantly reduced viability after treatment with ZnO at doses of 5 μg/cm^2 or higher. For all conditions tested, the simulated digestion of the particles did not significantly influence their toxicity as evaluated by the WST-1 assay.

To address potential pro-inflammatory effects of the particles, the mRNA expression and protein secretion of IL-8 was determined in both undifferentiated and differentiated Caco-2 cells. Results are shown in Figure 4.7 for SiO_2 and SiO_2-DS and in Figure 4.8 for ZnO and ZnO-DS, respectively.

In the undifferentiated Caco-2 cells, SiO_2 strongly induced mRNA expression of IL-8, with levels that were similar to those provoked by the positive control IL-1β. The upregulation was even higher after treatment with the SiO_2-DS particles. In differentiated cells, however, SiO_2 incubation could only induce a slight albeit non-significant upregulation of IL-8 mRNA expression. The effect was significantly lower than that induced in undifferentiated cells. IL-8 protein release from undifferentiated cells after 24 h was significantly increased after treatment with both the native and the digested SiO_2 particles (Figure 4.7 B). In the differentiated cells, an increase of IL-8 upon treatment with SiO_2 and SiO_2-DS was also observed, but this did not reach statistical significance.

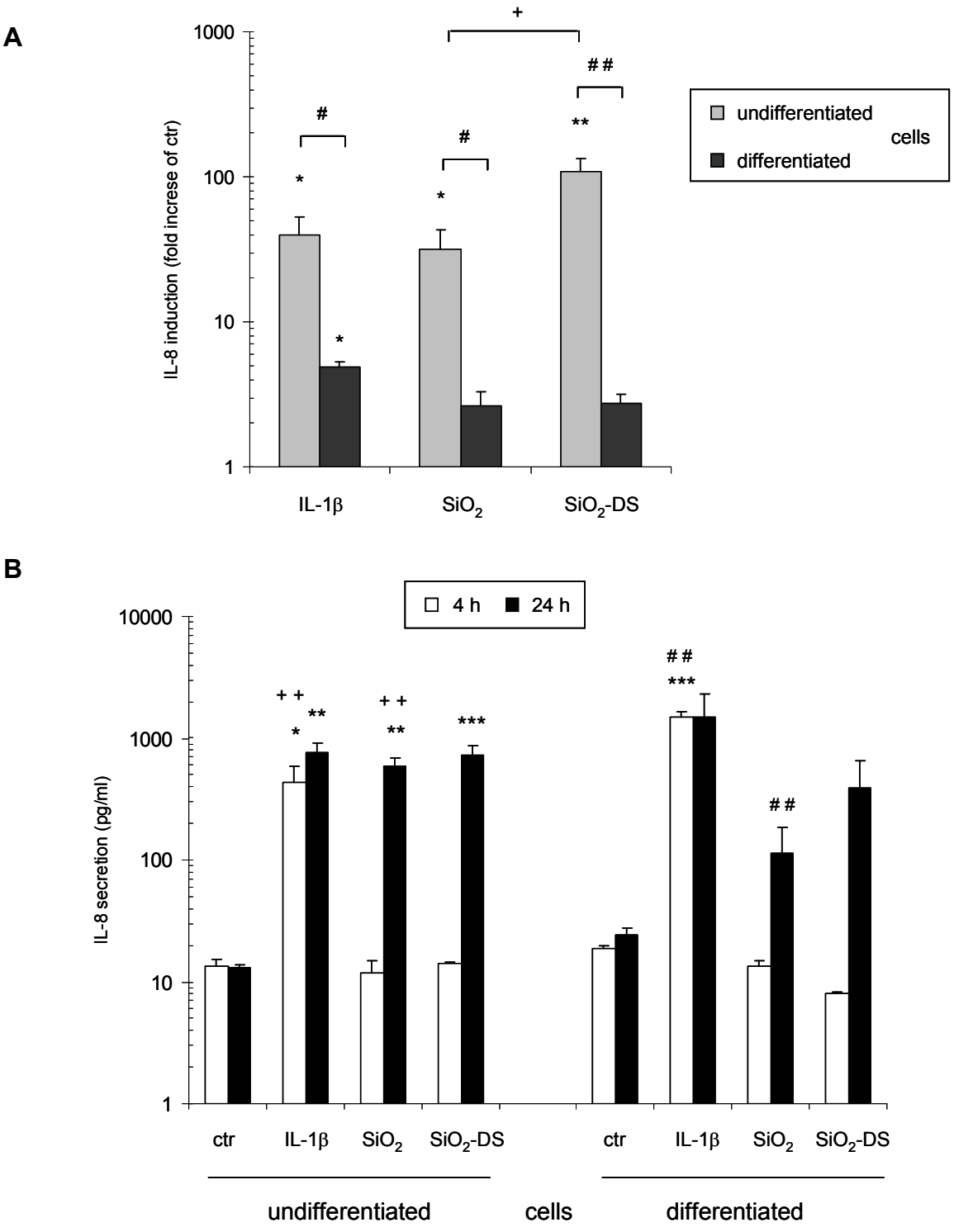

Figure 4.7 **Effects of SiO_2 particles on IL-8 mRNA expression and protein secretion after treatment of undifferentiated and differentiated Caco-2 cells.** Induction of IL-8 mRNA after 4 h incubation with 20 µg/cm^2 native or pH-treated SiO_2 was determined in undifferentiated and differentiated Caco-2 cells via qRT-PCR (A). The secretion of IL-8 after 4 and 24 h incubation with 20 µg/cm^2 native or pH-treated SiO_2 was determined in undifferentiated and differentiated Caco-2 cells by ELISA (B). IL-1β was used as a positive control. Values are expressed as mean and standard deviation, n=3.
* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ versus control
$^{+}$ $p < 0.05$ and $^{++}$ $p < 0.01$ native versus pH-treated particles
$^{\#}$ $p < 0.05$ and $^{\#\#}$ $p < 0.01$ differentiated versus undifferentiated cells

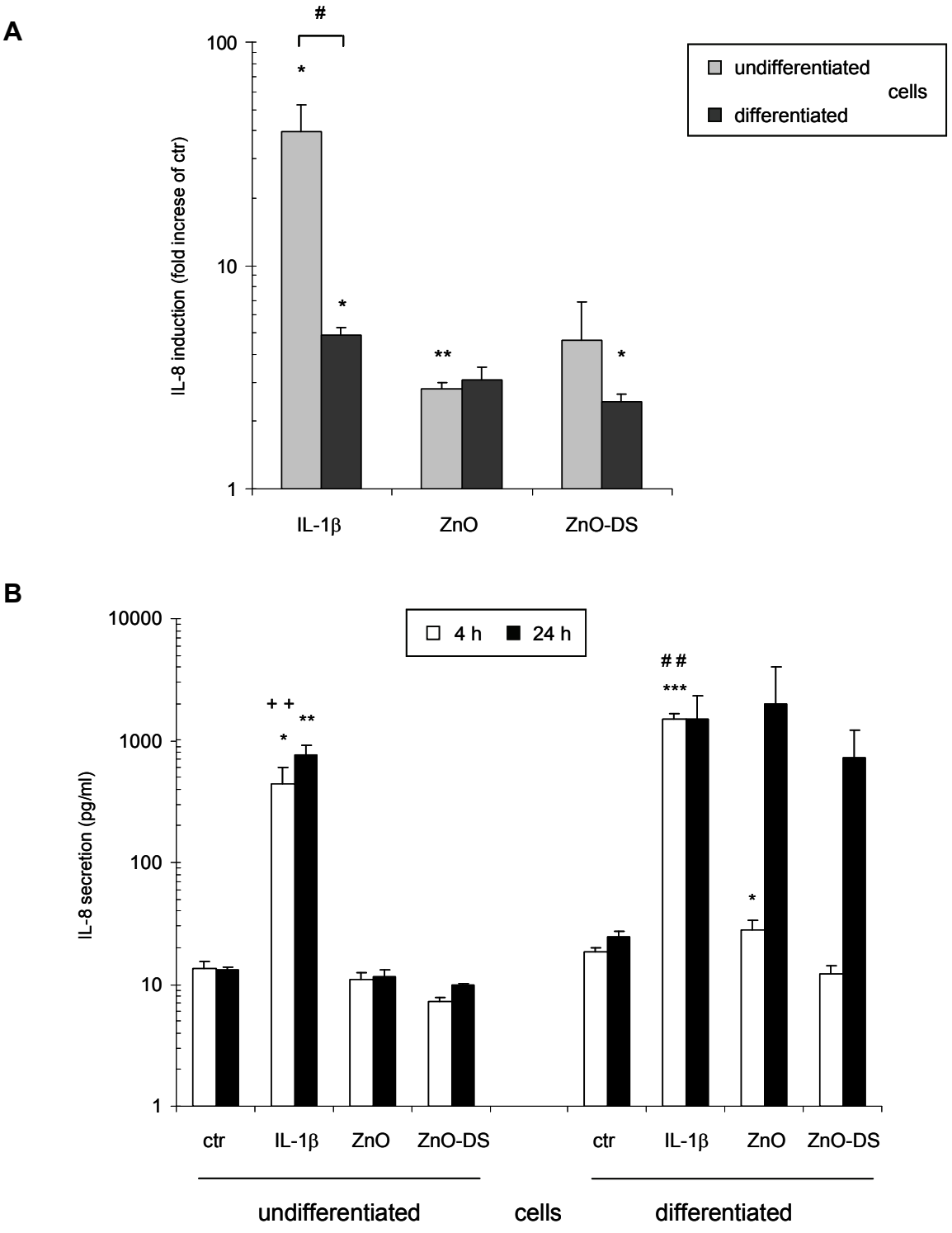

Figure 4.8 Effects of ZnO particles on IL-8 mRNA expression and protein secretion after treatment of undifferentiated and differentiated Caco-2 cells. Induction of IL-8 mRNA after 4 h incubation with 20 µg/cm^2 native or pH-treated ZnO was determined in undifferentiated and differentiated Caco-2 cells via qRT-PCR (A). The total secretion of IL-8 after 4 and 24 h incubation with 20 µg/cm^2 native or pH-treated ZnO was determined in undifferentiated and differentiated Caco-2 cells by ELISA (B). IL-1β was used as a positive control. Values are expressed as mean and standard deviation, n=3.
* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ versus control
$^{++}$ $p < 0.01$ native versus pH-treated particles
$^{\#}$ $p < 0.05$ differentiated versus undifferentiated cells

Incubation with ZnO resulted only in a slight upregulation of IL-8 mRNA levels in both undifferentiated and differentiated cells (Figure 4.8 A).

Significant increases in IL-8 mRNA expression were only found after treatment with the native ZnO in undifferentiated cells, and after treatment with the digested material (ZnO-DS) in differentiated cells. In contrast to the effects induced by SiO_2, undifferentiated cells did not respond to stimulation by ZnO in terms of IL-8 secretion (Figure 4.8 B). Surprisingly however, non-significant but notable IL-8 levels were observed in differentiated cells after 24 h incubation upon treatment with ZnO and ZnO-DS.

4.4 Discussion

In the present study, we characterised particle suspension of silicon dioxide (SiO_2) and zinc oxide (ZnO) particles and investigated their uptake, cytotoxic potential and pro-inflammatory properties on the intestinal Caco-2 cell line. Additionally, to mimic the behaviour of the particles after oral uptake, we have developed an assay to simulate the gastric and intestinal pH-conditions.

Particles were characterised by two independent methods, namely fractionated filtration combined with elemental analysis and laser diffraction. The first method revealed that both particle types where mostly present as large aggregates and/or agglomerates when suspended in serum free cell culture media. Less than 8 % of the material was soluble and only a negligible amount was present as small particles, aggregates and/or agglomerates (< 200 nm). Concordantly, size distribution measurement by using the Mastersizer revealed that both SiO_2 and ZnO particles are highly aggregated and/or agglomerated.

The uptake of both materials by the differentiated cells was clearly dependent on the treatment dose. Interestingly, ZnO particles were taken up more efficiently than SiO_2. Possible uptake mechanisms include adsorptive (Apodaca, 2001) and clathrin-mediated endocytosis (San Martin *et al.*, 2008; Kalgaonkar *et al.*, 2009). Involvement of endocytotic processes was determined by microtubules depolymerising and lowering of the energy availability, thus slowing down the whole process, applying a low incubation temperature of 4°C (Moriya *et al.*, 2005). The latter was more specifically inhibited by introducing hypertonic conditions via addition of sucrose, since it is known that such circumstances prevent the interaction of clathrin with AP2 (San Martin *et al.*, 2008; Kalgaonkar *et al.*, 2009). Interfering with either of the uptake mechanisms did not significantly reduce particle internalisation of both SiO_2 and ZnO, favouring adsorptive mechanisms as the chief form of uptake. In the gut, natural dispersants (like acids, mucin etc) may help to keep particles disaggregated but apical cell adsorption will at least first occur, making these data physiologically relevant.

The intestinal uptake of soluble Zn^{2+} ions can also be mediated by various other mechanisms, including transport via the human ZnT-like transporter 1 (hZTL1), which is also present on the apical membrane of Caco-2 cells (reviewed in Ford, 2004). However, involvement of this uptake pathway could only explain part of the

observed Zn uptake, since the solubility of the material used in our study is below 8 % whereas about 20 % of the ZnO was found to be associated with the cells. Moreover, this uptake mechanism could not explain our SiO_2 data since the uptake of the already low soluble SiO_2 fraction was found to be negligible (data not shown). This leads to the conclusion that presumably other mechanisms play an important role in the uptake of both materials, including phagocytosis, macropinocytosis or diffusion (Unfried *et al*., 2007).

To evaluate the cytotoxic and pro-inflammatory potential of our materials upon passage through the gastrointestinal tract, we developed an *in vitro* gastrointestinal assay based upon physiological pH conditions. The gastric pH ranges from 1.5-2.0 in the fasting state and might rise up to pH 7.0 after ingestion of a meal, and sodium and chloride are the most prominent elements within gastric acid (Powell *et al*., 1994; Lindahl *et al*., 1997; Hörter and Dressman, 2001). Therefore, we used a sodium chloride-based HCl buffer with pH 2.7, thus simulating the pH conditions that arise upon ingestion of a light meal. To mimic the subsequent pancreatic bicarbonate output, the suspension was neutralised with a carbonate/bicarbonate buffer to a pH of 7.5, which is representative of the intestinal conditions (Evans *et al*., 1988; Hörter and Dressman, 2001). To compare dispersibility of the digested particles to the undigested particles, fractionated filtration was performed after each digestion step as well as after preparing the final particle suspension. SiO_2 was only present as large aggregates and/or agglomerates at acidic pH, whereas at neutral pH around 8 % of the material was soluble. In the case of ZnO there was a small fraction of soluble material at acidic pH but again most of the material was present as large aggregates/agglomerates. In the final particle suspensions incubated with the cells, both particles showed similar dispersion behaviour (i.e. similar percentages of soluble, ca. 10 %; small aggregates/agglomerates, ca. 2 %; and large aggregates/agglomerates, ca. 90 %) and their dispersion was independent of the pre-digestion treatment.

The influence of the simulated digestion treatment on the behaviour of the particles in the cellular assay was investigated to determine if there were any alterations on the surface of the particle aggregates/agglomerates that could influence induction of ROS production, cytotoxicity or pro-inflammatory properties.

Firstly, we have observed that the superoxide-generating potential of both compounds was significantly reduced for the digested particles in comparison to the

native materials. This could be explained by surface modifications during acidic treatment, such as surface chemistry alterations and removal of possible contaminants including trace iron that might support $O_2^{\cdot -}$ formation by these materials (Fubini, 1998). Comparable effects have been reported for cobalt chrome particles, for which cyto- and genotoxic properties were found to be largely decreased upon macrophage phagocytosis, presumably due to surface modifications induced by the acidic conditions in the lysosomes of these cells (Papageorgiou *et al*., 2008). In contrast, for quartz particles (i.e. crystalline silica) an increased reactivity after chemical etching has been shown and this has been discussed as a relevant mechanism to explain its strong pulmonary toxicity after inhalation (reviewed in Fubini, 1998).

Secondly, in our study, the digestion treatment of the particles did not influence their cytotoxicity on Caco-2 cells as this was found to be mostly dependent on the cell's differentiation status. SiO_2 only reduced cell viability in undifferentiated cells whereas ZnO appeared toxic to both undifferentiated and differentiated Caco-2 cells after 24 h treatment. For differentiated cells, a higher dose of particles seems to be required to achieve comparable toxic effects on the cells. It should be noted however that upon confluency and subsequent differentiation, a 2 to 3-fold higher amount of cells was counted than in undifferentiated (semi-confluent) Caco-2 cell cultures. Therefore more cells face the same amount of particles and, as such, this influences the toxic effect measured by the WST-1 assay. Our observations stand in line with the work of Anelli and colleagues, showing a 2-fold difference in cytotoxicity to undifferentiated versus differentiated Caco-2 cells after treatment with lymphokine-activated natural killer cells (Anelli *et al*., 1993). This suggests that the state of differentiation might influence the response to toxic agents.

Differentiation of intestinal epithelial cells is a constant process in the healthy human gut (Huang *et al*., 1997; Ramachandran *et al*., 2000; Sancho *et al*., 2004), whereas ulcerative colitis and other inflammatory states of the intestine are, amongst others, marked by hyperproliferative epithelial cells, resulting in an expansion of an undifferentiated cell population (Huang *et al*., 1997). This process plays an important role in the immune response, as it is well known that differentiation of intestinal epithelial cells markedly reduces their pro-inflammatory response upon stimulation with IL-1β at early time points (2-12 hours) (Huang *et al*., 1997; Böcker *et al*., 2000). In our study we found similar effects after 4 h incubation with IL-1β, since the

induction of IL-8 mRNA expression was dramatically lower in differentiated Caco-2 cells in comparison to undifferentiated cells. However, these differential effects on the mRNA levels were not reflected on the secreted IL-8 protein level, as 24 h incubation with IL-1β led to a comparable IL-8 release for both differentiation states. Possibly, the gene expression of IL-8 in differentiated Caco-2 cells might be induced with slower kinetics than in undifferentiated cells, but nevertheless lead to comparable accumulated levels of secreted protein. Interestingly, we also observed a high pro-inflammatory potential of SiO_2 as the SiO_2-induced up regulation of IL-8 mRNA in undifferentiated cells at 4 h was comparable to (or for SiO_2-DS even higher than) the effects induced by IL-1β. After 24 hours, the IL-8 secretion induced by the native and digestion buffer treated particles was similar again. Although SiO_2 was capable of inducing IL-8 secretion from differentiated Caco-2 cells as well, this effect was not significant and lower than that found in undifferentiated cells. This low effect might be due to the increased amount of cells after confluency and differentiation, as discussed above. Surprisingly, ZnO only induced IL-8 secretion in differentiated cells, but mRNA up regulation was found to be low in both undifferentiated and differentiated cells.

The gastrointestinal assay applied in our current work allows a more reliable judgement regarding the possible hazards of particles that might be of relevance *in vivo*. Interestingly, the effects of conventionally prepared particles for cell culture experiments seemed to be comparable to more physiologically treated, (i.e. digested) particles for our endpoints. However, our current assay is limited to investigating pH effects of the gastrointestinal tract, and does not take into account the presence of proteins or various chemical or biological compounds including other endogenous or exogenous particles in the chyme, and bacterial flora that all might interact with our specifically tested materials. For example, it has been shown that bacterial components such as LPS can bind to nanoparticles which then, by serving as a carrier for these toxins, will lead to highly increased inflammatory responses, characterised by the release of IL-1β, IL-6 or TNF-α (Ashwood *et al*., 2007). Interestingly, the inflammation induced by the LPS-particle complex is more pronounced than that caused by incubation with either the particles or LPS alone. So, certain components might by able to even elevate the cytotoxic or pro-inflammatory potential of particles, and this needs to be considered in the hazard assessment of ingested particles.

In this study we have shown that simulated gastrointestinal digestion of SiO_2 and ZnO particles did not markedly alter the cytotoxic or pro-inflammatory potential of the materials. The reduction in the ROS generating properties, widely accepted to be relevant in nanoparticle toxicity, did not influence the endpoints measured in our study. This also suggests that the cytotoxic and pro-inflammatory potential of specific particles in intestinal epithelial cells is not driven by intrinsic ROS formation. However, it remains to be investigated whether the genotoxic potential (Gerloff *et al*., 2009) of the modified particles is altered. In contrast, cell differentiation appeared to play a far more important role in the toxic potential of SiO_2 and ZnO particles: undifferentiated Caco-2 cells were more sensitive towards particle toxicity and might therefore serve as an initial screening tool. The pro-inflammatory effects of particles on the cells appeared to be less dependent on cell differentiation than their cytotoxicity. Further research is needed to determine the relevance of the state of differentiation of intestinal epithelial cells for ingested particles *in vivo*, all the more since in patients suffering from inflammatory bowel disease this differentiation process is known to be imbalanced.

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Declaration

The manuscript is currently in preparation.
The experimental work presented was performed by Kirsten Gerloff. The impact on authoring this paper can be estimated in total with 80%.

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CHAPTER 5

In vitro and *in vivo* investigations on the effect of amorphous silica on DNA damage in the inflamed intestine

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Abstract

The discussion about possible adverse effects of ingested nanoparticles towards the human body is currently gaining much attention. One of the major contributors to regular nanoparticle ingestion includes amorphous SiO_2 which is already widely used in cosmetics, pharmaceutical products and foods. Recently we demonstrated that these particles are can cause cytotoxicity and oxidative DNA damage to human intestinal Caco-2 cells, as well as upregulation of the expression of the potent neutrophil chemoattractant interleukin-8. To elaborate whether the toxic potency of ingested nanoparticles differs between healthy individuals and those with (chronic) inflammatory bowel diseases (IBD) we have investigated the oxidative DNA damaging properties of SiO_2 (i) *in vitro* in Caco-2 cells during co-exposure to primary human neutrophils (PMN) and (ii) *in vivo* by evaluation of the effects of SiO_2 enriched chow in acute and chronic dextrane sulphate sodium (DSS) induced colitis in mice. Genotoxicity was evaluated by the formamidopyrimidine glycosylase (Fpg)-modified comet assay and immunohistochemistry for 8-hydroxydeoxyguanosine (8-OHdG). It was found that activated human neutrophils potently induce DNA strand breakage and oxidative lesions *in vitro*. However, SiO_2 did not lead to PMN activation, and no

PMN mediated DNA damage was determined in a co-culture model with Caco-2 cells. The outcomes of the *in vivo* study were in support of these findings: analysis of DNA damage in whole colonic tissue by the *in vivo* Fpg-modified comet assay did not reveal elevated strand breakage after receiving a SiO_2 enriched chow. However, a mild increase of 8-OHdG lesions was shown by immunohistochemical staining after chronic, but not after acute colitis induction.

5.1 Introduction

Throughout the last few years, the discussion about possible adverse effects of ingested nanoparticles towards the human body is gaining much attention. The use of nanoparticles, defined by a size smaller 100 nm, for food and food-related products becomes more popular (Oberdörster *et al.,* 2005; Chaudhry *et al*., 2008; Tiede *et al*., 2008; Schmid and Riediker, 2008; homepage nanotechproject.org). A daily ingestion of 10^{12}-10^{14} particles per day has been suggested already several years ago, with increasing tendency each year (Lomer *et al.*, 2001, 2004). One of the major contributors to regular nanoparticle ingestion includes the amorphous SiO_2 which is already widely used in cosmetics, pharmaceutical products and foods (Johnston *et al*., 2000; Chaudhry *et al*., 2008). SiO_2 in general is accepted as a common food additive (E551) and mainly used as an anticaking agent (homepage food.gov.uk). The uptake of such nanoparticles into the intestinal epithelium is still not fully understood and appears to vary with different particle compositions and applications (Unfried *et al*., 2007; Powell *et al*., 2010). In general, particle uptake by intestinal tissue is low (< 10 %) (Jani *et al*., 1994, Hillery *et al*., 1994) but reported to be a 15-250 fold higher for small particles (~110-120 nm) compared to larger particles (Desai *et al*, 1996). Intestinal particle internalisation is mainly driven by the so-called Peyer´s Patches, i.e. lymphoid tissue rich of specified M-cells (reviewed in Powell *et al*., 2010), but epithelial cells are also reported to be capable of considerable particle uptake (Hillery *et al*., 1994). Trouiller and co-workers recently showed marked systemic DNA damage and mutagenesis in mice after oral uptake of TiO_2 nanoparticles and suggested that this may be due to uptake and subsequent induction of systemic inflammation (Trouiller *et al*., 2009).

Recently, we have addressed the potential hazards of a selection of nanoparticles that are likely to find their way into engineered food or food packaging in the future. In these studies we observed for instance that nano-size SiO_2 and ZnO induce marked cytotoxicity, and also can cause oxidative DNA damage and the release of the pro-inflammatory mediator interleukin-8 from human intestinal Caco-2 cells. In contrast, TiO_2 only showed rather negligible cytotoxic and DNA damaging effects in our investigations (Gerloff *et al*., 2009, Gerloff *et al*., in preparation). Remarkably however and contrasting to findings in lung epithelial cells (e.g. Singh *et al*., 2007; Monteiller *et al*., 2007), the aforementioned effects could not be predicted

by the primary particle size or specific surface area of the different compounds (Gerloff *et al*., 2009, Gerloff *et al*., submitted). In the respiratory tract, particles of low solubility and toxicity, such as TiO_2 and carbon black, have been shown to cause inflammation in proportion to their specific surface area. This observation is of major importance for risk assessment of inhaled particles, since their adverse effects have been demonstrated to be associated with their intrinsic inflammatory potency (Oberdörster *et al*., 2005; Duffin *et al.,* 2007). In contrast, relatively little is known about possible inflammatory properties and possibly associated effects of nanoparticles in the gut. In the respiratory tract, the particle-driven inflammatory response is associated with tissue damage, remodelling and mutagenesis (Donaldson *et al*., 2005; Duffin *et al*. 2007; Schins and Knaapen, 2007). An important player in the relationship between inflammation and carcinogenesis is the formation of reactive oxygen species (ROS) during inflammatory phagocyte respiratory burst (Babbs, 1992; Knaapen *et al*., 2006). Indeed, activated polymorphonuclear neutrophils (PMN) have been shown to cause oxidative DNA damage in rat lung epithelial cells (Knaapen *et al*., 1999; Knaapen *et al*., 2002a), and the amounts of recruited neutrophils was shown to be correlated with genotoxic and mutagenic effects in the lung epithelium of particle-exposed rats (Driscoll *et al*., 1997; Knaapen *et al*. 2002b).

Inflammatory effects of particles might also play an important role in particle risk assessment for the intestine. For example, bacterial components such as LPS are shown to bind to TiO_2 particles which then, by serving as a carrier for these toxins, may lead to highly increased inflammatory responses in peripheral blood mononuclear cells, characterised by the release of various inflammatory cytokines. Interestingly, the inflammatory response induced by the LPS-particle complex is synergistic as it was shown to be more pronounced than that caused by incubation with either the particles or LPS alone (Ashwood *et al*., 2007). Since large amounts of bacterial compounds are present, such particle-endotoxin complexes might be easily formed within the colon. Transportation of these bacterial composites into the mucosa via binding to nanoparticles might lead to aggravation of inflammatory events induced by unintentional ingestion of nanoparticles via food uptake and exacerbate inflammatory bowel diseases (IBD) as ulcerative colitis. However, the influence of particles on the etiopathology of IBD is currently under discussion but not yet confirmed (Lomer *et al*., 2001, 2004; Schneider *et al*., 2007).

The main hallmark of colitis is the imbalance of the intestinal immune system. This is characterised by continuous migration of activated lymphocytes, granulocytes and macrophages into the mucosa as well as an increased production of various pro-inflammatory cyto- and chemokines (Mitsuyama *et al*., 1994; Rogler and Andus, 1998; MacDermott, 1999). In colonic tissue of colitis patients, increased levels of the potent chemoattractant interleukin-8 (IL-8), secreted by epithelial cells, macrophages or fibroblasts, are found. These elevated IL-8 levels are directly linked to the attraction and thus the infiltration of neutrophils, and therefore also correlate with the grade of local inflammation (Kunkel *et al*., 1991; Mazzucchelli *et al*., 1994; Mitsuyama *et al*., 1994; Kucharzik and Williams, 2002-2003). The respiratory burst of activated neutrophils will lead to an increased production of reactive oxygen species (ROS) that can further enhance inflammation and may introduce DNA damage. Persistent activation of neutrophils and ROS has been considered to contribute to carcinogenesis in the respiratory tract as well as the intestine (Itzkowitz *et al*., 2004; Knaapen et al., 2006; Westbrook *et al.,* 2009).

Recently, we have found that amorphous SiO_2 is capable of inducing cytotoxicity, oxidative DNA lesions and pro-inflammatory responses in Caco-2 cells, including the upregulation of mRNA expression and protein secretion of IL-8 (Gerloff *et al*., 2009, Gerloff *et al*., in preparation), one of the most prominent cytokines expressed by these cells (Jung *et al*., 1995). In the present study, we aimed to investigate the potential consequences of the pro-inflammatory properties of SiO_2 for DNA damage induction in intestinal cells using both an *in vitro* and *in vivo* model of intestinal inflammation. For the *in vitro* experiments, we used a co-incubation model composed of Caco-2 cells and primary human neutrophils. Using this co-incubation, we analysed the potential genotoxic effects of SiO_2 nanoparticles in Caco-2 cells in the presence or absence of neutrophils. For the *in vivo* experiments, we used a colitic mouse model using dextrane sulphate sodium (DSS), known to induce experimental ulcerative colitis by disturbance of the epithelial integrity (Wirtz and Neurath, 2007). Upon inducing either acute or chronic inflammation, the colon was analyzed for DNA strand breakage and oxidative DNA damage.

5.2 Methods

Materials. Trypsin, Dulbecco's Ca^{2+}/Mg^{2+}-free phosphate buffered saline (PBS), agarose, low melting point (LMP) agarose, Triton X-100, DMSO, ethidium bromide, fetal calf serum (FCS), phorbol-12-myristate-13-acetate (PMA) and deferoxamine (DFO) were all purchased from Sigma (Germany). Minimum essential Medium (MEM) with Earle´s salts, Hanks´ Balanced Salt Solution (HBSS), penicillin/streptomycin were purchased from Invitrogen (Germany). Lymphoprep was obtained from Axis-Shield (Norway). 1-hydroxy-3-carboxy-pyrrolidine (CPH) was purchased from Alexis Biochemicals (Germany), lucigenin (N,N´-Dimethyl-9,9´-biacridinium dinitrate) was obtained from Fluka and RNeasy Fibrous Tissue Mini Kit was from Qiagen (Germany). Dextran sulfate sodium (DSS; mol wt 36.000-50.000) was obtained from MP Biomedicals (USA). Hemocare was from Carediagnostica (Germany). The antibody against 8-OHdG was obtained from the Japan Institute of Aging, mouse IgG was purchased from Vector Laboratories (USA). Histomouse^{TM}-SP Kit was sourced from Zymed Laboratories (USA) and DePex was obtained from Serva (Germany). Formamidopyrimidine-glycosylase (Fpg)-enzyme was kindly provided by Dr. Andrew Collins (Institute for Nutrition Research, University of Oslo, Norway). All other chemicals were from Merck (Germany).

Nanoparticles. SiO_2 (amorphous Silica, fumed; Sigma, Germany) was used for all experiments in present study. The sample has been reported to have a surface area according to according to Brunauer, Emmett and Teller (BET) of 200 m^2/g and a mean primary particle diameter of 14 nm. For the *in vitro* studies, SiO_2-nanoparticles were suspended in HBSS, sonicated for 10 min (Sonorex TK52 water-bath; 60 Watt, 35 kHz) and then directly added to the cells at the indicated concentrations. Although the primary size of the SiO_2 particles is well in the nano-size range, the material is well-known to occur as aggregates/agglomerates. This was also confirmed by Mastersizer analysis in the suspensions used for cell treatment (Gerloff *et al*., submitted). For the *in vivo* study, chow was purchased from Ssniff (Germany), either supplemented with or without 0.1 % w/w SiO_2 (SiO_2-chow).

Animals. C57BL/6 mice were originally purchased from Harlan-Winkelmann and bred at the IUF animal facility under specific pathogen free (SPF) conditions. The

animals were housed and maintained in an accredited on-site testing facility under SPF conditions, according to the guidelines of the Society for Laboratory Animals Science (GV-SOLAS). Food and water were available ad libidum. Female mice were used at 9 weeks of age. Mice were painlessly sacrificed in a CO_2 chamber, according to German guidelines. The experiment was performed with the permission of the Regierung von Nordrhein-Westfalen, Germany.

Cell culture and co-incubation. The human colon adenocarcinoma cell line Caco-2 was obtained from the *Deutsche Sammlung von Mikroorganismen und Zellkulturen* (DSMZ) GmbH**,** Germany and grown in MEM with Earle´s salts and Non Essential Amino Acids, supplemented with 20 % FCS, 1 % L-glutamine and 30 IU/ml penicillin–streptomycin. For experiments, cells were trypsinised at near confluency and $4x10^4$ cells per cm^2 were seeded into 60 mm culture plates and grown overnight. Experiments were performed in HBSS between cell passages 5 to 30 after starvation of the cells for 20 h in serum free medium. Neutrophils (PMN) were isolated freshly from blood of healthy, non-smoking volunteers as described in Knaapen *et al*. (1999) using Lymphoprep. PMN were suspended in HBSS (+ Ca^{2+}/Mg^{2+}) and counted using a Neubauer chamber. Cell viability was tested via Trypan Blue staining (viability > 95%). PMA (100 ng/ml) was used for PMN-activation. For the co-incubation experiments, PMN were incubated with PMA or SiO_2 and added directly to the Caco-2 cells for 30 min at 37°C at the indicated ratios. SiO_2 particles were administered to the cell cultures at the indicated concentration immediately after sonication as described above. After treatment, cell monolayers were rinsed twice with PBS to remove excess of extracellular particles, neutrophils and detached (dead) cells or cell debris.

ROS measurements by luminescence and Electron Paramagnetic Resonance (EPR). ROS production was measured using either lucigenin-enhanced chemiluminescence or EPR spectroscopy. For luminescence, PMN (2.5 mil/ml) were stimulated with SiO_2 (31.25 and 312.5 µg/cm^2) or PMA (100 ng/ml) in a white MaxiSorp^{TM} 96-well plate (Nunc, Germany). Subsequently, 0.25 mM lucigenin was added to preferentially detect superoxide formation and chemiluminescence was recorded directly for 50 min at 37°C using a Luminometer (Multi-Bioluminat, Berthold,

Germany). Results were expressed as the area under the curve (RLU=relative light units).

For EPR spectroscopy the spin probe CPH was used. Caco-2 cells were previously seeded in a 96 well plate and grown to 70-80 % confluency before 30 min co-incubation with PMN (1.3 million/ml) at 37°C in the presence of 0.5 mM CPH in 10 µM DFO. Furthermore, PMN alone were preincubated with 20 or 80 µg/cm^2 SiO_2 for 30 min at 37°C in the presence of 0.5 mM CPH in 10 µM DFO. ROS generation was evaluated using a MiniScope MS200 Spectrometer (Magnettech, Berlin, Germany) at room temperature using the following instrumental settings: Magnetic field: 3360 G; sweep width: 97 G; scan time: 60 sec; number of scans: 1; modulation amplitude: 2000 mG. Data shown are calculated from the average peak amplitude of CPH characteristic triplet spectrum and expressed in arbitrary units (AU).

Colitis induction in C57/BL6 mice. Induction of chronic and acute colitis was performed by a method previously described, with some modifications (Okayasu *et al*., 1990). In short, mice were pre-treated with conventional or SiO_2-enriched chow for 14 days. Chronic colitis was induced by 3 cycles of DSS-treatment. Each cycle consisted of one week of 2 % (wt/vol) DSS in acidified drinking water ad libidum, while the mice received normal chow, followed by two weeks on normal drinking water and SiO_2-chow. After the last cycle, mice were sacrificed on day 77 for further analysis. For induction of acute colitis, mice were pre-treated with SiO_2-chow for 17 days. Then colitis was induced by 2 % DSS in drinking water for six days. During DSS-treatment, mice were offered normal breeding chow ad libidum. After day six, mice were left for additional two days, in which they were set on SiO_2 chow and normal drinking water, after which they were sacrificed for further analysis. Thus, both the acute and the chronic exposure model represented each 4 different treatment groups, i.e. the i.e. (i) animals without any treatment, (ii) animals treated with DSS, (iii) animals treated with SiO_2, and finally, (iv) animals exposed to both DSS and SiO_2. To evaluate the severity of disease, colon length was assessed as an established marked. A clinical score according to Cooper *et al*. (1993), which consists of bleeding intensity, weight loss and stool consistency was determined. Scores of these three categories were added and divided by three. In short, rectal bleeding was scored as 3, hemocare+ (i.e. the detection of minor amounts of haemoglobin in the faeces) as 2 and no bleeding was scored as 1. For weight loss,

more than 20% weight loss was scored as 4, 10 - 20% as 3, 0 – 10% as 2 no weight loss was scored as 1. For liquid stools 3 points were given, pasty, soft stools were scored as 2 and well-formed pellets were scored as 1.

Detection of oxidative DNA damage in Caco-2 cells and in cells obtained from mouse small intestine or colon tissues by Fpg-modified comet assay. The Fpg-modified comet assay was used to determine DNA strand breaks and alkali labile sites as well as specifically oxidative DNA damage in the Caco-2 cells, based on the method by Speit *et al*., (2004) and modifications as described earlier (Gerloff *et al*., 2009). Comet appearances were analyzed using an Olympus BX60 fluorescence microscope at 400× magnification. A comet image analysis software program (Comet Assay II, Perceptive Instruments, Haverhill, UK) was used for quantification of DNA damage by analysis of % DNA in tail. A total of 50 cells were analyzed per slide per experiment. A detailed description of the method in Caco-2 cells is provided in Gerloff *et al.* (2009). For the *in vivo* comet assay, after sacrificing the colons of the mice were removed and flushed with ice cold PBS. The entire colon was divided into 3 equal pieces, and one third of each piece was used to assess DNA strand breakage and oxidative DNA lesions. The tissue pieces of each colon were then pooled and processed for the *in vivo* Fpg-modified comet assay as described in Risom *et al*., 2003 with minor modifications. Shortly, the tissue samples were minced with the plunger of a syringe in 1ml ice cold *in vivo* comet assay buffer (IVCAB, 0.14 M NaCl, 1.47 mM KH_2PO_4, 2.7 mM KCl, 8.1 mM Na_2HPO_4 and 10 mM EDTA, pH 7.4). The cell-homogenate was filtered through a 40 µm sieve and subsequently centrifuged at 180 g for 10 min at 4°C. Supernatant was discarded and the pellet resuspended in 50µl IVCAB. Afterwards, 25 µl of the suspension were mixed with 235 µl 0.5 % low melting point agarose and applied to pre-coated slides (coated with 1.5 % agarose). From here, the Fpg-modified comet assay was performed as described for the *in vitro* measurements (Gerloff *et al*., 2009). Data are shown as % comet tail values for each individual animal in the presence or absence of Fpg, i.e. % tail DNA_{+Fpg} and % tail DNA_{-Fpg}, respectively. Moreover, results are depicted as the calculated group means and standard deviations (SD) of the differences in % tail DNA as measured in the presence or absence of the Fpg enzyme: delta (∆)Fpg = [% tail DNA_{+Fpg}] – [% tail DNA_{-Fpg}].

Colon fixation and immunohistochemistry of 8-hydroxydeoxyguanosine. Three colonic sections of each animal were removed, fixed in 4% paraformaldehyde/PBS and paraffin embedded. The tissue sections were mounted on slides and stained for 8-OHdG. Mouse IgG staining was used as a negative control. RNA digestion was performed using RNase (100 µg/ml) in Tris buffer (5 mM Tris, 1 mM EDTA, pH 7.5; 60 min at 37 °C) and DNA-denaturation was conducted by 70 mM NaOH with 0.14 M NaCl and 40 % Ethanol. Zymed Histomouse^{TM}-SP Kit was used according to the supplier's manual to block unspecific binding. The sections were then incubated over night with a primary antibody against 8-OHdG (1:250) or against IgG (1:250) as a negative control and counter stained with hematoxylin. After washing with water slides were dehydrated and covered in DePex. Slides were analysed using a light microscope (Olympus BX60).

Statistics. For all experiments, all means were calculated from three independent experiments, with the error bars representing standard deviation (SD). Analysis of statistical significance was done by unpaired Student's t-test with *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ as levels of significance.

5.3 Results

The potential of human primary blood derived neutrophils (PMN) to induce DNA strand breakage and/or oxidative DNA damage in human intestinal Caco-2 cells was evaluated using a co-incubation model consisting of both cell types. As depicted in Figure 5.1, only activated neutrophils were able to induce both DNA strand breaks and, more pronounced, oxidative lesions. A non-significant induction of oxidative damage was already observed at a 1:1 ratio of both cell types, whereas a 3:1 ratio (PMN:Caco-2) resulted in significant DNA strand breakage and oxidative DNA damage.

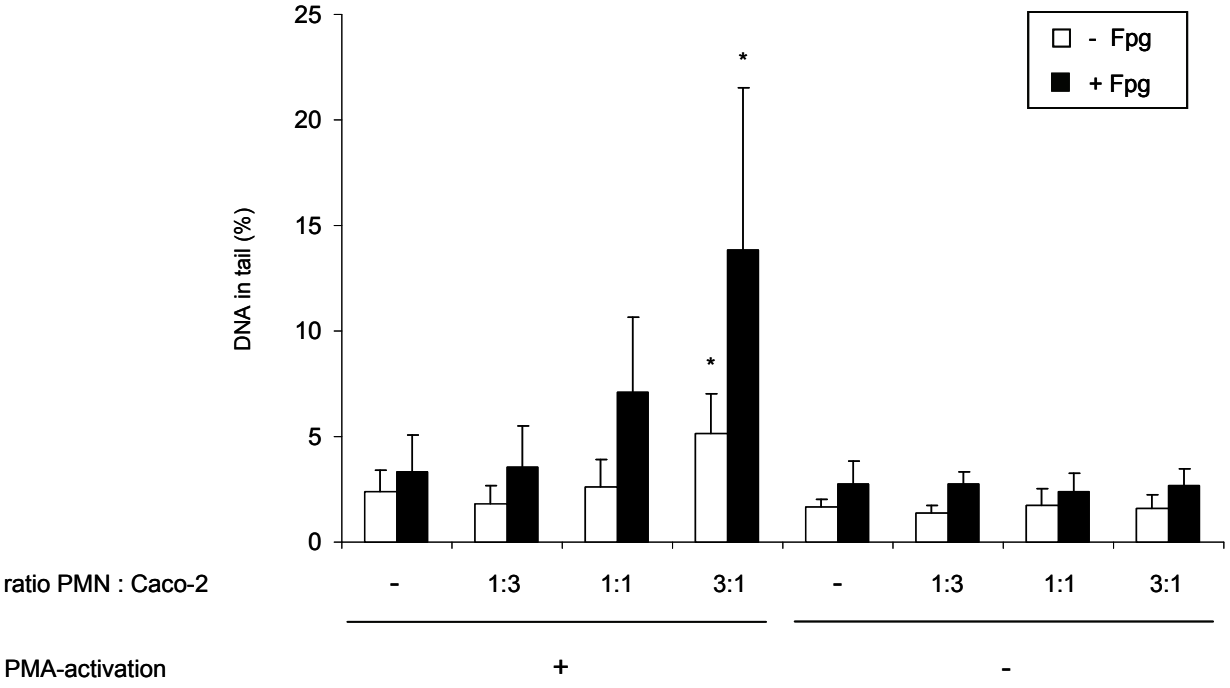

Figure 5.1 **DNA strand breakage and oxidative DNA damage in Caco-2 cells upon co-exposure to activated PMN.** DNA strand breakage and oxidative DNA damage were determined in Caco-2 cells using the Fpg-modified comet assay following 30 minutes co-incubation with PMA-activated or non-activated primary human neutrophils (PMN) at the indicated PMN:Caco-2 ratios in $HBSS^{+/+}$. PMA=phorbol-12-myristate-13-acetate. Values are expressed as mean and standard deviation, n=3.
* $p < 0.05$ versus control

Next, we investigated the ROS formation in this co-incubation model using EPR with the spin probe CPH, which has been introduced for the specific detection of superoxide anion radicals ($O_2^{\cdot -}$). Caco-2 cells alone, either with or without the addition of PMA, did not induce any detectable $O_2^{\cdot -}$ production (Figure 5.2). Non-

activated PMN were capable of mild ROS-formation, but this production could no longer be detected in the unstimulated co-incubation with Caco-2 cells. PMA-activated PMN did cause a pronounced and significant ROS generation, both in the absence and presence of Caco-2 cells. Interestingly, this PMN-induced ROS generation was slightly reduced in the presence of the intestinal epithelial cells although this reduction was not significant.

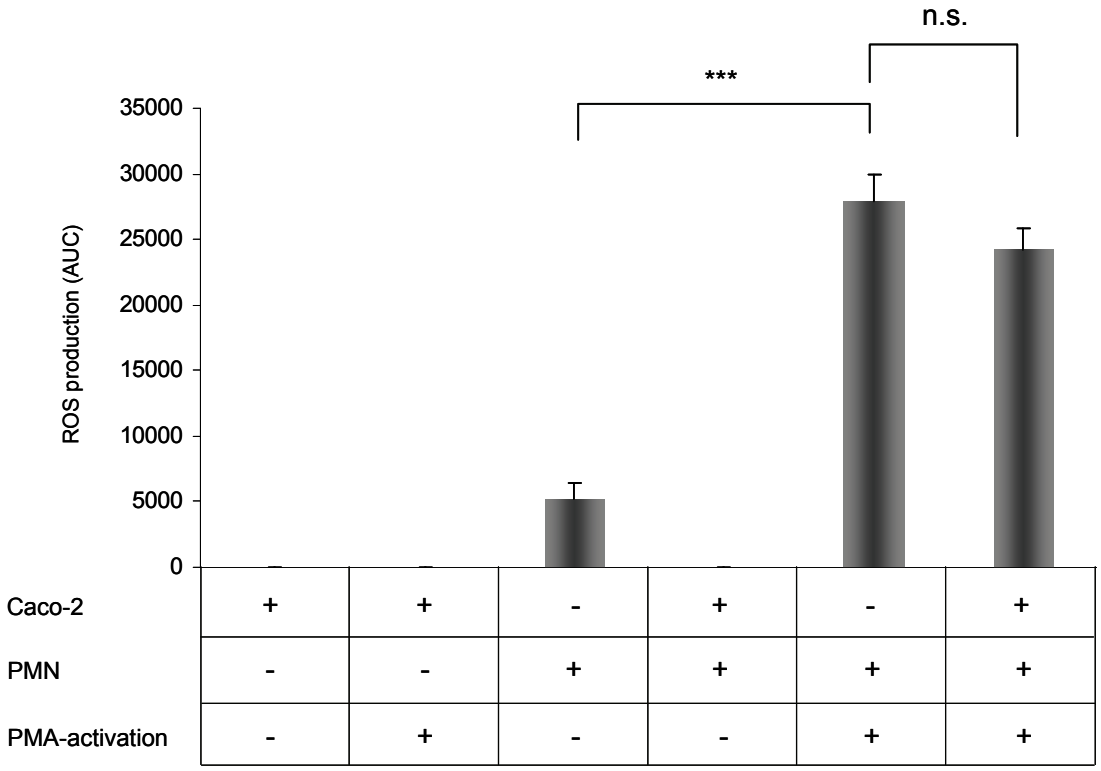

Figure 5.2 **ROS formation by PMN and Caco-2 cells.** The formation of superoxide anion radicals by Caco-2 cells alone, PMA activated or non-activated primary human neutrophils (PMN) alone or Caco-2 cells and PMN in co-incubation was determined via ESR after 30 minutes at 37°C using the spin probe CPH. PMA=phorbol-12-myristate-13-acetate. AU=arbitrary units. Values are expressed as mean and standard deviation, n=3. n.s.=not significant
*** $p < 0.001$ versus control

The effect of PMN on SiO_2 induced DNA damage in Caco-2 cells is shown in Figure 5.3. The SiO_2 particles caused a significant induction of DNA damage in Caco-2 cells in the absence of PMN. Notably, the level of induction observed with the SiO_2 in the Caco-2 cells was similar to the effect observed with PMA-treated PMN used as positive control in these series of experiments. Surprisingly, the presence of PMN did not augment the SiO_2-induced DNA strand breakage, but in fact resulted in markedly reduced effect in the intestinal epithelial cells. Application of the Fpg

enzyme revealed an increased, albeit not significant induction of oxidative lesions after SiO_2 incubation alone. In the presence of PMN however, a significant increase in oxidative DNA damage was detected.

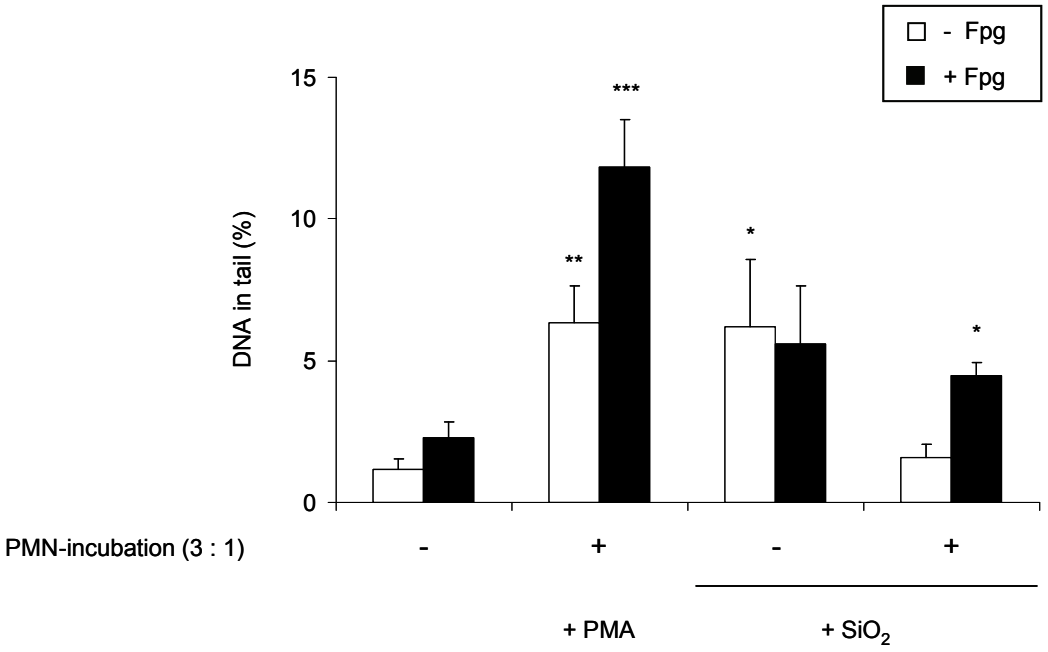

Figure 5.3 DNA strand breakage and oxidative DNA damage in Caco-2 cells by activated PMN and SiO_2. DNA strand breakage and oxidative DNA damage were determined in Caco-2 cells using the Fpg-modified comet assay. Cells were (I) not incubated as controls, (II) 30 minutes co-incubated with PMA-activated primary human neutrophils (PMN), (III) 30 minutes incubated with SiO_2 alone or (IV) 30 minutes co-incubated with SiO_2 and PMN. SiO_2 concentration: 20 µg/cm^2. PMN:Caco-2 ratio: 3:1. PMA=phorbol-12-myristate-13-acetate. Values are expressed as mean and standard deviation, n=3.
* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ versus control

Possible effects of SiO_2 on PMN-induced $O_2^{\cdot -}$ generation were analysed using two independent methods, i.e. both lucigenin-enhanced chemiluminescence (Figure 5.4 A) and EPR spectroscopy with CPH as spin probe (Figure 5.4 B). ROS detection via chemiluminescence occurs via constant measurement over 50 minutes and results in a typical curve as depicted in Figure 5.4 a. Compared to non-stimulated PMN, the presence of SiO_2 did not affect the overall formation of superoxide (area under the curve). In contrast, stimulation with PMA resulted in a significant ROS induction (Figure 5.4 A). EPR spectroscopy allows for a quantification of the amount of CPH that has reacted with superoxide anions over the 30 min incubation. Similar to our findings in chemiluminescence measurement, ESR confirmed that SiO_2 did not activate neutrophilic burst, since no increase in ROS formation was detected (Figure

5.4 B). In contrast, a clear effect was observed with the well-known neutrophil activator PMA. Figure 5.4 b shows representative EPR spectra of ROS formation.

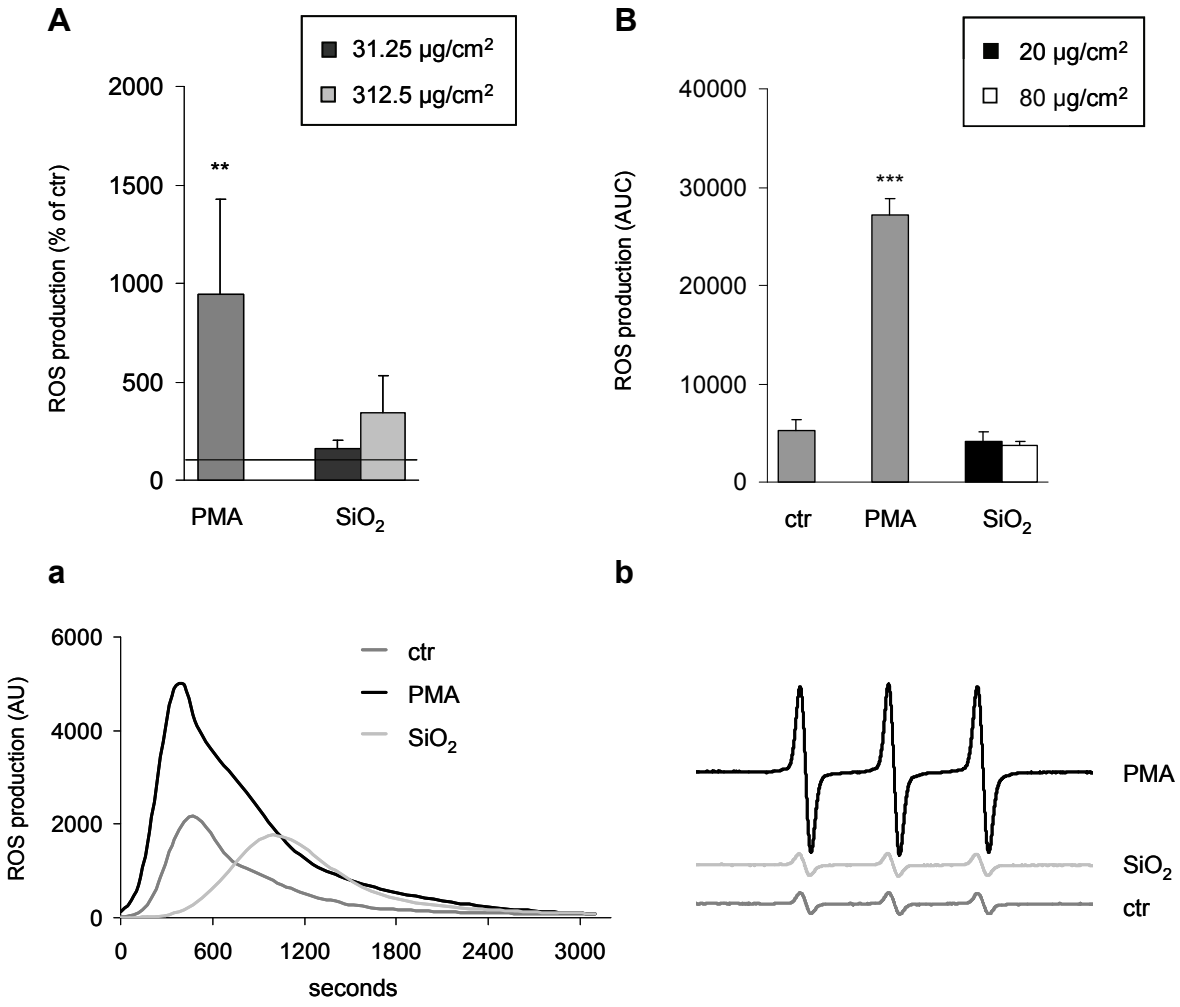

Figure 5.4 **ROS generation from PMN upon treatment with SiO_2.** The formation of superoxide anion radicals by primary human neutrophils (PMN) was determined by chemiluminescence using lucigenin over an incubation period of 50 minutes (A) and ESR after 30 minutes pre-incubation with the spin probe CPH (B). (a) Representative luminescence curve and (b) representative ESR spectra by non-activated PMN (ctr=control), PMA-activated PMN (PMA) and SiO_2-treated PMN (SiO_2; (a) 312.5 µg/cm^2, (b) 20 µg/cm^2). AU=arbitrary units. Values are expressed as mean and standard deviation, n=3.
** $p < 0.01$ and *** $p < 0.001$ versus control

To analyse the genotoxic properties of SiO_2 *in vivo* in healthy intestine as well as during inflammation, mice were fed normal or SiO_2 chow for 14 days, followed by 3 alternating cycles of DSS or SiO_2 treatment for the induction of colitis. The occurrence of symptoms of colitic inflammation was confirmed by measure of colon length (Figure 5.5 A) and the clinical score, with 3 indicating severest symptoms (Figure 5.5 B). The colon lengths were reduced markedly after colitis induction,

whereas mice receiving SiO_2 exhibited normal colon lengths. The clinical score, reflecting bleeding intensity, weight loss and stool consistency, revealed an increase in symptoms after each DSS treatment followed by states of remission.

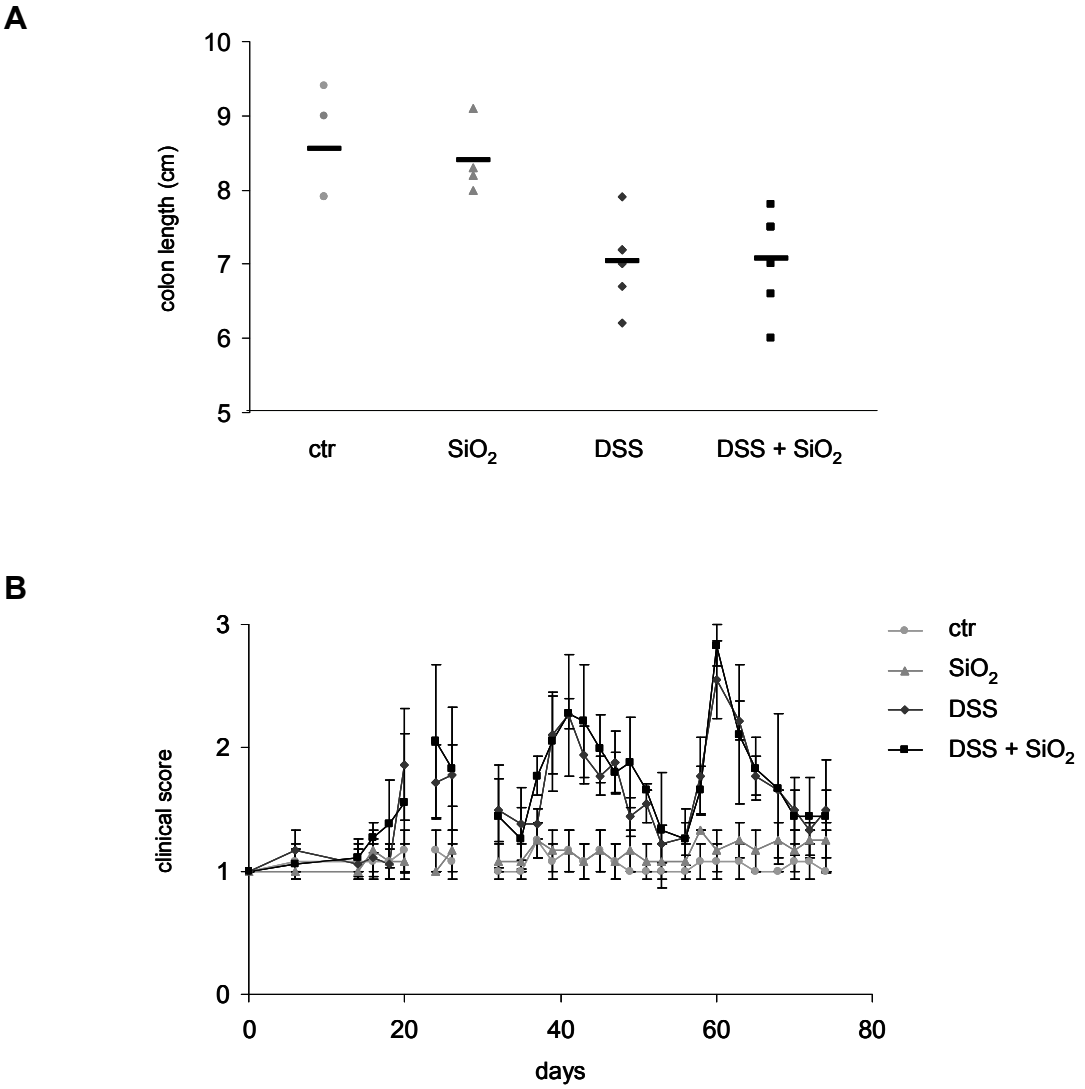

Figure 5.5 **Evaluation of the severity of the induced chronic colitis.** Colon lengths were determined as marker of colitic symptoms (A). The clinical score as a combined measure of changes in bleeding intensity, weight loss and stool consistency. 1 = no effect detectable to 3 = strongest colitic effects (B).

Interestingly, DNA damage as measured by *in vivo* Fpg-comet assay (see Figure 5.6) was already high in the colons of the control animals, i.e. the mice that were neither treated with SiO_2 nor with DSS. Consequently, chronic colitis did not result in an increased DNA damage in the colon of DSS-treated animals compared to these high controls (Figure 5.6 A). The SiO_2-enriched diet even led to a reduced oxidative DNA damage, both in the colon tissues of healthy and colitic mice.

However, calculation of the ΔFpg showed that the reduction in oxidative lesions was not statistically significant, as a result of high individual variations (Figure 5.6 B).

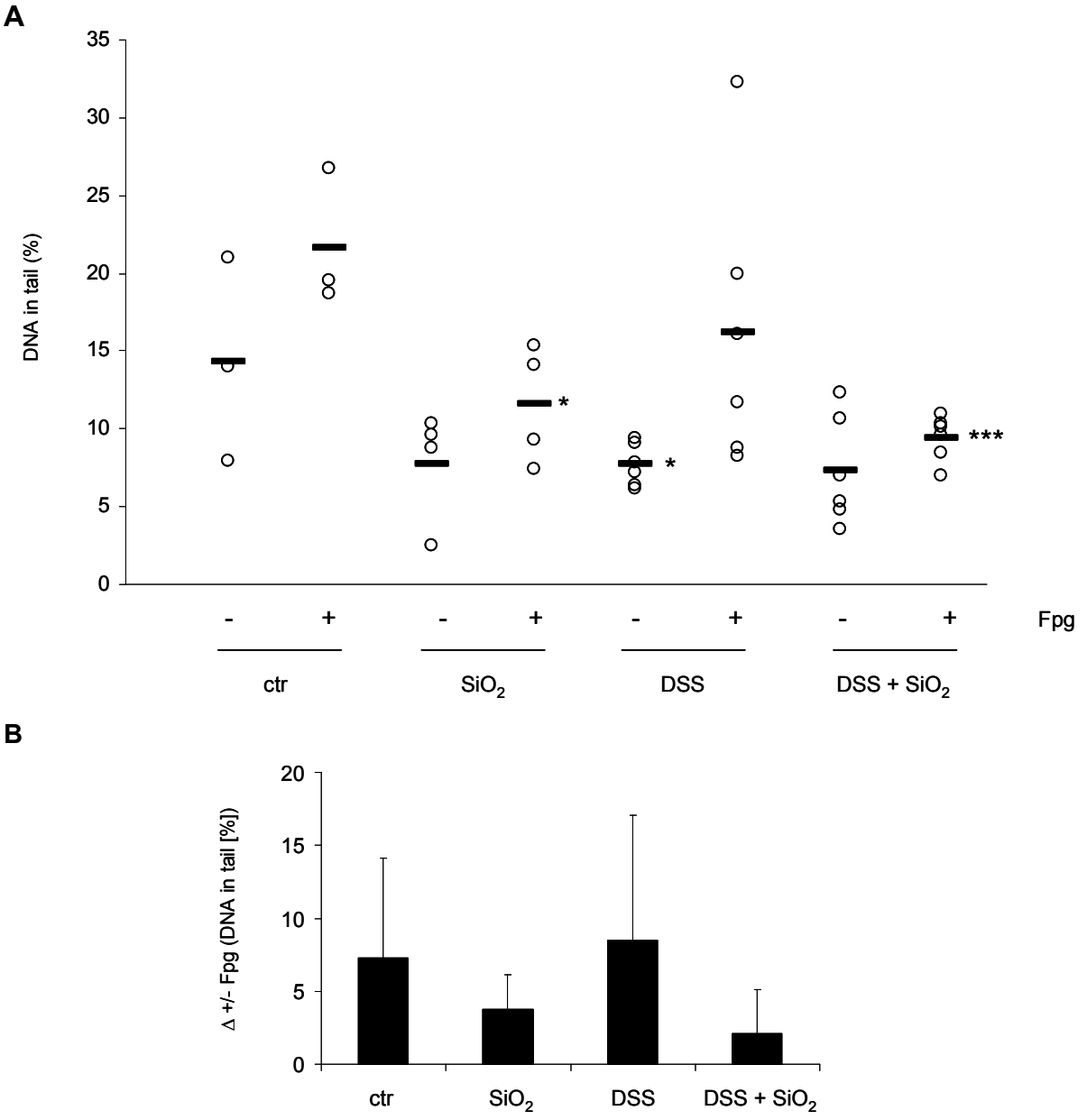

Figure 5.6 **Effect of oral SiO_2 on DNA strand breakage and oxidative DNA damage in murine colonic tissue in a chronic colitis model.** DNA strand breakage and oxidative DNA damage were determined in whole murine colonic tissue using the *in vivo* Fpg-modified comet assay (A). The total amount of oxidative DNA damage is depicted as ΔFpg, calculated by the difference between + Fpg and – Fpg values (B). Treated mice were fed with either DSS to induce chronic colitic symptoms, SiO_2 or both DSS and SiO_2. ctr=control, DSS=dextrane sulphate sodium. Values are expressed as mean and standard deviation, n=3 (ctr), n=4 (SiO_2) and n=6 (DSS, DSS+SiO_2).
* $p < 0.05$ and *** $p < 0.001$ versus control

Interestingly, detection of 8-OHdG by immunohistochemistry (IHC, Figure 5.7) revealed a clear increase of oxidative lesions, mainly located in the nuclei of epithelial cells, in both SiO_2 (Figure 5.7 B) and DSS (Figure 5.7 C) treated mice when compared to the control animals (Figure 5.7 A). Combined treatment with SiO_2 and DSS (Figure 5.7 D) also induced increased 8-OHdG sites compared to the control, however, these appeared less pronounced than the lesions induced by SiO_2 or DSS alone. All IgG stained (control) slides were found to be without marked nuclear staining (not shown).

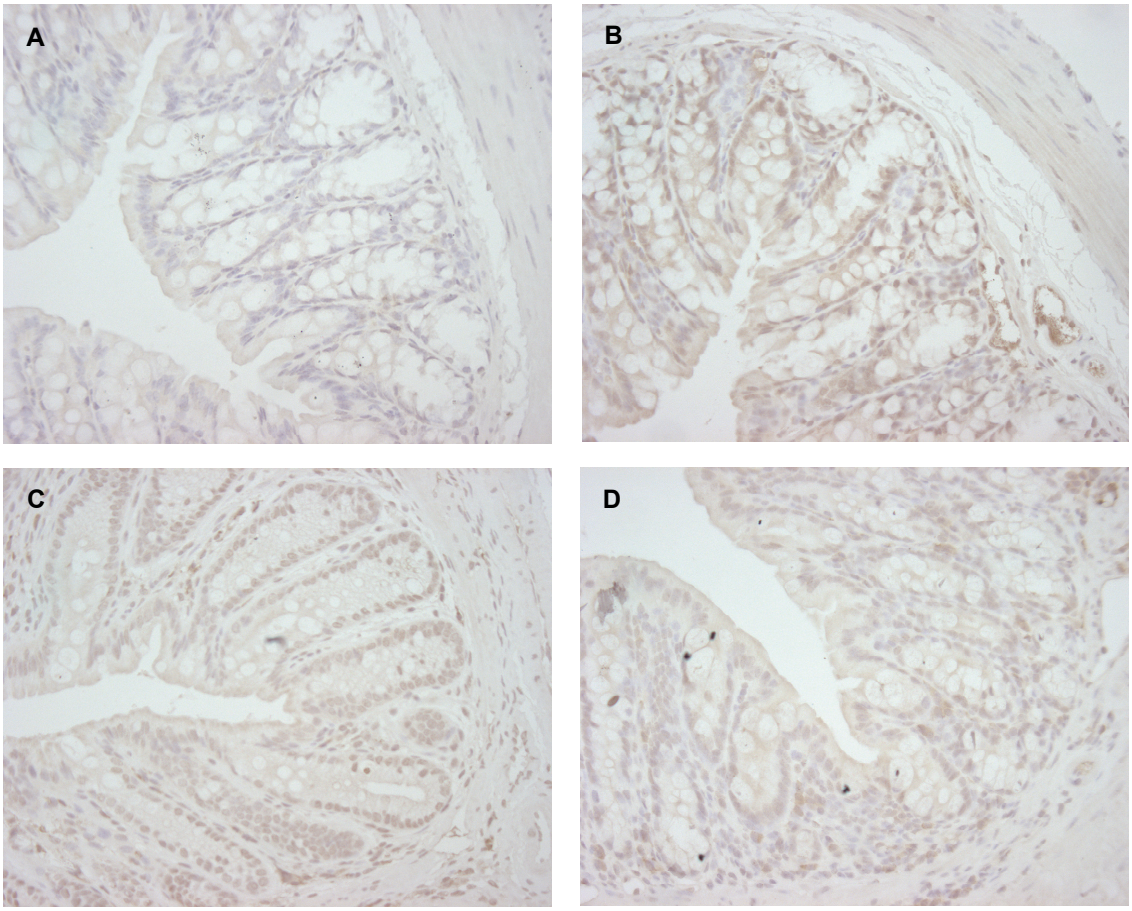

Figure 5.7 **Immunohistochemical analysis of 8-hydroxydeoxyguanosine in a murine colonic tissue in a chronic colitis model.** Representative images of colonic sections, obtained from controls (A), mice after receiving SiO_2 enriched-chow (B) DSS treated mice receiving normal chow (C) or DSS treated mice receiving SiO_2 enriched-chow (D), stained with an antibody against 8-OHdG (original magnification × 400). n=3 (ctr), n=4 (SiO_2) and n=6 (DSS, DSS+SiO_2).

We also evaluated the small intestines from the same animals. In contrast to colon, no differences in DNA damage or induction of oxidative lesions were observed between any of the various treatments in the small intestine by Fpg-comet assay (Figure 5.8 A and B).

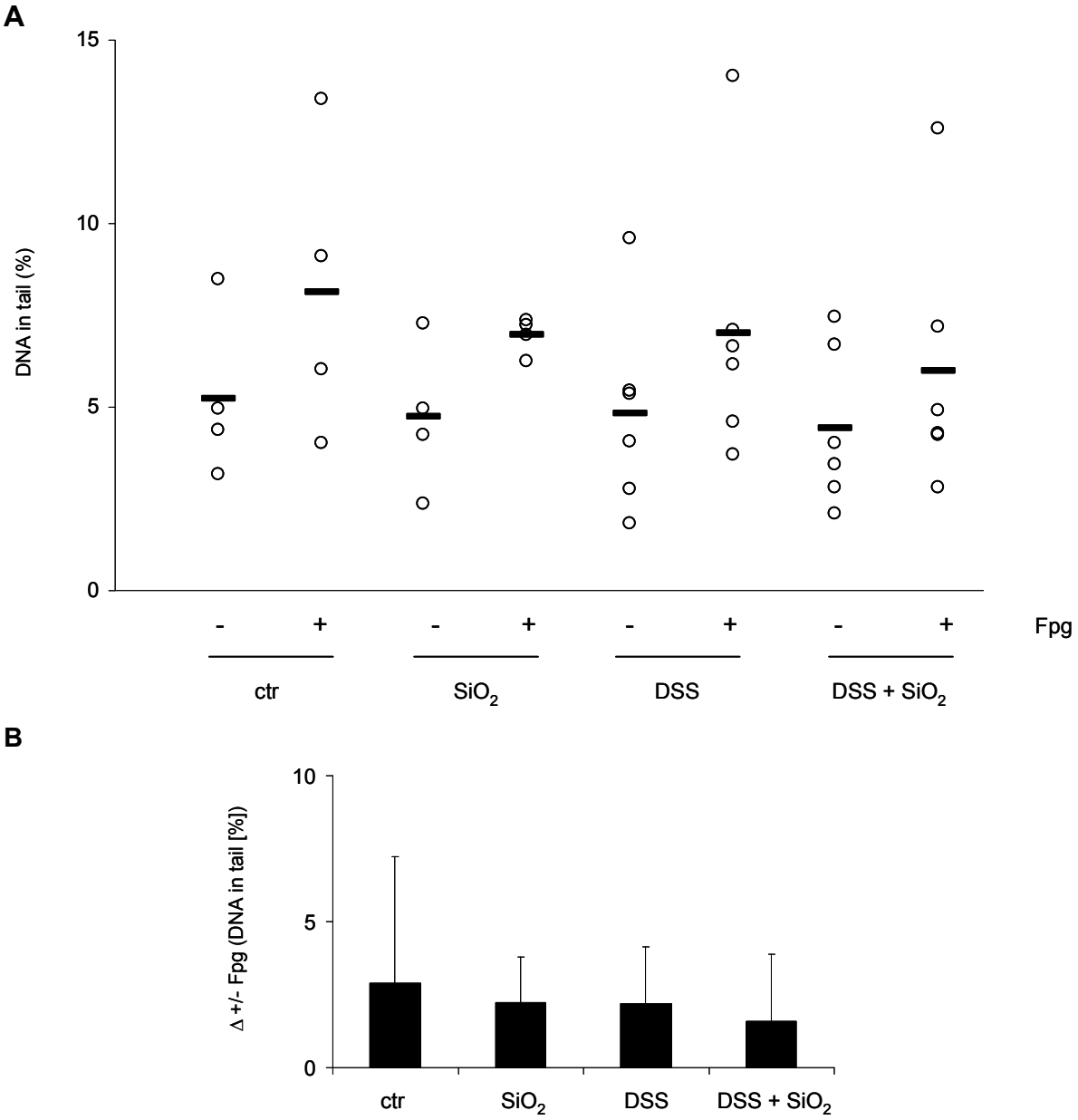

Figure 5.8 **Effect of oral SiO_2 on DNA strand breakage and oxidative DNA damage in murine small intestinal tissue in a chronic colitis model.** DNA strand breakage and oxidative DNA damage were determined in whole murine small intestinal tissue using the *in vivo* Fpg-modified comet assay (A). The total amount of oxidative DNA damage is depicted as ΔFpg, calculated by the difference between + Fpg and - Fpg values (B). Treated mice were fed with either DSS to induce chronic colitic symptoms, SiO_2 or both DSS and SiO_2. ctr=control, DSS=dextrane sulphate sodium. Values are expressed as mean and standard deviation, n=4 (ctr, SiO_2) and n=6 (DSS, DSS+SiO_2).
* $p < 0.05$ and *** $p < 0.001$ versus control

Since the above findings in colon tissue during chronic colitis might have been due to feedback and/or adaptive response mechanisms we also evaluated the genotoxic effects of SiO_2 after shorter particle exposures and/or acute colitis. The treatment consisted of administration of DSS for 6 days (acute colitis animals)

following 17 days of normal or SiO_2 chow. Results of these investigations are shown in Figure 5.9.

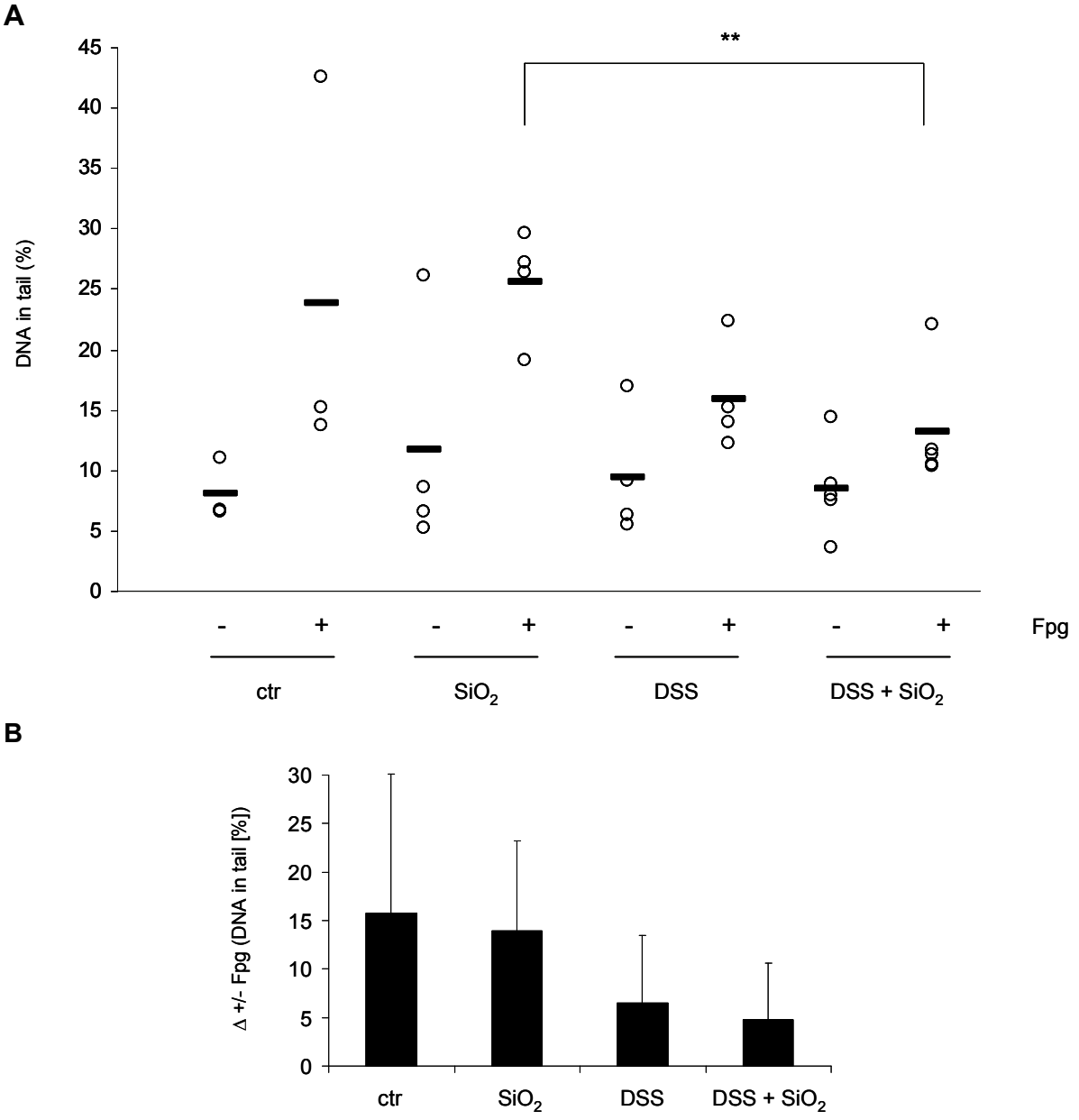

Figure 5.9 **DNA strand breakage and oxidative DNA damage in murine colonic tissue using *in vivo* Fpg-comet assay in an acute colitis model.** DNA strand breakage and oxidative DNA damage were determined in whole murine colonic tissue using the *in vivo* Fpg-modified comet assay (A). The total amount of oxidative DNA damage is depicted as ΔFpg, calculated by the difference between + Fpg and – Fpg (B). Treated mice were fed with either DSS to induce acute colitic symptoms, SiO_2 or both DSS and SiO_2. ctr=control, DSS=dextrane sulphate sodium. Values are expressed as, n=3 (ctr), n=4 (SiO_2, DSS) and n=5 (DSS+SiO_2).
** $p < 0.01$

Slightly elevated levels of oxidative lesions were observed after a SiO_2 containing diet, although they did not reach a statistical significance. Remarkably, in the presence of Fpg, the % of tail DNA was found to be significantly lower in the DSS-SiO_2 group when compared to the SiO_2 groups (Figure 5.9 A). However, no significant reduction was found for the ΔFpg values indicating that there was no significant reduction of oxidative DNA damage (Figure 5.9 B). The absolute levels of DNA damage in the DSS and DSS-SiO_2 groups of the acute colitis study were comparable to the damage in the corresponding groups of the chronic study, but revealed higher interindividual variations.

Moreover, IHC staining of the respective colonic tissue did not show marked differences in staining for 8-OHdG within the nuclei, as shown by representative images in Figure 5.10.

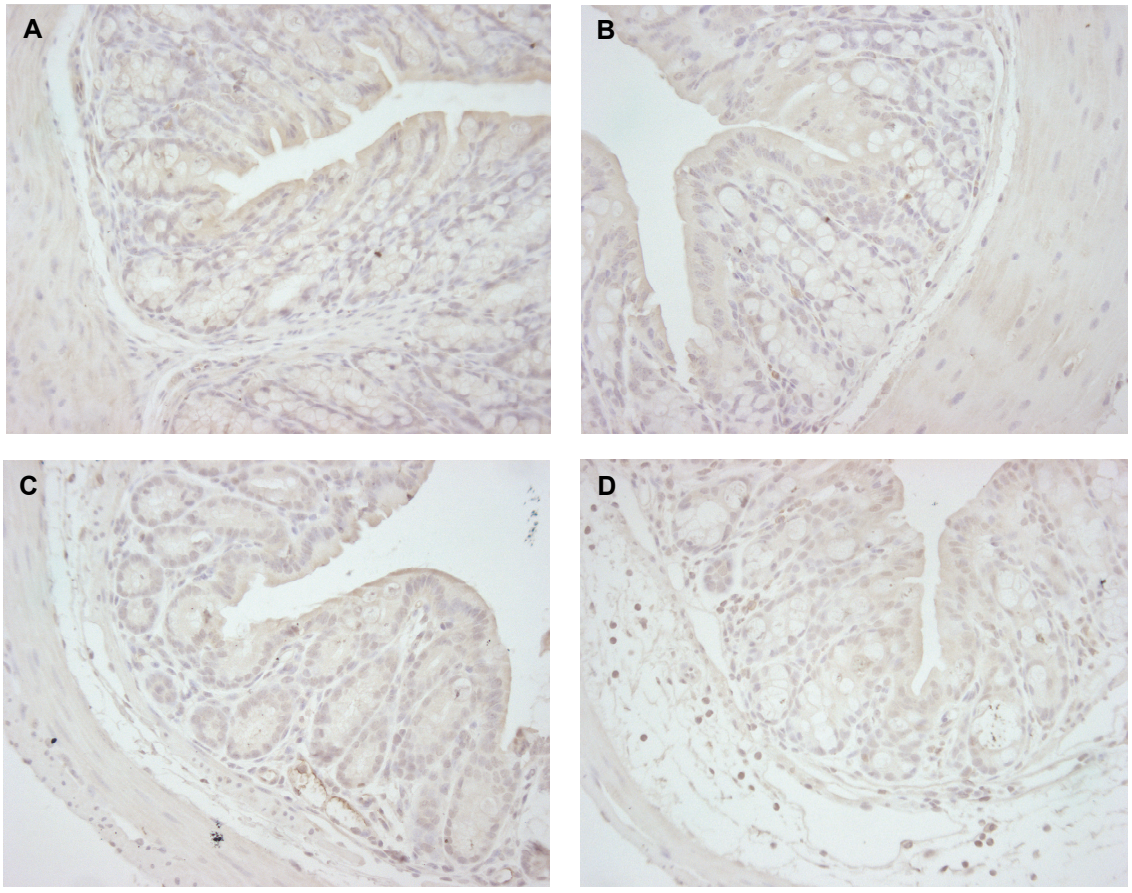

Figure 5.10 Immunohistochemical analysis of 8-hydroxydeoxyguanosine in a murine colonic tissue in an acute colitis model. Representative images of colonic sections, obtained from controls (A), mice after receiving SiO_2 enriched-chow (B) DSS treated mice receiving normal chow (C) or DSS treated mice receiving SiO_2 enriched-chow (D), stained with an antibody against 8-OHdG (original magnification × 400). n=3 (ctr), n=4 (SiO_2, DSS) and n=5 (DSS+SiO_2).

5.4 Discussion

In this study we evaluated the DNA damaging potential of SiO_2 in the presence or absence of neutrophils on intestinal epithelial cells *in vitro*, as well as in a colitic mouse model *in vivo*.

Our *in vitro* model for intestinal inflammation consisted of a co-culture of Caco-2 cells and human primary neutrophils (PMN) at direct cell-cell contact. The model is similar to the one introduced by Vermeer and co-workers (Vermeer *et al*., 2004) and an established model in our laboratory to address effects of PMN on alveolar epithelial cell lines (Knaapen *et al*., 1999, 2002a, 2006; Boots *et al*., submitted). The presently applied co-incubation model allowed us to investigate the effect of activated neutrophils on the genome integrity of human intestinal cells and by varying the number of neutrophils present, different degrees of neutrophilic inflammation could be simulated. Importantly, we used non-confluent undifferentiated Caco-2 cells, which are still capable of cell division and differentiation. As such the influence of a neutrophilic inflammation on the potential genotoxic effects of SiO_2 could be addressed in an optimal manner, since intestinal inflammation is marked by the presence of hyperproliferative epithelial cells (Huang *et al*., 1997; Arai *et al*., 1999). Our results clearly show the potential of activated PMN to induce both DNA strand breakage and oxidative lesions in Caco-2 cells. Interestingly, this effect was shown to be dependent on both the number of PMN present and their activation.

A likely explanation for the observed PMN-driven DNA damage is the marked ROS production which is initiated during the respiratory burst of these inflammatory cells. Indeed, it is known that increased ROS formation also occurs in the inflamed tissue of patients suffering from ulcerative colitis (Babbs, 1992) accompanied by a reduced antioxidant potential, as lower levels of glutathione (Sido *et al*., 1998). Since an increased amount of superoxide anion radicals could be found after activation of neutrophils alone or in the co-culture, we hypothesise that these ROS are the main cause of DNA damage in the Caco-2 cells.

Our previous studies demonstrated the potential of SiO_2 alone to induce oxidative lesions in Caco-2 cells after 4 hours incubation (Gerloff *et al*., 2009). In the present work, we adapted the application of this nanoparticle to the co-incubation model and determined possible interference of PMN and SiO_2 on DNA damage in the co culture. Surprisingly, the SiO_2 particles alone now induced only significant DNA

strand breaks, but no oxidative lesions. The most likely explanation for this apparent discrepancy between the results of both studies is the difference in treatment time and cell culture conditions. In the present study, to adapt to the conditions of the co-incubation model a shorter incubation time was applied and all experiments were performed in HBSS instead of serum free cell culture media in order to minimise scavenging effects of culture medium constituents. Interestingly, the effects of SiO_2 were not enhanced in the co-incubation, i.e. when neutrophils were also present. Current results indicate that the SiO_2 particles do not activate the neutrophils to induce comparable DNA damage as detected in the presence of PMA, but that the presence of neutrophils in fact decreases the potential of SiO_2 to induce DNA damage in Caco-2 cells.

SiO_2-induced ROS formation by PMN was determined by two independent methods. No potential of SiO_2 to induce neutrophilic burst could be revealed. This absence correlates nicely with the observed absence of DNA damage within the co-incubation model. The observed abrogation of the DNA damaging effect of SiO_2 in the presence of PMN may be explained by the increased number of total cells in culture (i.e. a 3-fold number of PMN), which thereby reduces the particle number dose per Caco-2 cell. The SiO_2 might preferentially interact with the phagocytosing PMN and thereby the amount of particles faced by Caco-2 cells is reduced, which in turn leads to reduced direct SiO_2 effects on Caco-2 cells. Moreover, the neutrophils are known to contain various antioxidants including superoxide dismutase (Zakhireh *et al*., 1979), which may scavenge to moderate oxidative effects of the SiO_2 to the Caco-2 cells.

The actual influence of SiO_2 on DNA integrity *in vivo* was investigated in a mouse model of colitis via the application of SiO_2 in the chow. The polysaccharide DSS was used for induction of experimental colitis as it reduces the integrity of the mucosal barrier by causing a direct toxicity to gut epithelial cells (Gaudio *et al*., 1999; Wirtz and Neurath, 2007). Our *in vivo* comet assay analyses of fresh whole colonic tissue in this model surprisingly revealed reduced DNA lesions after SiO_2 ingestion in both healthy and colitic animals compared to control animals fed a SiO_2-free diet. The finding that DNA strand breaks were markedly reduced in the colitic mice leads to the assumption that DNA repair mechanisms might be upregulated in colitis. Such upregulation would be in accordance with the results of a previous study performed by Wessels and co-workers in our lab in an experimental model of pulmonary

exposure to nanoparticles (Wessels *et al*., in press). In this study, no induction of DNA strand breaks or oxidative lesions could be detected in the lungs of mice upon inhalation of spark generated carbon nanoparticles, while several genes regulating DNA repair enzymes tended to be upregulated within this tissue.

In our current study however, immunohistochemical staining of 8-OHdG revealed a clear induction of oxidative lesions in the DNA of epithelial cells after treatment with SiO_2 or DSS. Moreover, the intensity of the induced staining tended to be slightly lower after combined DSS-SiO_2 treatment compared to treatment with either SiO_2 or DSS alone. The discrepancies between the immunohistochemical findings and the comet assay data might lie within the fact that not only epithelial cells are analysed by the latter method, but whole colon tissue. Thus, this also includes other cell types present in the colon, including infiltrated immune cells or small muscle cells, which might lead to a “dilution” of the overall determined effect. Moreover, one should take into account that the *in vivo* comet assay method requires processing time to isolate cells and nuclei from the tissue homogenates, during which potential artefacts may be induced. In contrast, IHC staining allows for the specific detection of 8-OHdG formation in individual cells.

Caco-2 cells, although isolated from a colon adenocarcinoma, form an apical brush border and microvilli upon differentiation (Chantret *et al*., 1988) and thus show several properties of the small intestine. To investigate the influence of ingested SiO_2 on the gene integrity of the small intestine *in vivo*, the comet assay was also performed in whole small intestinal tissue. In line with our findings within the colonic tissue, no DNA damage was detectable in the small intestine after chronic ingestion of SiO_2. In contrast to a short-term study of Hong *et al*. (2005) who administered DSS in the drinking water for 48 hours, we also could not find any oxidative DNA damage in whole tissue samples of the small intestine after DSS treatment in our chronic study by Fpg-modified comet-assay analysis.

To address whether the observations were due to the chronic exposure conditions we also performed an acute colitis study. Comparably to our results of the chronic study, no induction of DNA strand breaks or oxidative lesions were found in colons of DSS treated mice. However, oxidative DNA damage tended to be increased after treatment with SiO_2 alone, although this did not reach statistical significance and may be due to the limited number of animals investigated. Remarkably, the oxidative DNA damage in the SiO_2 treatment group was significantly

higher than the damage found in the DSS+SiO_2 treatment group when the absolute amount of strand breaks plus oxidative lesions were considered (Figure 5.9 A), whereas IHC staining for 8-OHdG lesions did not show marked differences within the four treatment groups. However, the comet assay results are in concordance to our *in vitro* results, where the DNA damaging potential of SiO_2 seemed to be reduced in an inflammatory environment. The mechanisms of these *in vivo* effects require further investigation in view of their relevance for hazard and risk assessment of ingested SiO_2 and other nanoparticles, but also because of potential preventive or therapeutic strategies in patients suffering from inflammatory bowel diseases.

As already mentioned it should be emphasised that the *in vivo* comet assay used in our study reflects alterations in the whole tissue. Therefore it determines not exclusively the DNA damage of epithelial cells which are considered relevant target cells for colon carcinogenesis (Itzkowitz *et al*., 2004). The DNA damage measured by the *in vivo* comet assay also may reflect integrity changes in endothelial cells, smooth muscle cells or, most importantly inflammatory cells, which might dilute a possible damage of the epithelial DNA. Earlier studies detected increased levels of 8-hydroxydeoxyguanosine (8-OHdG) in isolated colonic mucosal cells of DSS treated rats by HPLC (Tardieu *et al*., 1998) or in whole colonic rat tissue, measured by quantitative immunohistochemistry (Hong *et al*., 2005). The latter finding could be confirmed in our chronic colitis model for treatment with DSS, but not after co-treatment with DSS and SiO_2. This suggests that the *in vivo* comet assay of whole tissue might not be sensitive or specific enough to detect slight alterations in epithelial DNA damage. Additionally, Westbrook *et al*. (2009) could detect systemic DNA damage after chronic DSS treatment in mice, as found in peripheral leukocytes, detected with the alkaline comet assay during acute colitis. However they also reported a slight decrease of DNA strand breaks during remission cycles, in concordance with the observations in our current study of chronic colitis.

Here we highlighted the effects of amorphous SiO_2, possibly present in food or pharmaceuticals, on the genome integrity of the healthy or inflammatory intestinal epithelium *in vitro* and *in vivo*. We analysed the effects of activated human primary neutrophils on the genome integrity of Caco-2 cells in an *in vitro* model of intestinal inflammation. The induction of oxidative lesions was dependent on the amount of activated inflammatory cells and associated with the neutrophilic burst. In the presence of SiO_2 no significant neutrophil-activation was detected, as measured by

reactive oxygen species formation. In line with these observations, the concurrent incubation of Caco-2 cells with neutrophils and SiO_2 did not lead to an augmented DNA damage when compared to the effect of SiO_2 alone. We further analysed the DNA damaging properties of ingested SiO_2 on the intestine in a DSS-induced colitic mouse model. DSS alone did not induce DNA strand breakage in the colons in both acute and chronic colitis studies. Treatment with SiO_2 in healthy or colitis mice also did not lead to a significant enhancement of DNA damage measured by the *in vivo* Fpg-comet assay, whereas slight induction of 8-OHdG lesions was shown in immunohistochemical staining in the chronically, but not in the acutely treated colons. We conclude that the risk of SiO_2 ingestion on the genome integrity of the intestine is low, also in the inflamed gut, taken into account that a rather high concentration was used. Importantly however, slightly increased oxidative lesions were found locally after chronic SiO_2 treatment. Moreover, systemic or long-term damage could not be excluded, and extrapolation of our current results to the human situation is limited and should be done with caution. Concern about the potential genotoxic hazard of nanoparticles in food was recently expressed on the basis of the investigations by Trouiller and co-workers, who showed marked systemic DNA damage and mutagenesis in mice after oral uptake of TiO_2 nanoparticles (Trouiller *et al*., 2009). In contrast to our investigations no data were provided in their study on local (geno)toxic effects in the intestine. In our present study, the mice chow contained 0.1 mass % of SiO_2 (estimated daily intake: 200 mg/kg body weight), which is in the range of the concentrations of TiO_2 within the drinking water as applied by Trouiller *et al*. (2009). The different methods of administration of the tested particles also need to be considered in the interpretation of both studies for risk assessment purposes. An appropriate extrapolation of our current findings to human risk assessment requires a thorough evaluation of both quantitative (mass and number dose) and qualitative (size, aggregation/agglomeration, etc.) particle properties in consumer products such as food and beverages. Presently such information is still very limited.

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Declaration

The manuscript is currently in preparation.
All experimental *in vitro* work, processing of the isolated tissue samples for the *in vivo* Fpg comet assay and the IHC staining as well as the performance and analysation of the *in vivo* Fpg comet assay was performed by Kirsten Gerloff. The impact on authoring this paper can be estimated in total with 90%.

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CHAPTER 6

6 General Discussion

Presently, very little is known about potential toxic, genotoxic or pro-inflammatory effects of food-relevant metal oxide nanoparticles (NP) in the human intestine. This thesis aimed to contribute to a better understanding of possible adverse effects and their underlying mechanisms of several of these materials, thus leading to improved hazard and risk assessment.

As hardly any information on the extent of NP usage in food and, if indeed used, the characteristics of these materials is available, the first intention of this work was to determine the toxic and DNA damaging potential of a choice of particles that might already find their way in modern food products (**Chapter 2**). The use of nano-sized titanium dioxide (TiO_2) is already described, and patents exist describing its potential for use in confectionary products, but the actual size and structure of the applied materials remains unclear (Chaudhry *et al*., 2008; Bouwmeester *et al*., 2009; Mars Inc.'s US Patent US 5741505). Amorphous silicon dioxide (SiO_2) is known to induce DNA fragmentation, increased influx of PMN and cytotoxicity in rat lungs upon subchronic inhalation (Johnston *et al*., 2000), whereas hardly any evidence on possible adverse health effects in the intestine has been reported. Accordingly, amorphous SiO_2 has a long-standing use in pharmaceutics (US Patent US 2002/0025964 A1), and general SiO_2 use in food is already described (Chaudhry *et al*., 2008; Schmid and Riediker, 2008; homepage nanotechproject.org). Zinc oxide (ZnO) and magnesium oxide (MgO) both can be used in food packages as well as in food directly (Chaudhry *et al*., 2008; homepage nanotechproject.org). Carbon black (CB) was chosen as a model of environmental nanoparticles; diesel exhaust e.g. contains carbonaceous nanoparticles and (I) these can be inhaled and subsequently transported into the gastrointestinal tract upon mucociliary clearance or (II) may deposit on vegetables grown on fields close to high-traffic roads or upon transportation (Dybdahl *et al*., 2003).

Upon treatment of Caco-2 cells with this selection of NP, a clear potential of ZnO and SiO_2 could be found to induce both cytotoxicity as well as DNA strand breakage and oxidative lesions, respectively, associated with induction of intracellular

oxidative stress, as further confirmed by reduction of the intracellular GSH content (**Chapter 2**). The adverse effects that could be determined are depicted in Figure 6.1. A DNA damaging potential of TiO_2 upon processing by light was already shown for various cells lines and confirmed in the present study using Caco-2 cells, but obviously photoactivation is not an issue within the human intestine (Nakagawa *et al*., 1997; Sayes *et al*., 2006; Johnston *et al*., 2009). Comparison of three samples of TiO_2 and ZnO respectively, each featuring different specific surface areas (SSA), revealed no relationship between the DNA damaging potential and a varying SSA for both materials tested. However, cytotoxicity appeared to be elevated when using the high-SSA TiO_2 samples.

To follow-up on this study two additional TiO_2 particles were included and all five samples were analysed for crystallinity, primary particle size, SSA and aggregation/agglomeration (**Chapter 3**). Two samples contained both anatase and rutile and were found to have a similar SSA, in contrast to three pure anatase powders with one having a very low and two a very high SSA respectively. Interestingly, only the anatase-rutile containing particles caused mild cytotoxicity and DNA oxidation under exclusion of illumination. Similarly to the first study (**Chapter 2**), the effects could not be explained by the different SSA of the samples. The results indicated that cytotoxic and DNA damaging properties of TiO_2 in Caco-2 cells are at least in part driven by their chemical composition. This highlights the need for thorough evaluation and labelling of the TiO_2 species within the final (food) product.

As SiO_2 and ZnO were found to be the most cytotoxic and DNA damaging particles (**Chapter 2**), their solubility and aggregational/agglomerational potential was further determined, as well as their uptake onto differentiated Caco-2 cells (**Chapter 4**). ZnO was found to be absorbed more effectively, and upon analysis of possible uptake mechanisms both microtubule-dependent and clathrin/AP2-mediated endocytosis did not seem to be of major importance in both systems. This suggests that further research is needed to discriminate between actual uptake into versus adhesion onto the cells. Moreover, phagocytosis, macropinocytosis or diffusion might play a role, always depending on the particle properties and the exposed cell type (Unfried *et al*., 2007). The influence of gastrointestinal digestion was simulated by means of pH changes (**Chapter 4**), and both materials were found to be highly aggregated/agglomerated before and after the simulated digestion. For this study, undifferentiated as well as differentiated Caco-2 cells were used to test cytotoxic and

pro-inflammatory effects of both the native and digestion-simulated (DS) particles. Pre-treatment under gastrointestinal conditions was found to markedly reduce the ability of both materials to generate acellular reactive oxygen species. However, native versus DS particles showed comparable effects on cell viability. ZnO decreased viability of both undifferentiated and differentiated cells in a dose and time dependent manner, whereas surprisingly SiO_2 was only cytotoxic to undifferentiated cells. Interestingly, interleukin-8 (IL-8) secretion after SiO_2 treatment appeared lower in differentiated compared to undifferentiated cells, whereas the secretion following ZnO incubation was markedly higher in the differentiated Caco-2 cells. The digestion simulation treatment of the particles did not affect their ability to induce IL-8 mRNA expression and secretion.

In **Chapter 5**, an inflammatory intestinal condition was simulated by incubating Caco-2 cells with primary human neutrophils, important phagocytes in the innate immune response. The effect of the PMN on the genome integrity of the intestinal cells and the influence of SiO_2 on this system was evaluated. When activated with PMA, neutrophils strongly induced DNA strand breakage and oxidative lesions in Caco-2 cells, going along with generation of superoxide anion radicals (Figure 6.1). SiO_2 however did not induce the oxidative burst of the PMN, and surprisingly the presence of SiO_2 in the co-culture appeared to even reduce the DNA damaging potential of PMN. Current *in vitro* findings might actually provide some insight to not only the adverse, but also potential beneficial effects associated with oral exposure to specific types of NP. Therefore, the significance of these interesting findings was additionally evaluated *in vivo*. Mouse studies were performed to determine the impact of ingested SiO_2 in the healthy and colitic gut. Chronic and acute colitis symptoms were induced by treatment with dextrane sulphate sodium (DSS). Immunohistochemical staining revealed an induction of oxidative lesions in the colonic epithelium of SiO_2 or DSS treated mice in the chronic, but not in the acute colitis experiment. Interestingly the co-treatment of SiO_2 and DSS did not induce 8-OHdG. Contrary, the *in vivo* comet assay, an assay well known for its high sensitivity for quantification of both overall DNA strand breakage and oxidative lesions (Liao *et al*., 2009), revealed neither elevated levels of DNA strand breaks nor enhanced oxidative lesions in the colitic colon, regardless of SiO_2 treatment. Moreover, in specific circumstances the damage appeared even alleviated compared to the control animals (in the chronic study) or compared to the SiO_2 treated mice (in

the acute study). Direct interactions between SiO_2 and DSS can be excluded as an explanation for this, since time-staggered treatment of both substances was performed. Notably, the *in vivo* comet assay did not only include epithelial cells into the analysis, but also infiltrated immune or smooth muscle cells e.g., thus most likely leading to a dilution effect in determining epithelial DNA strand break alteration. However, in future experiments, the use of more sophisticated models of colitis (e.g. knock out animals that develop spontaneous colitic symptoms, such as STAT3 deficient or VILLIN-HA x CL4-TCR mice) might add valuable information about these mechanisms (Westendorf *et al*., 2006; Reindl *et al*., 2007). Not surprisingly, no effect was observed in the small intestinal tissue after chronic colitis induction, as colitis usually only affects the large intestine.

Taken together, the present studies demonstrated a cytotoxic and/or DNA damaging potential of a variety of food relevant NP, and a simulated gastro- and intestinal digestion did not influence the cytotoxic potential of SiO_2 and ZnO. However, both materials strongly induced pro-inflammatory cytokine secretion that might lead to a subsequent infiltration and activation of neutrophils *in vivo*. Ongoing experiments on mouse colonic tissues will focus on this matter. The genotoxic potential of activated PMN towards human intestinal cells was demonstrated by elevated levels of DNA strand breakage and oxidative lesions, but SiO_2 clearly did not trigger this effect *in vitro*.

It should be emphasised that in all studies performed in the framework of this thesis, relatively high concentrations were chosen. The actual amount of ingested particles for consumers is considered to be markedly lower. The predominantly applied dose in the *in vitro* studies was 20 µg/cm^2. Considering an exemplary intestinal surface area of 200 m^2 (Wells *et al*., 1995) which equates to 2,000,000 cm^2, our dose represents a single dose of 40 g particles (assuming that all material distributes equally over this system). Lomer and colleagues calculated an average daily TiO_2 (micro)particle intake of around 2.7 mg and a daily ingestion of 4.5 mg mixed silicates per person (Lomer *et al*., 2004), which is expected to increase dramatically, but is still around 5000 times lower compared to the *in vitro* doses used in the present studies. However, ingested particles do not immediately dispense throughout the whole intestine, and the small intestine is the first segment of the gut facing the particles. It is more specialised for uptake than the colon and therefore might be burdened more than other intestinal tissues. Furthermore, a daily ingestion

of particles can lead to accumulation. This has already been shown for microparticles by the presence of so-called pigment cells (PC), mainly mature macrophages located at the base of Peyer's patches that contain particle-filled lysosomes (Thoree *et al*., 2008). Up to now these PC were mainly challenged with microparticles and are found widely unreactive, but an increase of NP in our diet, accompanied by elevated uptake (Desai *et al*., 1996) and an increased intrinsic reactivity (Oberdörster *et al*., 2005) might rapidly increase the amount of accumulated particles and possibly alter the activity of PC. Therefore, single application of relatively high doses enables a first screening and discrimination of the hazardous potential of the various materials tested. As no effects were found after treatment with MgO and CB (**Chapter 2**), even at these relatively high doses, further approaches focused on TiO_2 (**Chapter 3**), ZnO (**Chapter 4**) and SiO_2 (**Chapter 4, 5**).

Contrasting to various findings in the lung *in vitro* and *in vivo*, we could not find any relation of the SSA with the toxicity of TiO_2 in the Caco-2 cells (Oberdörster *et al*., 2005; Monteiller *et al*., 2007; Johnston *et al*., 2009). In our hands, this effect was found to mainly depend on the chemical composition, as the anatase-rutile containing particles were more reactive than pure anatase, when photoactivation was avoided. This effect however was in line with the studies from Gurr and colleagues (Gurr *et al*., 2005) and Grassian *et al*. (2007). The present study is the first to confirm this phenomenon to be true for particle treatment of intestinal cells. A thorough validation of the material properties herewith appears to be of major importance.

Agglomeration/aggregation of nanoparticles is a well-known issue, and nearly all studies report this instance, if particles were used without certain stabilisation. For the present studies it was deliberately refrained from stabilising the particles in suspension, as no information is available on the properties of particles or their stabilisation in food or within the human body. However, this raises the question if working with merely aggregated/agglomerated materials actually means analysis of nanoparticle behaviour. This issue is currently discussed controversial amongst scientists, but the aim of this thesis was not to analyse an artificial system but to approximate the *in vivo* situation. Herein, analysation and characterisation of NP within the body is presently complicated and aggregational behaviour not clearly described. Additionally even aggregated/agglomerated nanoparticles still feature a higher SSA compared to their micro-sized counterparts, and were found to induce

stronger oxidative and pro-inflammatory effects in lung epithelial cells (Singh *et al*., 2007).

The simulation of gastrointestinal digestion was a first approach to study altered NP behaviour by means of physiological pH changes. For ongoing studies this assay clearly can be modified to even more resemble the *in vivo* situation. For example, certain enzymes might be included to the buffers, such as pepsin to the gastric buffer, or artificial bile and glycoproteins such as mucin to the intestinal buffer (Zhao *et al*., 1998; Sripanyakorn *et al*., 2009). Nevertheless, this was the first study performed to analyse the influence of gastrointestinal pH changes on NP toxicity, and even though the ROS generating potential appeared reduced, it was even more surprising that no alteration in cytotoxicity or the pro-inflammatory potential could be found. These experiments also enabled us to conclude that the behaviour and toxic potential of particles used for “conventional” cell treatment is close to physiological particle behaviour, as we couldn’t find significant differences in treatment of DS or native particles. It will be interesting to determine the influence of the digestion simulation on the DNA damaging potential of the materials, even before extending the buffers with enzymes or (glyco)proteins.

Various other methods can be considered in the hazard screening of NP for the GI tract. For instance, investigations on the uptake of NP into the intestinal epithelium can be further enhanced by using a transwell system. Herein, cells grow on a permeable membrane to form a confluent cell layer, and are in contact with cell culture media within an underlying well and directly upon the cells. Using this system, differentiated cells express an apical, i.e. luminal aligned, and a basal side. This model allows studying not only particle uptake, but also active particle transport. Therefore, the cells would be incubated apically, which equals the physiological situation. After a certain period, the media on the basal side could then be analysed for the amounts of particles by quantification of the respective element via ICP-OES, as described in **Chapter 4**, or other methods, such as electron microscopy. Gullberg *et al*. developed an *in vitro* M-cell model by co-culturing Caco-2 cells and B-cell lymphoma Raji cells, whereupon the epithelial cells express various M-cell markers and an altered morphology (Gullberg *et al*., 2000). This model could be used to compare to the uptake capacity of “normal” differentiated Caco-2 cells and to further evaluate the underlying mechanisms, for example by use of specific uptake inhibitors (e.g. cytochalasin D to inhibit actin polymerisation) (Quaddoumi *et al*. 2004).

The co-incubation model of Caco-2 cells and PMN was first used as an in vitro model for intestinal inflammation by Vermeer and co-workers (2004), based on preceding studies using co-incubation models of primary human neutrophils and lung epithelial cell lines in the same laboratory to address the role of inflammation in pulmonary toxicity (Borm *et al*., 1997; Knaapen *et al*., 1999). In inflammatory bowel diseases such as UC, a clear correlation exists between the amount of infiltrated neutrophils and the grade of local inflammation (Mazzucchelli *et al*., 1994; Mitsuyama *et al*., 1994). Epithelial cells in active UC are known to be hyperproliferative (Huang *et al*., 1997; Arai *et al*., 1999), which is simulated in this assay by the use of semiconfluent, proliferating Caco-2 cells. However, studies on undifferentiated Caco-2 cells always need to be viewed critically, as they feature various properties of cancer cells. In future experiments, this co-culture model could be modified to investigate the influence of repeated incubation of a confluent Caco-2 cell layer, reflecting the healthy intestinal epithelium, with activated PMN to simulate an inflammatory onset. It will be interesting to determine whether this would lead to an altered permeability of the cell layer, when Caco-2 cells are grown in a transwell system, and whether this would influence NP toxicity and their pro-inflammatory potential, e.g. by determination of the transepithelial resistance (TEER) or intercellular communication (connexins). Investigation of the mRNA regulation of DNA repair enzymes (e.g. apurinic/apyrimidinic endonuclease [APE/Ref-1], 8-oxoguanine DNA glycosylase [OGG1], DNA-polymerase α [DNA-pol-α]) or markers for oxidative stress (such as heme oxigenase 1 [HO-1]) are further approaches for both the existing and the modified model.

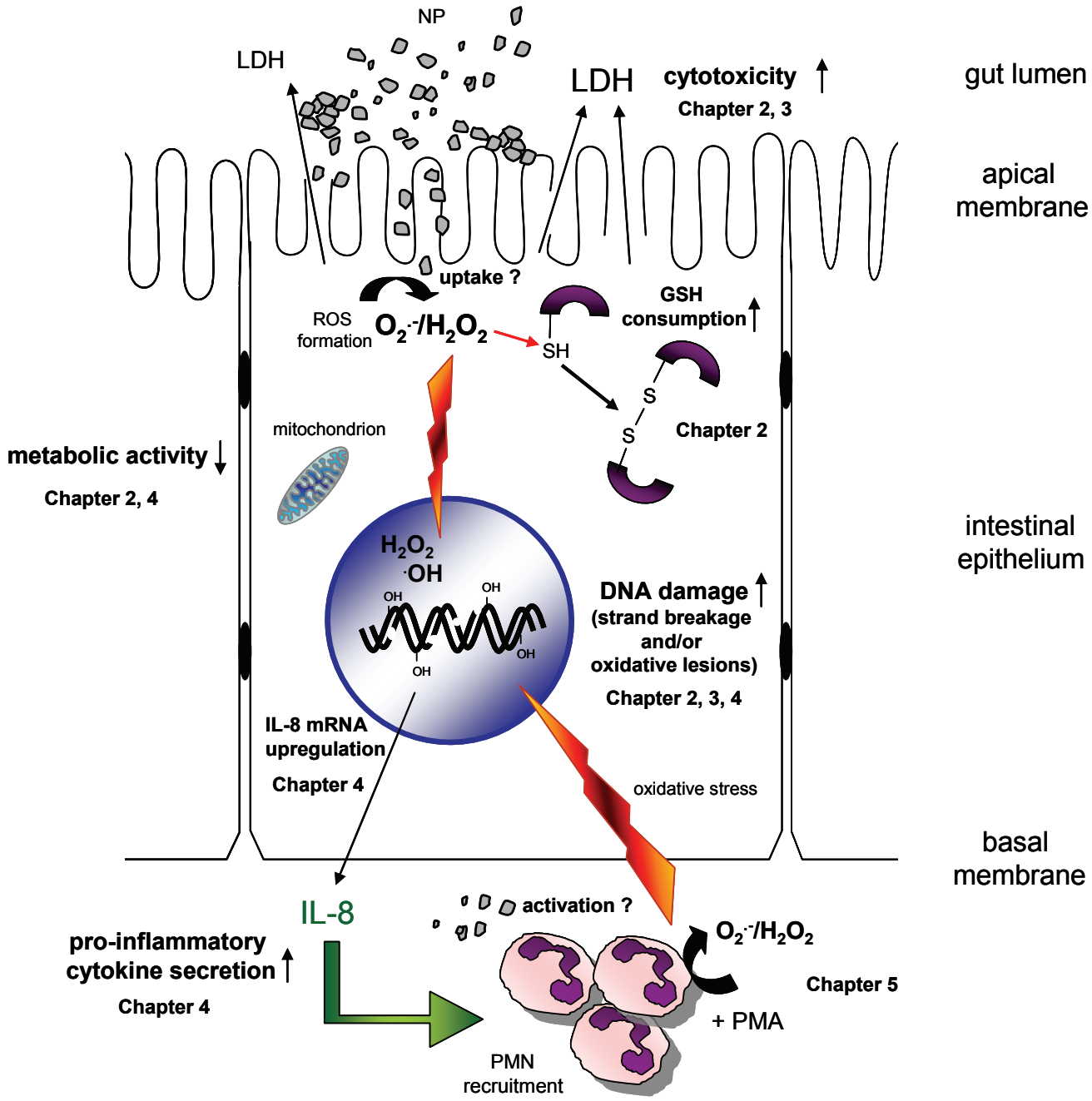

Figure 6.1 **Overview of the various possible adverse NP effects as determined in the present thesis.** Specific NP were found to cause cytotoxicity (LDH leakage following membrane damage and/or decreased mitochondrial metabolic activity), oxidative stress (consumption of glutathione [GSH] and induction of oxidative lesions, as depicted by DNA-associated OH-groups), DNA strand breakage and the mRNA expression and secretion of the pro-inflammatory cytokine interleukin-8 (IL-8). IL-8 is implicated in the recruitment of neutrophils (PMN) from the blood capillaries. PMN were found to induce DNA strand breakage and oxidative lesions in intestinal epithelial cells upon activation with phorbol myristate acetate (PMA), but not upon treatment with specific NP (SiO_2).

6.1 Conclusions

Taken together, the present thesis contributes to our understanding about possible hazards of food relevant nanoparticles, a knowledge which currently is rather limited. The potential of TiO_2, SiO_2 and ZnO to induce cyto- and/or genotoxicity in a human intestinal epithelial cell line was clearly demonstrated. The induction of oxidative stress was shown by reduction of the intracellular antioxidant glutathione and oxidative DNA damage induction. Ongoing studies should be performed to further confirm the importance of oxidative stress by the use of antioxidants (reduced oxidative lesions expected) or depletion of glutathione (increased oxidative lesions expected). In view of current research interest and focus on antioxidant applications in food, it may also be interesting to use non-toxic NP for pharmaceutical and neutraceuticals applications. For example, various antioxidants such as quercetin might be taken up more efficiently when coated with or bound to certain nanomaterials, thus leading to a more directed uptake to the target tissues and increased bioavailability (Boots *et al*., 2008; Lopes *et al*., 2010).

For TiO_2, it was shown that the hazardous potential was merely dependent on the chemical composition, and not on the SSA, as previously expected. Presently it is still too preliminary to recommend the preferential use of pure anatase particles and avoidance of the more reactive rutile-anatase particles in the food sector. However further screening of various TiO_2 formulations is advisable. Furthermore, the present work demonstrated that the physiological pH changes throughout digestion processes did not alter the cytotoxic and pro-inflammatory potential of SiO_2 and ZnO for the evaluated effects. The reduction of the ROS activating potential in the cell-free system further points towards an even reduced reactivity within the human body. This hypothesis was further underlined by the performed *in vivo* study. No DNA damaging effect of ingested SiO_2 was found in the colitic murine intestine. An *in vitro* intestinal inflammation model confirmed these findings. Moreover, SiO_2 appears to even provide certain beneficial effects when inflammatory cells are present, both *in vitro* and *in vivo*. This is an important observation in view of using NP in therapeutic or molecular preventive medicine approaches, and currently ongoing investigations focus on this topic.

A further aspect of the current thesis relates to the specific methods used. The *in vitro* assays developed and used, especially the gastrointestinal assay (**Chapter 4**)

and the co-culture model (**Chapter 5**), further provide tools for general *in vitro* screening of NP that are considered for oral uptake, either in food or for medical purposes. Therefore, the present work also contributes to animal alternative testing (3R concept: 'reduction, refinement, replacement' of animal experiments) (Richmond, 2002).

The broad diversity of engineered nanomaterials and their manifold use complicates a reliable hazard assessment. Nowadays, their widely unpredictable behaviour necessitates an individual evaluation for each particle (*case-by-case-approach*) in each target tissue (*target-by-target-approach*), depending on the application and main uptake route. The findings reported in this thesis offer insights into the hazard and possible mechanisms of some of the main food relevant NP within the intestinal epithelium.

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6.3 Summary

The toxicological mechanisms of nanoparticles (NP) in the respiratory tract are relatively well understood and are thought to arise mainly from their unique ability to generate reactive oxygen species (ROS). NP are characterised by a size range between 1 and 100 nm and can directly induce intracellular oxidative stress, e.g. resulting in cell death or oxidative DNA damage. Induction of pro-inflammatory signalling pathways, which is also triggered by ROS, can initiate or exacerbate inflammatory events by recruitment and activation of phagocytes such as neutrophils or macrophages. NP induced oxidative stress and inflammation in the lung is known to play a role in various lung diseases including chronic obstructive pulmonary disease (COPD) and asthma. Elevated DNA strand breakage and oxidative DNA damage by ROS is implicated in carcinogenesis and may provide an explanation for the associations between inhalation of ambient particulate matter, diesel exhaust particles or nano-sized metal oxide particles and lung cancer.

Industrial applications of nano-sized materials in food and food products have increased dramatically. However, possible adverse effects of particles in the human intestine are poorly investigated to date and these may play a role in chronic inflammatory bowel diseases or bowel cancer.

In the present thesis, a selection of five NP relevant as likely food additives or contaminants (e.g. for use in food packaging) was analysed for the potential to induce cytotoxicity and DNA damage in the widely used human intestinal epithelial cell line Caco-2. Carbon black, a model for environmental NP, as well as magnesium oxide did only slightly reduce the metabolic activity of the cells, and no DNA damaging potential was found. In contrast, titanium dioxide (TiO_2) revealed both cytotoxic and light-dependent DNA damaging potential. Upon detailed particle characterisation, these effects on intestinal cells were found to be mainly driven by the crystalline structure of the different TiO_2 samples and less on their particle size or specific surface area, both being major factors in TiO_2 induced lung toxicity.

Silicon dioxide (SiO_2) and zinc oxide (ZnO) were both found to induce not only strong cytotoxic effects in Caco-2 cells, but also oxidative stress accompanied by induction of (oxidative) DNA damage: Moreover, they showed a strong potential to upregulate the pro-inflammatory cytokine interleukin-8, which is known as a potent recruitment and activating factor for neutrophils. Both materials tended to form aggregates and/or agglomerates in suspension and were found to be taken up into Caco-2 cells independent of microtubule- and clathrin/AP2-mediated endocytosis. A simulated gastrointestinal digestion procedure was established; however, this treatment did not alter the cytotoxic or pro-inflammatory potentials of both SiO_2 and ZnO. This is relevant information for hazard assessment, when these particles are used in food products and as such enter the human intestinal tract.

Potential interactions between NP and neutrophils were investigated using a co-culture-model of freshly isolated primary human neutrophils with Caco-2 cells. In this model enhanced superoxide anion production by the neutrophils was observed and, in association with this, increased DNA damage and oxidative lesions in the Caco-2 cells. However, SiO_2 did not directly activate the neutrophils, and no induction of DNA strand breakage was detected when SiO_2 was applied in the co-culture model. The effect of ingested SiO_2 was therefore also determined *in vivo* in healthy as well as colitis induced mice. The induction of DNA strand breakage and oxidative lesions were analysed by using the highly sensitive Fpg-modified comet assay and immunohistochemical staining. Interestingly, SiO_2 only appeared to induce slight oxidative DNA damage after chronic but not after acute treatment and played no role in the modification of the genome integrity in colitic colons.

Taken together, the present thesis contributed to a better understanding of various adverse effects of relevant NP in the intestinal epithelium and thus offers valuable information for improved hazard and risk assessment. Furthermore, specific experimental models used in the framework of this thesis may serve as tools to screen for the potential hazards of novel engineered NP for which oral exposure can be anticipated.

6.4 Zusammenfassung

Die toxikologischen Mechanismen von Nanopartikeln (NP) im Respirationstrakt sind heutzutage verhältnismäßig gut untersucht. Diese besonders kleinen Materialien, charakterisiert durch eine Größenverteilung von 1-100 nm in ein oder mehr Dimensionen, weisen üblicherweise ein hohes Potenzial zur Generierung reaktiver Sauerstoffspezies auf. Diese wiederum sind häufig Ursache für die zelltoxischen, inflammatorischen und DNA-schädigenden Effekte von NP. DNA-Strangbrüche sowie oxidative DNA-Schäden spielen eine entscheidende Rolle in der Kanzerogenese, und die Inhalation von Feinstäuben, Dieselabgasen oder Nano-Metalloxiden stellt ein erhöhtes Risiko zur Entstehung von Lungentumoren dar.

Eine NP-induzierte Freisetzung von proinflammatorischen Zytokinen kann zudem zur Auslösung und/oder Exazerbation entzündlicher Erkrankungen führen, wobei die Rekrutierung und Aktivierung von Phagozyten wie neutrophilen Granulozyten (Neutrophilen) oder Makrophagen eine entscheidende Rolle spielen. Es konnte gezeigt werden, dass durch NP induzierter oxidativer Stress sowie Entzündungsreaktionen auch eine Rolle bei der Pathologie chronisch-entzündlicher Lungenerkrankungen wie Asthma oder chronisch obstruktiver Lungenerkrankung (chronic obstructive pulmonary disease, COPD) spielen.

Bereits seit Jahrzehnten werden größere, so genannte Mikropartikel als Zusatzstoffe für Nahrungsmittel verwendet, häufig ohne mengenmäßige Einschränkungen. Die veränderten chemischen und physikalischen Eigenschaften derselben Substanzen in Nano-Größe könnten jedoch zu unvorhersehbaren gesundheitlichen Risiken führen, würden diese Partikel in Nahrungsmitteln Verwendung finden. Bereits heutzutage wird umfangreich Forschung betrieben, die zu einer weiten Nutzung von NP in alltäglichen Lebensmitteln, aber auch in so genannten „functional foods“ oder auch Verpackungsmaterialien führen sollen. Vereinzelt sind derartige Produkte bereits auf dem Markt. Die Reaktivität dieser Partikel im menschlichen Körper und mögliche damit verbundene gesundheitliche Risiken sind derzeit jedoch noch weitgehend unerforscht. Besonders im menschlichen Darm könnten NP, vergleichbar mit der Lunge, eine Rolle bei der Entstehung von Darmkrebs oder chronisch entzündlichen Darmerkrankungen wie Colitis Ulzerosa spielen.

In der vorliegenden Arbeit wurden fünf verschiedene NP untersucht, die möglicherweise eine Rolle als Lebensmittel-Zusatzstoff oder in Verpackungsmaterialien für Lebensmittel spielen könnten. Ihr zelltoxisches sowie DNA-schädigendes Potenzial auf den Darm wurde zunächst an der vielfach genutzten humanen intestinalen Epithelzelllinie Caco-2 analysiert.

Es konnte gezeigt werden, dass sowohl Magnesiumoxid als auch Kohlenstoffpartikel, die als Modell für Feinstaub verwendet werden, nur einen schwachen Einfluss auf die metabolische Aktivität der Zellen sowie kein DNA-schädigendes Potenzial aufwiesen. Dagegen führte eine Belastung mit Titandioxid (TiO_2) sowohl zu einer dosisabhängigen Zelltoxizität als auch zu lichtabhängiger Erhöhung von DNA-Strangbrüchen. Fünf verschiedene TiO_2-Partikel wurden daraufhin vergleichend untersucht und die Materialeigenschaften detailliert charakterisiert. Hierbei zeigte sich, dass das toxische Potenzial hauptsächlich von der kristallinen Struktur der Partikel abhing, nicht jedoch von der Partikelgröße oder der spezifischen Oberfläche, welche als wichtigster Faktor der Partikelreaktivität in der Lunge bekannt ist.

Des Weiteren konnte gezeigt werden, dass Siliziumdioxid (SiO_2) und Zinkoxid (ZnO) nicht nur starke Zelltoxizität, sondern auch ein hohes Maß an oxidativem Stress, verbunden mit einer Erhöhung von (oxidativen) DNA-Schäden hervorriefen. Das zytotoxische Potenzial war dabei jedoch abhängig vom Differenzierungsstatus der Zellen. Beide Materialien induzierten zudem die mRNA-Expression und Proteinsekretion von Interleukin-8, einem proinflammatorischen Zytokin, welches besonders bei der Rekrutierung und Aktivierung von Neutrophilen aus dem Blutkreislauf eine Rolle spielt, welche nach Aktivierung erhöhte Mengen an reaktiven Sauerstoffspezies produzieren.

Sowohl SiO_2 als auch ZnO wurden von den Caco-2 Zellen über Mikrotubuli- und Clathrin/AP2-vermittelte Endozytose-unabhängige Mechanismen aufgenommen. Zudem zeigte sich, dass beide Partikel in Suspension in hohem Maß zur Bildung von Aggregaten und/oder Agglomeraten neigten. Ein simulierter pH-abhängiger gastrointestinaler Verdau der Partikel führte zudem zu keiner signifikanten Veränderung des zytotoxischen sowie proinflammatorischen Potenzials der Partikel. Dieses Ergebnis ist besonders relevant für eine Einschätzung möglicher Gesundheitsrisiken, da die verwendete Methode das Verhalten der Partikel im Organismus realistischer widerspiegelt als herkömmliche *in vitro* Partikelaufbereitungen.

Um den Einfluss von SiO_2 auf den inflammatorischen Darm vertiefend zu untersuchen, wurde ein Koinkubationsmodell aus Caco-2 Zellen und frisch isolierten humanen Neutrophilen verwendet. Aktivierte Neutrophile zeigten dabei eine signifikante Generierung reaktiver Sauerstoffspezies und induzierten sowohl DNA-Strangbrüche als auch oxidative DNA-Schäden in den Caco-2 Zellen. SiO_2 dagegen führte nicht zu einer Aktivierung der Neutrophilen, und die DNA-Schädigung in Caco-2 Zellen war zudem sogar leicht verringert im Verhältnis zu einer SiO_2-Inkubation ohne Kokultur mit Neutrophilen. Um diesen wesentlichen Effekt genauer zu untersuchen wurde sowohl gesunden als auch akut bzw. chronisch an Colitis erkrankten Mäusen SiO_2-angereichertes Futter verabreicht. In allen Gruppen wurde keine Induktion von DNA-Schäden im Gesamt-Kolongewebe nach SiO_2-Behandlung nachgewiesen. Immunhistochemische Analysen in Kolon-Gewebeschnitten zeigten dagegen eine leichte Erhöhung oxidativer Läsionen sowohl in SiO_2-behandelten gesunden Tieren als auch nach Colitis-Induktion im chronischen Modell.

Die vorliegende Arbeit leistet einen wichtigen Beitrag zum besseren Verständnis möglicher Risiken von NP in Lebensmitteln und deren Auswirkungen auf das Darmepithel. Die Ergebnisse dieser Arbeit tragen somit zu einer verbesserten Risikoabschätzung und damit erhöhten Lebensmittelsicherheit bei. Zudem können die hier beschriebenen experimentellen Modelle als *in vitro* Methoden zum Screening von neuartigen NP hinsichtlich potenzieller Gesundheitsrisiken verwendet werden.

6.5 Abbreviations

8-OHdG	8-hydroxydeoxyguanosine
°C	degree Celsius
Ag	Silver
Al_2O_3	alumina
AP2	adaptor protein 2
APC	adenomatous polyposis coli
APE/Ref-1	apurinic/apyrimidinic endonuclease
AU	arbitrary units
BCA	bicinchonnic acid- and copper(II) sulphate solution
BET	Brunauer-Emmett-Teller
BSA	bovine serum albumin
CB	carbon black
CD	Crohn´s disease
cDNA	complementary DNA
CGD	chronic granulomatous disease
CIN	chromosomal instability
cm^2	square centimetres
COPD	chronic obstructive pulmonary disease
CPH	1-hydroxy-3-carboxy-pyrrolidine
CRC	Colorectal cancer
CXCR	CXC chemokine receptor
DC	dendritic cells
DC assay	detergent compatible assay
DFO	deferoxamine
DLS	dynamic light scattering
DMSO	dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNA-pol-α	DNA-polymerase α
DPBS	Dulbecco's Phosphate Buffered Saline
DPP4	dipeptidylpeptidase 4
DS	digestion-simulated
DSMZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen
DSS	dextrane sulphate sodium
DTNB	5,5'-dithiobis-(2-nitrobenzoic acid)
Duox	dual oxidases
EDTA	ethylenediaminetetraacetic acid disodium salt dehydrate
EFSA	European Food Safety Autority
ELISA	Enzyme-linked immunosorbent assay
ENP	engineered nanoparticles
E-number	Europe-number (for food additives)
EPR	Electron paramagnetic resonance
F	fine
FCS	fetal calf serum
FDA	Food and Drug Administration
Fe_3O_4, Fe_2O_3	iron oxides
Fpg	formamidopyrimidine glycosylase
G	gauss
GALT	gut-associated lymphoid tissue
G-CSF	granulocyte-colony stimulating factor
GI	gastrointestinal
GSFA	Codex General Standard for Food Additives
GSH	glutathione
GSSG	glutathione disulfide

GTPase	guanosine triphosphate hydrolase
h	hours
HBSS	Hanks´ Balanced Salt Solution
HEI	Health Effects Institute
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HNO_3	nitric acid
$HO^{\cdot}$	hydroxyl radical
HO-1	heme oxigenase 1
HOCl	hypochlorous acid
H_2O_2	inflammatory bowel diseases
HRTEM	High-resolution transmission electron microscopy
HSA	high surface area
hZTL1	human ZnT-like transporter 1
IBD	inflammatory bowel diseases
ICAM-1	the intercellular adhesion molecule 1
ICDD	International Centre for Diffraction Data
ICP-OES	inductively coupled plasma optical emission spectrometry
IEL	intraepithelial lymphocytes
IHC	immunohistochemistry
IL-1β	interleukin-1β
IL-8	interleukin-8
iNOS	inducible nitric oxide synthase
IU/ml	International Units per milliliter
IVCAB	*in vivo* comet assay buffer
LDH	Lactate dehydrogenase
Lgr5	Leucine-rich repeat-containing G-protein coupled receptor 5
LMP	low melting point
LP	lamina propria
LPS	lipopolysaccharide
kDa	kilodaltons
kg	kilograms
kHz	kilohertz
kV	kilovolt
M	molar
m^2	square metres
m^{ϕ}	macrophages
mA	milliampere
M-cells	microfold cells
MDR1	Multi drug resistant 1
MEM	Minimum essential Medium
mG	milligauss
mg	milligrams
MgO	magnesium oxide
min	minutes
MLN	mesenteric lymph nodes
mM	millimolar
mm	millimetres
MPO	myeloperoxidase
mRNA	messenger ribonucleic acid
MSI	microsatellite instability
NADPH	nicotinamide adenine dinucleotide phosphate
NF-κB	nuclear factor-κB
ng	nanograms
nm	nanometer
$NO^{\cdot}$	nitric oxide
NP	nanoparticles

Ns	nano-sized
$O_2^{\cdot -}$	superoxide anion radical
$^1O_2$	singlet oxygen
OGG1	8-oxoguanine DNA glycosylase
$ONOO^-$	peroxynitrite anione
PAF	platelet activating factor
PAMP	particulate matter
PBS	phosphate buffered saline
PC	pigment cells
PCR	polymerase chain reaction
PMA	phorbol myristate acetat
PMN	polymorphonuclear neutrophils
PP	Peyer's patches
ppm	parts-per-million
PSP	poorly soluble low toxicity particles
qRT-PCR	quantitative Real-Time reverse-transcription PCR
RLU	relative light units
RNS	reactive nitrogen species
Ro19-8022	[R]-1-[(10-Chloro-4-oxo-3-phenyl-4H-benzo[*a*]quinolizin-1-yl)-carbonyl]-2-pyrrolidinemethanol
ROS	reactive oxygen species
rpm	revolutions per minute
SCC	sporadic colorectal carcinoma
SD	standard deviation
SiO_2	silicon dioxide
SOD	superoxide dismutase
SPF	specific pathogen-free
SSA	specific surface area
STAT3/4	activator of transcription 3/4
TD_{50}	median toxic dose
TEER	transepithelial resistance
TEM	transmission electron microscopy
Th1/2	type 1/2 helper cell
TiO_2	titanium dioxide
TLR	Toll-like receptors
TNF-α	tumor necrosis factor-α
Tris	tris(hydroxymethyl)aminomethane
UC	ulcerative colitis
UV	ultraviolet
V	Volt
W	Watt
WST-1	water-soluble tetrazolium salt
WT	wild type
XRD	X-ray diffraction
µm	micrometer
ZnO	zinc oxide

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Gerloff K, Albrecht C, Boots AW, Förster I, Schins RPF. Cytotoxicity and oxidative DNA damage by nanoparticles in human intestinal Caco-2 cells.
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In preparation

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Oral presentations

DNA damage in human colon cells by nanoparticles and activated neutrophils. 3rd Symposium of the GRK 1427 “Nanoparticles and the gastrointestinal tract”. 5-6 March 2009, Düsseldorf, Germany.

Nanoparticle-activated neutrophils cause oxidative DNA damage in human colon epithelial cells. 50th Annual meeting of the German Society for Toxicology and Pharmacology (DGPT). 10-12 March 2009, Mainz, Germany.

Poster presentations

Gerloff K, Boots A, Förster I, Albrecht C, Schins R. Effects of engineered nanoparticles on oxidative stress and DNA damage in human colon epithelial cells-lines. 1st Symposium of the GRK 1427 “Food, gut, and the immune system: A challenge in environmental medicine”.
4-5 October 2007, Düsseldorf, Germany

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Gerloff K, Boots A, Förster I, Albrecht C, Schins RPF. Oxidative stress and DNA damage in human colon epithelial cells by engineered nanoparticles. NANOTOX2008, 2nd International Nanotoxicology Conference.
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2-4-June 2010

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Eidesstattliche Erklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit eigenständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe. Ferner versichere ich, dass ich weder an der Heinrich-Heine-Universität Düsseldorf noch an einer anderen Universität versucht habe, diese Dissertation einzureichen. Ebenso habe ich bisher keine erfolglosen Promotionsversuche unternommen.

Düsseldorf, den 19.05.2010

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