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Biologic effects of inorganic Titanium Nanoparticles in Ocular Surface pathology.

Thesis subject

To explore the potential use of Titanium Dioxide Nanoparticles for Ocular Surface diseases

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Table of contents

Abstract

Titanium dioxide is abundant in nature and has found wide use in a variety of applications. TiO_2 nanoparticles' small size is responsible for their unique physicochemical properties. Nanoparticles have demonstrated superb antimicrobial efficacy due their photocatalytic properties. They have also been tried as a photosensitiser for photodynamic therapy of various tumor cells. Activation of the nanoparticles with light has been a prerequisitive for reactive oxygen species production that are responsible for cell distruction. Photoactivated titanium dioxide has been effective against various microorganisms including gram positive, gram negative bacteria, fungi and protozoa, providing an alternative treatment modality in view of the evolution of antibiotic resistant microorganisms. Ocular surface infections such as corneal ulcers can potentially have a devastating impact on vision and ocular integrity. UVA activated TiO_2 can become a promising adjunctive treatment to accomplish faster disinfection and recovery. Ocular surface neoplasias can also present a treatment challenge due to their recurrence rate as well as the difficulty to be completely excised at healthy margins. Photoactivated TiO_2 can be helpful in reducing tumor size or even as a sole treatment to small tumors. Nevertheless the issue of toxicity remains. The ocular surface can sustain UVA irradiation of relatively high dose as known from various existing ophthalmic treatments. Topical, intraoular and systemic absorption of the nanoparticles cannot be ignored. Several studies have reported the toxic effects of TiO_2 on ocular tissues as well as brain and other organs where they can reach through various routes. Although promising, treatment parameters have to be carefully studied and potential ways for toxicity to be eliminated need to be explored so that TiO_2 can find use in ocular surface diseases.

1. Introduction

Titanium dioxide (titanium, titania, TiO_2) is an inorganic compound that is in abundance in nature . TiO_2 is biologically and chemically stable, biocompatible, corrosion-resistant and inexpensive. These properties make it an ideal candidate for biomedical applications. It owes its recent rise in scientific interest to photoactivity. After the illumination in aqueous media with UV light, TiO_2 produces an array of reactive oxygen species (ROS). Production of ROS and thus induction of cell death is the mainstay of photodynamic therapy (PDT). PDT has become an emerging and promising treatment of a wide range of conditions, from antibiotic-resistant bacterial infections to malignant tumours. Neat TiO_2 nanoparticles, as well as their composites and combinations with other molecules or biomolecules, have been successfully tried as photosensitizers in PDT. Due to their nano size and thus increased surface to volume ratio, TiOs NPs have unique properties and are more active than their micro counterparts.

The ocular surface is exposed to various environmental insults and therefore prone to different pathologies. Both microbial infections of the cornea and neoplasias can potentially lead to permanent visual loss. Traditional treatments need to be very intensive and prolonged to control the disease. Even then, sometimes they prove to be inadequate to completely eradicate it. Therefore alternative approaches that replace or complement the existing therapies need to be developed. PDT appears to be a feasible treatment especially as the ocular surface is readilly accessible to UV irradiation.

2. Theory

The question explored is if titanium oxide nanoparticles can be effectively used to treat common ocular surface pathologies namely corneal ulcers and neoplasias without major toxicity side effects.

3. Methods

This thesis is based on a systemic literature review. Only articles in english language are included that are either published or in press with no time frame. Main medical search engines were used such as pubmed and Cochrane library, Google scholar,

Science Direct, as well as medical manuscripts with relevant information (eg ocular anatomy & physiology books). Articles chosen were review papers, original research papers but not case reports or letters to the editor. Data were sought for: ocular surface anatomy, corneal ulcers, ocular surface neoplasias, titanium oxide physicochemical properties, antimicrobial effect, anticancerous effect and toxicity. Search was performed using appropriate key words including titanium nanoparticles, titanium oxide, toxicity, photocatalysis, brain, ocular surface, microbiocidal, cornea, infection, tumor, treatment etc on their own or in combination. Further search for eligible articles was performed among the bibliography of each article chosen. Results are reported for the three variables, bacteriocidal effect, anti tumor effect and toxicity and the strength of evidence assessed. Based on the existing evidence the potential for application of titania to treat ocular surface disease is discussed.

4. Results & Discussion

4.1 Ocular Surface

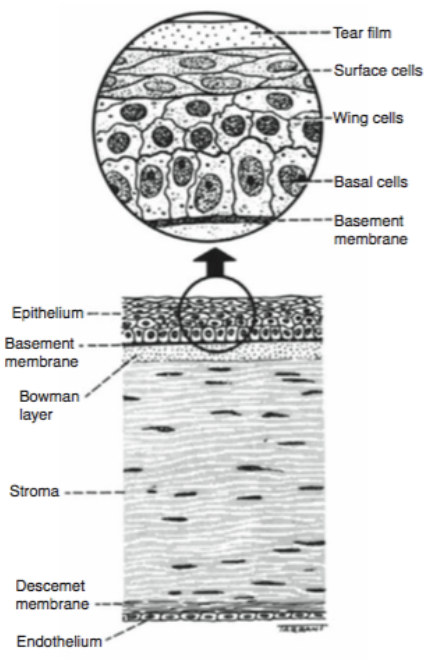

Fig 1. Anatomy of the cornea, demonstrating all layers and details of the epithelium. From J Kanski, Clinical Ophthalmology, A systemic approach, 8th edition.

The ocular surface is composed anatomically of the mucosa that lines the globe and palpebral surfaces, the corneoscleral limbus, the cornea and the tear film (1). The accepted definition of ocular surface was introduced by Thoft and Friend in 1979. They suggested that the conjunctiva, the cornea, the lids and the lacrimal glands work as an integrated unit called ocular surface (2).

The conjunctiva is a membrane covering the globe from the cornea limbus to the fornices (bulbar conjunctiva) where it reflects back to underline the inside part of the eyelids forming the palpebral conjunctiva. On histology, it is composed of squamous cell epithelium attached on a vascularized stroma. Goblet cells in the epithelium are responsible for mucin production, the inner layer of the tear film.

The cornea (fig 1) is an avascular tissue that acts predominantly as a refractive surface converging light rays to the retina and also as a barrier to infections and other harmful stimuli. It is a multilayer structure consisting of five layers: the epithelium, Bowman's membrane, stroma, Descemet's membrane and endothelium. Cornea is thinner in the center (550-565mm) and gradually thickness increases towards the periphery. Anterior curvature is maintained by the anterior stroma rigidity. The cornea consists of cellular components such as epithelial , endothelial cells and Keratocytes, and acellular components namely collagen fibrils and glycosaminoglycanes.

The epithelium is stratified and non keratinized. It comprises of a single layer of basal columnar cells attached with desmosomes to the underlying basement membrane, two to three rows of wing cells, two rows of squamous surface cells the outermost of which has numerous microvilli for mucin attachment. Superficial cells form tight junctions with the adjacent cells through desmosomes. Desmosomes also form attachments between lateral membranes of all epithelial cells, while hemidesmosomes are responsible for attachment of basal calls to the basement membrane. These attachments offer the cornea its barrier function. Other types of junctions are adherence junctions between apical cells and gap junctions between the lateral aspects of all epithelial cells that permit small molecule diffusion. Epithelial stem cells are principally located in the superior and inferior limbus. They are the source of all epithelial cells and maintain the integrity and health of the epithelium. Damage of these cells either from exogenous factors such as irradiation, toxicity or even surgery, or from endogenous factors such as congenital limbal stem cell deficiency associated with genetic disorders, results in corneal decompensation.

Basement membrane consists of collagen type IV and laminin. Bowman layer is the superficial layer of the stroma. It consists of collagen type I and V and proteoglycans. It does not regenerate.

Corneal stroma accounts for the 80% of corneal thickness. It's transparency is due to the precise organization of stroma fibers and extracellular matrix. The fibrils consist of collagen I. Type V and XII are also found in stroma. Those fibrils are arranged in lamellae more densely packed in the anterior stroma. It also contains keratocytes and extracellular matrix (ECM). Keratocytes secrete collagen fibers, glycosaminoglycanes such as dermatan sulfate, keratan sulfate and chondroitin sulfate as well as matrix metalloproteinases (MMPs). Under UVA irradiation keratocytes of the irradiated area die and repopulation may take few weeks.

Descemet's membrane is made of collagen IV and laminin. It is secreted by endothelial cells. Descemet's membrane is very resistant to enzymatic lysis and thus protects the cornea from full thickness perforation when other layers have been destructed by an infection.

Endothelium is a single cell layer. Those hexagonal cells do not regenerate in adults. They are attached to Descemet's membrane via desmosomes. Adjacent cells form interdigitations and possess gap and tight junctions along the lateral boarders. These cells contain two very important ion transport systems: the membrane bound $Na^{+}K^{+}$ATPase and the intracellular carbonic anhydrase pathway. Both of them are very important for the flux of ions from the stroma to the aqueous humour, therefore maintaining the cornea transparency.

The cornea is heavily innervated by the nasociliary branch of the first division of the trigeminal nerve. Normally it is avascular except from tiny vessels that reach the limbus. There are no lymphatic vessels.

Drugs enter the aqueous humour via corneal penetration. The epithelium limits adsorption of hydrophilic substances with its tight junctions and favors the penetration of lipid soluble hydrophobic substances. If epithelium is damaged both substances and microbes get easy access to the intraocular compartments. Water-soluble ionized forms penetrate the stroma. Penetration through the endothelium is determined mostly by molecular size.

4.2 Ocular surface diseases

There are two main categories of ocular surface diseases that warrant special therapeutic management and constitute a therapeutic challenge. A) Corneal infections and B) Ocular surface neoplasias. These diseases can become resistant to conventional treatment so that development of alternative and novel therapeutic interventions are needed.

4.2.a. Corneal infections

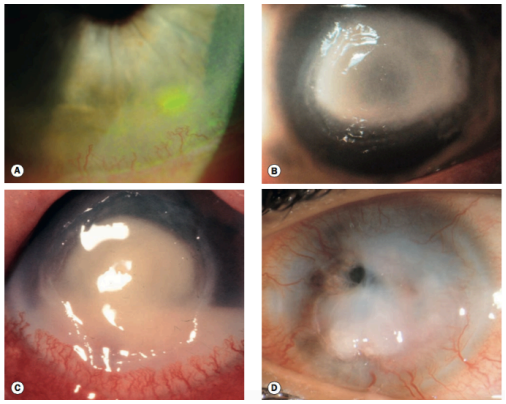

Fig 2. A-D Stages of microbial corneal ulcer. From Kanski J, Clinical Ophthalmology textbook

According to microbiology studies the commensal bacterial flora of the ocular surface comprises mainly of gram positive species such as Staphylococci, Corynebacterium, Propionibacterium and Streptococci (3). A pilot study using DNA-sequencing based detection and identification of bacteria revealed ubiquitous and diverse genera with different prevalence to the one reported in culture based studies. The 16S rRNA gene sequencing showed the highest prevalence of *Pseudomonas*, *Propionibacterium*, and *Bradyrhizobium*, with only 4% of *Staphylococcus* spp. The disparity between the microbiologic and molecular approaches was attributed to the fact that culture-based detection is biased toward fast-growing bacteria that can be easily cultivated on standard media (4). As many of the known ocular pathogens belong to the ocular surface flora, it is possible that ocular infections can be due to a change in their virulence under various circumstances rather than only invasion by an external factor.

Early and accurate diagnosis of infectious keratitis is very important for the treatment and fast resolution of the infection so that the minimum damage to the cornea can be acheived. Gram stain and cultures in appropriate medium have been succesfull in identifying the causative microorganism in most cases, although new methods such as PCR and in vivo confocal microscopy can be used to confirm a diagnosis. Once the causative microorganism is identified appropriate adjustement of antibiotic treatment is required to disinfect the cornea.

Nevertheless two main problems are to be dealt with namely resistance to treatment and corneal distruction resulting in corneal thinning and irregular astigmatism due to severe and prolonged infection. The number of infections related to antibiotic resistant bacteria is increasing worldwide. It is indicative that 80% of the MRSA resistant Staphylococci is fluoroquinolone resistant. As global consumption of antibiotics rise, along with their overuse and inappropriate use, multi-drug resistant and pan-drug resistant microbes are emerging. Those are extremelly difficult to treat due to limited therapeutic options.(5)

Fungal infections can be more persitent and generally have worse outcomes than bacterial. Treatment includes natamycin, amphotericin B and voriconazole. Efficacy can be limited by insufficient penetration and toxicity although new formulations are emerging.

Viral keratitis is also very common. Herpes Simplex Virus is the most common cause of uniocular infectious keratitis. In addition herpes zoster and also CMV can cause keratitis. Herpetic keratitis can reccur and even when not active seem to impact on the patient's quality of life. Protozoan infections are more rare and most of the times contact lens related.

In view of this wide range of potential infectious microganisms, it is obvious that there is a growing need to develop not only antibiotic related approaches but also novel non antibiotic treatments to treat infectious disease.

An innovative method to inactivate pathogenic and resistant microbes is a light-based approach. Although bacteria have been the most widely studied, fungi and parasites have also been killed by these techniques.

4.2.a.1 Light based techniques

Light based techniques use a UV light and a dye called chromophore. Their mechanism of action depends on the production of reactive oxygen species, mainly O^{-}_{2}. Those are very reactive and destroy tissue and cells at the location they are produced. The photodynamic procedure with UVA and riboflavin has been used to inactivate microorganisms in various medical fields and sterilization of blood and water products for transfusion (6). In addition to the chromosomal damage caused by activated chromophore, UV radiation itself has sporocidal and virucidal effects. In ophthalmology the technique of corneal cross linking (CXL) that involves UVA irradiation of the cornea in the presence of fluorescein has been widely used. It has been introduced in 2003 by Wollensak initially for treatment of corneal ectatic diseases in order to stiffen the cornea and halt disease progression. Recently it has been used for the treatment of infectious keratitis (PACK-CXL) as a sole therapy or in addition to antibiotics. The combination of elimination of pathogen load and strengthening of the cornea against enzymatic digestion has a beneficial role in corneal infection treatment (6). Strengthening of the corneal stroma increases it's resistance to proteolysis induced by enzymes produced by polymorphonuclear leukocytes participating in the inflammatory process and also reduces penetration of toxins. (7). As a result corneal healing and restoration of corneal architecture is promoted. Another effect of CxL is keratocyte apoptosis followed by repopulation by proliferating cells.

Efficacy of PACK-CXL in vitro and in vivo has been shown against Staphylococcus Aureus, Pseudomonas Aeruginosa, Streptococcus Pneumoniae and Candida Albicans, Fusarium species and Aspergillus fumigatus (8). Nevertheless the anti fungal and antiparasite effects against Acanthamoeba have not been confirmed in other studies and efficacy is yet to be proved (7, 9).

Downsides of this treatment are reported reduced penetration of antimicrobials, possible reactivation of herpes simplex virus due to damage of corneal nerves and intraoperative thinning that may cause corneal endothelial damage from UV radiation (7). Further more the penetration of UV radiation into the inflamed tissue is not yet known as penetration depth of UVA radiation of 400μm has been confirmed in studies involving healthy corneas (9). As a result, thinning of the cornea tissue due to the infection may

result in direct irradiation of the endothelial cells and irreversible damage of this non-regenerating layer of cells.

Light based approaches have been tried in many fields to assess their microbiocidal effect. Nevertheless those approaches must draw a balance between microbe killing, penetration depth and harm to healthy tissue. Photodynamic therapy (PDT) involves production of different reactive oxygen species through a Type I or II photochemical reaction (5). When excited with UV radiation the triple state of the photosensitive structure transfers an electron to oxygen to form superoxide anion (type II photochemical reaction). Most of these photosensitive (PS) substances tested for their antimicrobial effect possess intrinsic cationic charges like cationic porphyrins, chlorins, bacteriochlorins, phthalocyanines, phenothiazinium dyes, fullerenes, BODIPY-dyes, as well as some natural products. Electron transfer occurs from the PS substance to the inorganic anions to produce free radicals. TiO_2 has known antimicrobial photocatalysis of type I (production of hydroxyl radicals, hydrogen peroxide, and superoxide anion).

4.2.b Ocular surface neoplasias (fig 3)

4.2.b.1. Ocular surface squamous neoplasia

4.2.b.2. Ocular surface melanoma

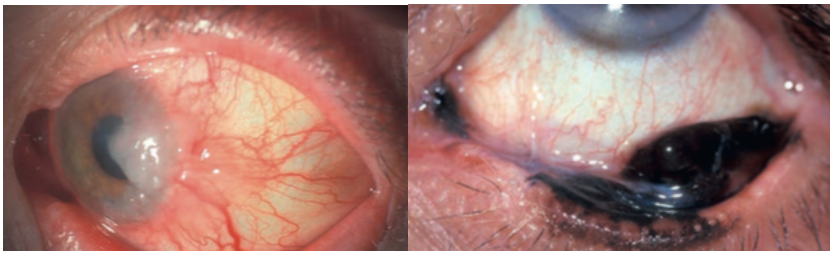

Fig 3. Conjunctival squamous cell carcinoma-Melanoma
From J Kanski: Clinical Ophthalmology textbook 8th edition.

4.2.b.1. **Ocular surface squamous neoplasia (OSSN)** includes a range of entities emanating from the squamous epithelium of the conjunctiva and the cornea. From dysplastic lesions to tumors such as in situ neoplasias and true invasive carcinomas (10), it is the most common neoplasia of the ocular surface. It has an incidence of 0.03-1.9 per 100.00/year in the Caucasian population (11). It is usually unilateral although it can be bilateral in immunocompromised patients.

UV exposure, immunosuppression, male gender, age, smoking, ocular surface injury and vitamin A deficiency as well as genetic predisposition have all been accused as putative risk factors (12,10). The role of Human Papilloma Virus is not yet certain in OSSN. Most likely it acts as a co-factor in already susceptible individuals. It is supposed to be a disease of the elderly therefore if present in young people immunosuppression or genetic predisposition such as xeroderma has to be excluded. Patients are usually asymptomatic although a sensation of grittiness can be present as well as redness. Pain is more rare.

OSSN can present on the conjunctiva or the cornea as a subtle lesion, but in more advanced cases it has the appearance of a gelatinous mass, a nodule or an elevated growth with leukoplakia, localized or diffuse usually with feeder vessels. The corneal lesions are flat and opalescent. (12) Their usual location is the interpalpebral area involving the bulbar conjunctiva and more rarely the tarsal.

Microscopically OSSN presents as mild to severe dysplasia. When the basal membrane is intact then this is the characteristic of in situ carcinoma, when it is involved then the tumor acquires the features of an invasive carcinoma (11). Lesions that are diffuse, multifocal, with large basal diameter > 10mm, thickness > 1mm, brown pigmentation are more suspicious of malignant transformation.

There are several lesions that can mimic OSSN, including pinguecula, pterygium, amelanotic melanoma, pyogenic granuloma, corneal pannus etc (12). Those either benign or malignant can masquerade OSSN.

Diagnosis and grading of OSSN is based on biopsy with histology of the specimen. Excisional biopsy can be possible for small lesions. The advantage in this case is that the whole lesion can be assessed. Nevertheless it might be impossible for large diffuse lesions where incisional biopsy has to be applied. Biopsy of recurrent lesions has the risk of limbal stem cell deficiency, symblepharon and scarring (12).

Other diagnostic modalities are exfoliative cytology, in vivo confocal microscopy and high-resolution spectral domain optical coherence tomography (HR-OCT). Exfoliative cytology can only be helpful in superficial lesions, but cannot be used in grading and invasive tumors. Confocal microscopy requires technical expertise and the efficacy in diagnosis has been variable (10). Small or suspicious lesions can be diagnosed with HR-OCT. It is non invasive, non contact and it can also help in the follow up of lesions on treatment and their recurrence.

Treatment of OSSN can be either surgical or medical. Cryotherapy is also an option. While small lesions can be completely removed with excision, excision of large, multifocal, annular lesions can induce symblephara or limbal stem cell deficiency. In those cases topical medical therapy can be used.

Topical therapy includes interferon a2b drops, 5-FU, MMC 0,02-0,04% depending on tolerance and ocular irritation. Topical chemotherapy can be used as primary therapy, chemoreduction of the tumor or post operative adjunct when the margins are positive. The role of topical chemotherapy in invasive disease is not yet established. (12)

There are a few case reports that used photodynamic therapy coupled with verteprofin in small OSSN with promising results. Reduction of tumor has been attributed to occlusion of the tumor's vasculature as well as the direct phototoxic effect due to reactive oxygen species. (13) Although effective in same case reports, PDT has yet to prove its efficacy in OSSN treatment. Combination with other possible photosensitizers may result in better efficacy.

4.2.b.2. **Conjunctival melanoma (CM)** is a rare but life threatening tumor. It accounts for 2% of all ocular malignancies with rising incidence. It is most common in non-Hispanic white population. It usually arises from Primary Acquired Melanosis (PAM), but it can arise de novo from nevi. The latter carry a higher risk of metastasis and death (14). When amelanotic or non pigmented (20% of CM) clinical diagnosis may be difficult and delayed.

Typically is a nodular, pigmented lesion, located on the bulbar conjunctiva, although it can also present on the palpebral conjunctiva and the caruncle. It is usually surrounded by feeder vessels.

Metastatic disease is not uncommon and 10 year risk is up to 50% depending on the origin. Risk factors are increased tumor thickness, local recurrence and de novo origin (12). Unlike OSSN when CM is diagnosed a full systemic work up is warranted to exclude lymph nodes involvement and more distant metastasis.

Diagnostic imaging can also be helpful. Anterior segment optical coherence tomography, in vivo confocal scanning microscopy, UBM and impression cytology are valuable diagnostic tools.

Definite diagnosis of CM is by histology after complete excision and cryotherapy of margins. Incisional biopsy is better to be avoided. The importance of full excision cannot

be overstressed as remaining tissue can result in high risk of recurrence, metastatic disease and tumor related death. Margins are examined for atypical cells and invasive disease.

Melanomas arise from atypical melanocytes in the epithelial basal layer. Those atypical cells invade the basement membrane into the substantia propria. Four types of cells have been identified: spindle cells, balloon cells, small polyhedral cells and large epithelioid cells, which is the type with the higher morbidity (14). Recent studies have shown molecular commonalities between conjunctival and cutaneous melanoma. Biological data can be useful not only in understanding disease pathophysiology but also has implications for therapy.

Therapy of CM is by complete surgical excision. The conjunctival margins should be treated with cryotherapy. Intraoperatively pure alcohol or sodium hypochloride can be used as cytotoxic agents although rather irritating for the ocular surface.

Orbital enucleation or exanteration is needed in very extended tumors that cannot be controlled with local excision and cryotherapy. Cryotherapy is only reserved as an adjunctive treatment as it has high recurrence rates in cases of multinodular CM. (14).

Topical chemotherapy is also used as an adjunct to surgical treatment. It is mainly used when the margins of the tumor show PAM with atypia. Agents mainly used are Mitomycin C, Interferon-2a-beta. Others such as 5FU are tried. PDT treatment has not been used so far for CM.

5. Titanium nanoparticles

5.1 General characteristics of TiO2

Titanium dioxide (titania, TiO_2) is a metal oxide that is abundant in nature. It has four polymorph crystal structures, metastable anatase (tetragonal, a = b = 3.782 Å, c = 9.502 Å), stable rutile (tetragonal, a = b = 4.584 Å, c = 2.953 Å), brookite (rhombohedral, a = 5.436 Å, b = 9.166 Å, c = 5.135 Å) and TiO_2 (B) (monoclinic, a = 12.16 Å, b = 3.74 Å, c = 6.51 Å). The last two forms are less studied. (15,16). The anatase and the rutile crystals are formed by a basic building block consisting of a titanium atom surrounded by six oxygen atoms in an octahedral configuration. The octahedron shares two, three, and four edges with adjacent octahedra to give rutile, brookite and anatase, respectively (15,16). (fig 4.)

In rutile forms the octahedron is slightly distorted so that the unit cell is stretched beyond a cubic space. In anatase forms the unit is significantly distorted so that there is no orthorhombic symmetry. Anatase and brookite are metastable, and transform irreversibly to a stable rutile phase by heating at 500–700 °C. Rutile is the most stable form for nanoparticles of more than 35nm while anatase form is the most thermodynamically stable form for nanoparticles 10-20nm (16). The physicochemical properties of TiO_2 and their photocatalytic activity are governed not only by their intrinsic electronic status and crystalic form but also from their size, shape and surface area and doping. The equilibrium shape of anatase consists of a truncated bipyramid constructed by {101} and {001} facets. According to the Wulff construction, the {001} facets constitute nearly 6% of the total exposed surface of anatase TiO_2, while stable {101} facets contribute to more than 94% of the surface area. However, the {001} facets of anatase TiO_2 have a higher photocatalytic performance than {101} facets (17). TiO_2 NPs with a larger surface area and smaller size than their bulk counterparts generate more ROS during photoexcitation.

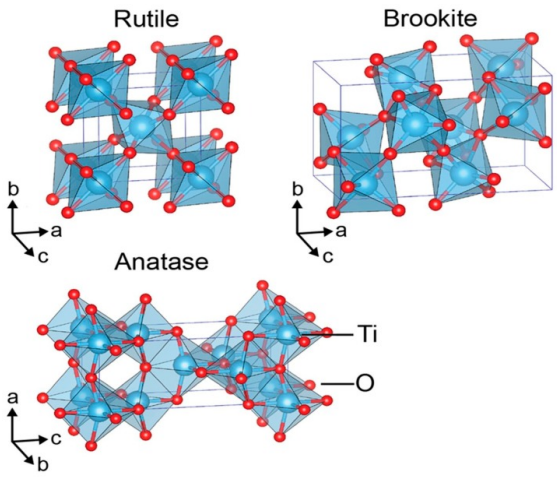

Fig 4. TiO_2 isomorphs.(Reproduced from Haggerty J.)

5.2 Production of titania

Titania is produced by various ways such as purification of rutile mineral and chloride or sulfate process of ilmenite ($FeTiO_2$). Both routes yeld pure titania. Heating of amorphous titania to 400 ^{0}C produces the anatase and the brookite form, a process called calcination. When these polymorphs are heated above 600 ^{0}C, they convert to rutile form (18).

TiO_2 nanoparticles can be produced in the form of thin films, nanocrystals and powder. There are three main categories of fabrication techniques. A)Physical deposition techniques such as thermal evaporation, reactive sputtering and pulsed laser deposition. B) chemical gas-phase atomic layer deposition (ALD) process and C) wet chemical deposition methods such as dip-coating, spin-coating, spray coating and sol-gel, that have been employed by researchers to prepare TiO_2 thin films.

Chemical wet solution process techniques are the more convenient for production of large quantities of titania especially for bacteriocidal uses . Sol gel and hydrothermal technique are the most frequently used for Titania production (16). New green methods and microwaves are also becoming more popular.

With the sol-gel method titania colloids can be synthesized through the hydrolysis and condensation reaction of titanium alkoxide in the presence of water, and these reactions are catalyzed by an acid. The sol-gel process involves the transformation of metal alkoxide or metal salt into a solid by adding an excess of water to give a metal–oxo linkage (M–O–M)(28). The hydrolysis facilitates the formation of original nuclei TiO_2, and the subsequent condensation promotes the growth of a crosslinked network of TiO_2 nuclei. This strategy allows the formation of TiO_2 NPs with a high level of chemical purity . For fabricating metal-doped TiO_2 nanopowders, an additional metal source reagent is needed, and added to titanium precursors during the sol-gel process.

The hydrothermal/solvothermal method is a useful tool for fabricating TiO_2 nanostructures involving chemical reactions in a solvent (water/nonaqueous) medium at an elevated temperature >100 °C and a pressure higher than 1 atm, within a closed system using an autoclave. As the sol-gel process generally produces amorphous or low crystalline materials, a subsequent annealing at high temperatures for crystallization is needed. In this context, hydrothermal or solvothermal processing is beneficial for improving the crystallinity of titania synthesized by the sol-gel technique.

The organic solvents in solvothermal treatment help to control the morphology of synthesized nanocrystals. Thus, this process enables better control of the shape, size distribution and crystallinity of TiO_2 NPs in comparison with the hydrothermal method. (17)

Titania nanostructures with specific physicochemical properties can be obtained and tailored by carefully selecting and modifying the parameters of the process such as titanium substrates used, temperature, solvents and time. Further modification can occur with binding of surfactants, dopants or other organic molecules on their surface, resulting in improvement of properties such as lower band gap, charge separation and light absorption shift. (18).

Fabricated Titanium nanoparticles can be characterized in terms of size and morphology by transmission electron microscope (TEM), crystal type with an X Ray diffractometer (XRD) and size distribution with techniques such as light scattering. Infrared spectroscopy allows analysis of the chemical groups present on the surface. Diffuse reflectance UV-Vis (UV-Vis DRS) spectroscopy determines the light absorption spectrum of the functionalized materials thus their bandgap (15).

5.3 Photocatalytic properties

Titanium dioxide is a semiconductor material. The energy gap of the rutile form is 3,06mV and 3,23mV for the anatase polymorph. The energy gap is the key property of a semiconductor material. TiO_2 nanoparticles can only be activated by UV light, corresponding to an onset of the absorption band at about 350nm. That accounts for less than 5% of the solar spectrum (19). Photocatalysis of the TiO_2 involves three stages. Excitation, bulk diffusion and surface transfer of photoinduced charged carriers. Firstly when absorption of photons with energy higher than the energy gap occurs, titanium enters an excited state where released electrons move to the conduction band, leaving positively charged holes in the valence band. Secondly the separated electrons and holes separate and migrate to the surface of the crystal. This is affected by the crystallinity, the crystal structure and the particle size of the photocatalyst. Thirdly those free electrons react with surface oxygen to yield superoxide radical anions while the holes can react with H_2O to produce hydroxyl radicals (fig 5). Those species in solutions can further react

to give more ROS including hydrogen peroxide and peroxy radicals. Those highly unstable oxygen species react with cell membanes causing apoptotic or necrotic cell death.

The isomorphs can affect the extend of ROS production. Anatase isomorph is more active than rutile (20). Very small nanoparticles < 10nm may fail to generate $^1O_2$ as due to their small size they cannot maintain charge separation. In that case, recombination of holes and electrons can occur so photocatalytic activity is decreased. Recombination occurs when excited electrons return to the valence band without interacting with the adsorbed surface species. This recombination produces energy in the form of light or heat.

Photocatalytic activity can be enhanced either by reducing the energy gap therefore shifting activation to the visible light spectrum, or minimizing the recombination rate. To achieve this, surface modification with various elements has been investigated. Doping with both nonorganic and organic materials has been tried. These include metal and non-metal doping, coupling with semiconductors, and modification with graphene oxide or carbon nanotubes (17).The incorporation of those dopants into titania affects its electronic band structure greatly, thereby promoting visible light absorption and a red shift in the bandgap.

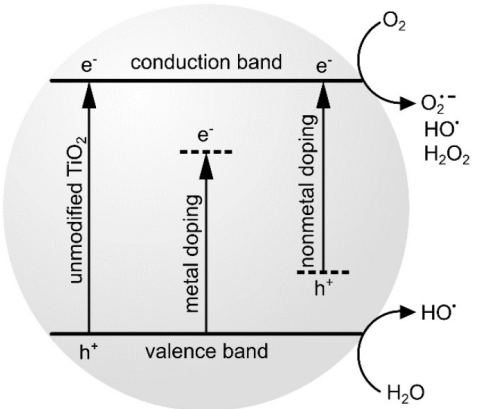

Fig 5. Simplified mechanism of Reactive Oxygen Species generation. Reproduced from Zaleska A.

5.3.1.Doping

Titanium or oxygen ions' sites of titania lattice can be substituted with either metal or nonmetal dopants to alter their optical and photocatalytic properties. The cationic doping of titania with transition metals, rare earth metals and noble metals is typically used to improve its photocatalytic performance under visible light excitation. The dopant energy level is typically located below the conduction band (CB) of TiO_2, acting as an electron or hole trap, and thus allowing suppression of recombination and transportation of more carriers to the surface . The photocatalytic activity of metal-doped titania depends on several factors, including the dopant concentration and distribution withinin the particles, type of metal dopant, d-electron configuration and energy band level of dopant in the titania lattice and incident light intensity (21). Significant improvement in photocatalytic activity can occur only at optimal dopant content. Above this level rapid recombination rate of photogenerated charge carriers can occur from a reduction in the distance between the trapping sites by increasing the number of dopant ions (17).

Titania can be self-doped with Ti^{3+} ions to improve its visible-light absorption and avoid the incorporation of other impurities into its lattice. Apart from Ti^{3+} ions, other transition metals, such as copper (Cu), vanadium (V), chromium (Cr), manganese (Mn), iron (Fe) and nickel (Ni) can be intoduced to the crystalic structure to enhance the visible-light photocatalytic activity of titania. Generally, two or more types of metal cations can be incorporated into the TiO_2 lattice. This is typically termed the 'co-doping'.The visible light response of titania can also be achieved by doping with noble metals such as gold (Au), silver (Ag), platinum (Pt) and palladium (Pd).

Graphene has also been used as a dopant. Graphene provides a network to facilitate the rapid transfer of excited electrons therefore promotes the separation between electron–hole pairs and inhibit their recombination. Under visible light illumination, electrons located in high-energy graphene states are delocalized into the conduction band of TiO_2.

Non-metal elements such as carbon, nitrogen and boron, with an atomic radius close to that of the O_2 atom, can be utilized as anionic dopants for replacing lattice oxygen anions. In this respect, non-metal doping appears to be an alternative route for enhancing visible light efficiency due to the introduction of a new valence band (VB) with an upshifted position compared to the titanium .

Titania can be coupled with other semiconductor metal oxides (e.g., Cu_2O, Fe_2O_3, WO_3) and chalcogenides (e.g., CdS, MoS_2 and WS_2) that have a lower energy band level. Combination of semiconductors enhance charge separation with visible light absorption. Photoexcited electrons in the CB and holes in the VB of a sensitizer semiconductor can be transferred to the CB and VB of TiO_2 NPs .

In conclusion mixed polymorphs have been shown to be more active. This additive effect can be attributed to the reduction of energy gap and consequently to reduction of the recombination of photo generated holes and electrons, thus increasing the generation of reactive oxygen species. ROS act at a close distance from their production. Of those, sort life ROS such as $^1O_2$ and OH^- can interfere with intracellular pathways to induce local lipidic membrane peroxidation, while the long life ROS such as H_2O_2 diffuse intracellularly and induce apoptosis by interacting with the neighbouring cell signal pathways (20).

Another approach to improve the photocatalytic properties of TiO_2 beyond modification of chemical composition with various dopants has been proposed by Aprile C et al. (19) This is based on manipulation of titanium nanoparticle size and spatial organization (physical approach).

Other modifications of TiO_2 such as to improve solubility in water has been achieved by Seo J et al (16) through a high temperature non-hydrolytic method. By doing so aggregation and agglomeration that leads to loss of photocatalytic potential could be prevented. Fabrication of such nanoparticles that showed high toxicity in the presence of UV radiation could be used in biological applications.

5.4 Photodynamic therapy

Photodynamic therapy (PDT) is a technique that involves three main components: a photosensitizer (PS), a light source and an oxygen molecule. The photosensitizer can be either a macromolecule or a nanosized organic or inorganic nanoparticle. The photosensitizer is excited by photon absorption and transfers energy to the oxygen or water molecule to produce reactive oxygen species. In detail, the ground state of oxygen is excited to $^1O_2$ and then to the triplet state. Photoactivated PS can produce ROS for up to 18 hours, time enough to cause irreversible damage to the target cells but also adverse effects to the surrounding environment (22). The hydrophilic nature and their delivery process are the two most important features of PSs for PDT (23). Due to the hydrophilic

nature and formation of hierarchical structures of TiO_2, it is a very suitable material for the photodynamic treatment as production of ROS and consequently cell apoptosis can be used for skin disease, microbial infections and tumors (18,22). Nevertheless, TiO_2 in its pure form can only be excited by UV radiation with short wavelength (385nm) due to its energy gap. (15,16). However UV radiation has low penetration, can cause tissue overheating, destruction of proteins and DNA, and as such can be harmful to the human body. Therefore titania induction to photodynamic therapy has been relatively limited.

5.5 Antimicrobial properties

The antimicrobial activity of UVA-activated TiO_2 was first demonstrated by Matsunaga and coworkers in 1985. Since then, there have been many reports on the use of photocatalysis for the destruction of gram positive and gram negative bacteria, fungi, algae, protozoa and viruses as well as microbial toxins and even prions (24). NPs acheive direct contact with the bacterial cell wall, and thus penetrate the cell; this raises the hope that NPs would be less prone to promoting resistance in bacteria than antibiotics. According to existing research, the major processes underlying the antibacterial effects of NPs (fig 4) are as follows: 1) disruption of the bacterial cell wall 2) generation of ROS; 3) penetration of the bacterial cell membrane; and 4) induction of intracellular antibacterial effects, including interactions with DNA and proteins. Titanium dioxide nanoparticles' antibacterial activity is attributed to their photocatalytic action that results in enhanced cell permeability, causing accelerated photo-oxidation of the intracellular components and cell death (25) (fig 6).

Titania NPs are negatively charged at the point of zero charge (pzc) at pH = 6.2. Therefore, they exhibit low bactericidal activity in neutral and alkaline solutions by repelling negatively charged bacteria in the absence of light. At acidic pH, positively charged TiO_2 NPs strongly interact with the bacterial cells, resulting in bacterial membrane penetration and inducing oxidative damage accordingly. The photocatalytic power of titania due to ROS generation depends on the power and duration of UV-A radiation.

Cell walls and membranes are important defensive barriers for bacterial resistance to the external environment. The components of the cell membrane produce different adsorption pathways for NPs for Gram-positive and Gram-negative bacteria (17).

Bacteria exhibit a negative charge on their cell wall surface. The cell wall of Gram-positive bacteria is relatively porous and thick (20–80 nm) , thus allowing various substances to penetrate and cause cell damage and death. It consists of several layers of peptidoglycan that is negatively charged, interspersed with teichoic and lipoteichoic acids, so a large number of NPs are attracted. In contrast, the cell wall of Gram-negative bacteria is thinner (<10 nm) with a single peptidoglycan layer, surrounded by an outer membrane with a very complex structure. Lipopolysaccharides (LPS) and lipoproteins are located in the outer leaflet, while phospholipids are found in the inner leaflet of the outer membrane. The phosphate groups of LPS increase the overall negative charge. Thus Gram-negative bacteria have a higher negative charge than Gram-positive . The structural variations in the cell walls between these two bacterial strains lead to their different interactions with photocatalysts. As such, Gram-negative bacteria is more resistant to attack from the superoxide anion and hydroxy radical with a negative charge. Moreover, LPS also creates a permeability barrier at the cell surface, thus contributing to its resistance against many antibiotics and substances . Many studies have shown that NPs have greater activity against Gram-positive bacteria than against Gram-negative bacteria due to the structure of the cell wall. In fungi and yeast, cell walls are mainly composed of chitin and polysaccharides (26).

Foster et al (24) confirmed that titanium dioxide NPs can adhere to the surface of bacterial cells to produce ROS. ROS generated on the titania surface are very effective at killing bacteria through lipid peroxidation, depletion of glutathione, DNA damage and finally disintegration of the cell membrane. This results in a leakage of cellular contents, thus causing cell lysis and eventual cell death (27). Negatively charged superoxide and hydroxyl radicals generally reside on the membrane and do not penetrate into the bacterial cytoplasm, while electrically neutral H_2O_2 can pass through the cell membrane. Hydrogen radicals can abstract hydrogen atoms from the fatty acids of bacterial membrane lipids, causing lipid peroxidation and damaging the respiratory electron transport chain located in the membrane. Oxidative damage generates lipoperoxidation also of internal cell membranes due to their lipid nature, so the respiratory chain, which takes place in the double-membrane mitochondria, is also affected. This organelle is a natural source of ROS in aerobic metabolism because superoxide anions are produced in the electron transfer respiratory chain process. Mitochondria can control this fact by

converting them into H_2O_2 by superoxide dismutase (SOD), and finally into water by glutathione peroxidase and catalase. Overproduction of ROS depletes the cells anti-oxidants.

Damage at molecular level in DNA affects all regulatory microorganism functions such as metabolism, replication, transcription, and cell division (26). DNA is particularly sensitive to oxidative damage because oxygen radicals, especially $OH^{\cdot}$ produced by Fenton reaction, may attack the sugar-phosphate or the nucleobases and cause saccharide fragmentation and as a consequence strand break. The enzymatic detoxification system (SOD, glutathione and catalase) as well as other systems are upregulated in order to cover DNA injury. Those systems are related to post-translational modification, protein turnover, chaperones (related to folding) and DNA replication and repair. These are all significantly over-expressed in the presence of TiO_2 NPs.

In terms of genetic issues, there is evidence that the bacteria change the level expression of certain genes encoding for proteins involved in lipopolysaccharide and peptidoglycan metabolism, pilus biosynthesis, and protein insertion related to the cell wall which values are lower-expressed after exposition to TiO_2 NPs .In contrast with the lower expression of genes related to the cell wall seen before, the level expression of genes encoding for enzymes involved in metabolism of lipid essential for the cell membrane structure, are over-expressed. It would be concluded that cells compensate the initial cell wall damage by reinforcing the second defense barrier, the cell membrane, in a way to provide support against the oxidation produced by ROS. (26).

The physicochemical properties of NPs such as size, charge, zeta potential, surface morphology, and crystal structure are significant factors that regulate the actions of NPs on bacterial cells. Anatase forms are more active than rutile and mixted forms are reported more active than anatase alone. This is attributed to the interaction between the two forms reducing the amount of recombination (28). Moreover, environmental conditions like pH and temperature, the bacterial strain, and the exposure time are some other major factors that influence the antibacterial effects of NPs (27). The presence of light is important to the antibacterial properties exhibited by titanium. Although an

increase in nano-TiO_2 concentration may lead to an increase in bactericidal performance, antibacterial effect may decrease at very high concentrations. A possible explanation of this paradox can be that although an increase in nano-TiO_2 concentration results in an increase of contact with the bacteria, at very high concentrations the nano-TiO_2 has less contact with light, resulting in a decrease in photocatalytic activity, therefore its antibacterial effects. It is important to note that an increase in temperature may change the anatase structure into rutile, deteriorating the photocatalytic activity.

Lin et al. prepared TiO_2 NPs with smaller particle sizes which produced high contents of intracellular reactive oxygen species. The small surface area of nanoparticles resulted in superior membrane damage and internalization (25).

Morphology also affects activity. The photocatalytic activity of one-dimensional Titanium nanotubes (TNT) is considerably higher than that of TiO_2 NPs because of their large surface area, high aspect ratio, and good light-harvesting properties (29) . Recently, Podporska-Carroll et al. (30) reported that TNTs exhibit very high bactericidal efficiency against *E. coli* (97.53%) and *S. aureus* (99.94%) under 24 h of UV irradiation. Moreover, anodic TNTs exhibit a higher photocatalytic inactivation of bacteria than commercial Degussa P25 TiO_2 powders. They attributed this high efficacy to the nanotube architecture and inherent desirable features such as high-aspect ratio, enhanced active surface area and improved light harvesting and trapping. As with nanoparticles, to extend the optical absorbance to the visible-light region and improve bacteriocidal performance in this optical regime, noble metal dopants are added to TNTs accordingly.

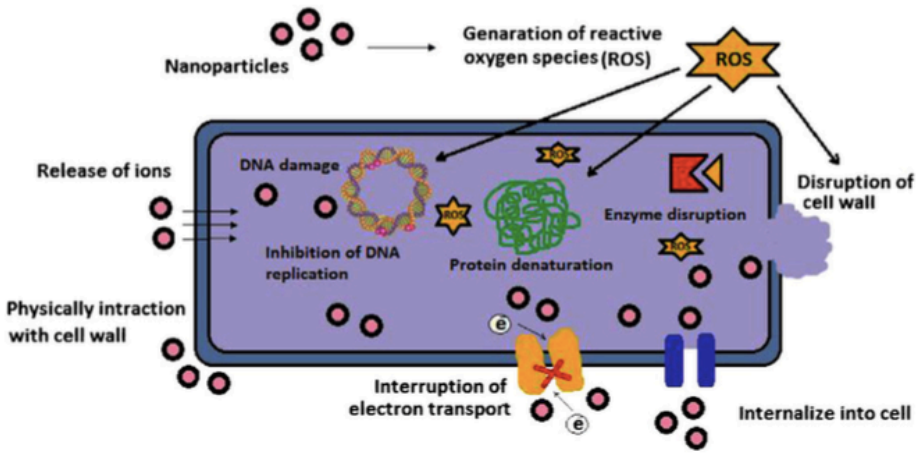

Fig 6. Mechanism of antibacterial action of nanoparticles by Dijah et al 2014.

The majority of antimicrobial studies have been performed with Escherichia Coli and it's subtypes. Lin et al (31) investigated the antibacterial effect of five types of TiO_2 NPs with various crystal phase and particle size against E Coli. They concluded that anatase TiO_2 NPs are more prone to attaching on the bacterial surfaces than rutile NPs as shown by TEM, and the larger NPs interact weaker with cells compared to the smaller NPs. As particle size decreases, the ratio of surface area to mass increases and changes in the physicochemical properties (e.g., surface atom reactivity, electronic and optical properties) of the nanoparticles occur, consequently, the smaller particles tend to agglomerate to a greater extent, which can further influence their reactivity and binding characteristics and even cell death. If TiO_2 NPs are sufficiently small, they can penetrate in the cells. They can then induce the potential photocatalytic process inside the cells , adsorb and deactivate biomolecules such as proteins. Therefore, the physical NP-cell attachment and interaction could substantially contribute to the observed nanotoxicity. Carrey et al (32) conducted an in vitro study where E Coli was treated with TiO_2 NPs irradiated for 30 minutes. He concluded that the antibacterial photocatalytic activity was accompanied by lipid peroxidation that caused enhancement of membrane fluidity and disruption of cell integrity.

In the environment, nanoparticles form compounds consisting of nanoparticles, their aggregates and agglomerates greater than 100 nm. These compounds are defined NOAA (nano-objects). An aggregate is a particle that is comprised of strongly bonded, or fused, single primary particles whereas an agglomerate is a collection of weakly bonded single primary particles, aggregates or a mix of the two. Yamada et al (33) assessed the effects of TiO_2-NOAAs on microbes under UV irradiation by using E. coli and Saccharomyces cerevisiae. Their findings suggest that the amount of ROS generated by TiO_2- NOAAs was not sufficient to show growth inhibition and inactivation of microbial growth. Their results also suggested that TiO_2- NOAAs protect microbes during UV irradiation. Furthermore, it was suggested that this phenomenon was caused by an adsorption effect on TiO_2-NOAAs.

Allahverdiyef et al (34) demonstrated that TiO_2 nanoparticles (100 μg/ml) inhibited *Leishmania* parasites, one of the most important eukaryotic pathogens after

irradiation with UV light. Maness *et al.* reported that ROS which occurred on the surfaces of TiO_2 carried out lipid peroxidation reaction and caused the death of *E. coli* K-12 cells (35). Ahadrami A. in his study found TiO_2 nanorods to have superior antibacterial activity at 30 minutes following UVA exposure. Tsuang *et al (*36) investigated the effects of TiO_2 nanoparticles on different bacteria cells such as *E. coli*, *Pseudomonas aeruginosa*, *S. aureus*, *Enterococcus hirae* and *Bacteroides fragilis* under UV light 365nm, at 8mW/m^2 for 50 minutes. They concluded that TiO_2 nanoparticles under UV light inhibited approximately all bacterial cells indicating that TiO_2 nanoparticles are very effective as antimicrobial agents.

Liu P et al (37) assessed the activity of TiO_2 against E coli and the damage of it's outer membrane after irradiation with UV light for one hour. They concluded that the outer membrane was irreversibly damaged after the treatment and this effect was due to the photoactivation of TiO_2. UVA irradiation alone or exposure to NPs only had a minimal effect. Huang YY (38) for the first time in his study showed that TiO_2 antimicrobial photocatalysis is strongly potentiated by addition of the nontoxic salt potassium iodide. The magnitude of the effect was surprisingly large, giving up to six logs of additional killing. The extent of microbial killing depended on the concentration of TiO_2, the delivered fluence of UVA light, and critically on the concentration of added KI. The killing was broad spectrum in nature, with the Gram-negative *E. coli* being the most susceptible, followed by the Gram- positive MRSA and then the fungus *C. albicans*.

Roy et al. (27) evaluated in vitro the effect of 20nm TiO_2 nanoparticles with different antibiotics against methicillin-resistant S. aureus (MRSA). They reported that TiO_2 nanoparticles improved the antimicrobial effect of beta lactams, cephalosporins, aminoglycosides, glycopeptides, macrolids, lincosamides and tetracycline against MRSA but not of nalidix acid. In another experiment of the same group their results showed that antimicrobial resistance of MRSA against various antibiotics decreased in the presence of TiO_2 nanoparticles. (26,39).

Haghighi et al (40) investigated antifungal effect of TiO_2 nanoparticles on the fungal biofilms from fluconazole resistant standard strains of Candida albicans (C.

albicans). According to their results, the synthesized TiO_2 nanoparticles had improved antifungal effect. The authors suggested that TiO_2 nanoparticles could effectively inhibit the fungal biofilms especially those formed on the surface of medical devices.

In studies concerning solar catalytic water disinfection with TiO2 by Lonnen (85), the ease of inactivation for the various pathogens decreases in the following order: E.coli (DH5a) > P.aeruginosa >Acantamoeba polyphaga(trophozoite) > C.albicans > F.solani >B.subtilis(spore) >A. polyphaga (cyst). This result indicates that photocatalysis with TiO2 can be effective with bacteria and protozoa but not with their resistant forms, spores and cysts. Similarly, in a review by Foster H et al (24), it is concluded that most fungi, algae and protozoa have been shown to be susceptible to photocatalysis. Fungal spores were generally more resistant than vegetative forms, and *Trichoderma harzianum* spores in particular were resistant to killing under the conditions tested .Cysts of *Acanthamoeba* showed only a 50% reduction during the treatment time . The authors speculated that further killing might have been acheived if the treatment time had been extended.

Lipovski A et al (41) conducted an in vitro study using TiO_2 and ZnO NPs against Staphylococcus aureus and Staphylococcus epidermis. He used non-UV light (blue and visible light) irradiation for 5minutes. Combination of illumination with the nanoparticles resulted in a mean reduction of 80–90% of the microorganisms. He concluded that TiO_2 nanoparticles can serve as good exogenous photosensitizers for bacteria killing, especially for skin wound disinfection.

As mentioned doping with metals has shown enhanced photocatalytic properties. Especially the combination with AgNPs exhibits excellent antibacterial activity against various microorganisms, including *S. aureus*, MRSA, Bacillus subtilis, *E. coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, and *Acinetobacter baumani*. (42)

Visible light-responsive TiO_2 doped with non-metals exhibit bactericidal activity against a wide variety of bacterial species including Gram-negative *E. coli*, Acinetobacter baumannii, Shigella flexneri, and Gram-positive *S. aureus*, Bacillus subtilis, Listeria monocytogenes, as well as Bacillus anthracis spores (43). In a study by Ananpattarachai J et al (44) , N-doped TiO_2 was far more effective in inactivating S aureus and E Coli under

visible light than neat TiO_2 . Those photocatalysts can be used for the disinfection of pathogenic bacteria. The bactericidal activity of polymer-TiO_2 nanocomposites under visible light also depends on the type of polymers employed. In particular, natural chitosan (CS) with biodegradable behavior can bind to TiO_2 NPs through its amino and hydroxyl groups, thereby extending the optical absorption of TiO_2 NPs into the visible region. The CS/TiO_2 nanocomposites exhibit a red shift in absorption in their UV-vis spectra .The bandgap of TiO_2 NPs in CS/TiO_2 nanocomposites is then reduced from 3.20 to 3.00 eV (24) .

As well as their considerable antimicrobial efficacies, it is also thought that bacteria may be difficult to develop resistance to metallic nanoparticles simultaneously since these nanoparticles attack different components inside the bacterial cells. Therefore microbial cells will need to acquire multiple mutations to develop resistance toward NPs. Despite this fact resistance to NPs is always a clinical concern. Wu at al(45) found that after exposure to Cu^{++} and Cu-doped TiO_2 NPs, reduced antimicrobial activity of TiO_2 NPs to *Shewanella oneidensis* was noted. He proposed that this effect is likely to be associated with decreased uptake and/or increased efflux of Cu^{++} and Cu-doped TiO_2 . He raised the concern for careful use of nanostructures as antibacterial agents.

Based on the existing evidence TiO_2 NPs are excellent antimicrobial agents. However, toxicity generates the most important disadvantages of metal oxide nanoparticle use and has prevented their wide spread clinical applications to date.

5.6 Anti-tumor effect

The oxidative stress is the underlying factor for adverse biological effects caused by TiO_2 NPs in cancer cells. In the cells this is reflected by the increase of the level of ROS and oxidative products, as well as by the depletion of cellular antioxidants (46). It is assumed that TiO_2 NPs, when exposed to the UV radiation, induce, depending on the intensity of the oxidative stress and their subcellular position, one of the cell death pathways: apoptosis, autophagy, or necrosis, whereas the mechanism of those processes is still insufficiently examined. Plasma membrane damages lead to cell necrosis, nucleus and mitochondria damages induce apoptotic cell death, and endoplasmic reticulum damages cause cell death by autophagy. In a molecular level ROS can inflict cell injury as

an effect of reaction with lipids in cellular membranes, nucleotides in DNA, or sulfhydryl groups in proteins. ROS generated have as a short half-life (<0.04 μs). Therefore, the occurrence of ROS-induced oxidative injuries is limited to a small distance (<0.02 μm) from the subcellular position of the sensitizer NPs (47).

The NPs with size ≤ 10 nm are able to deeper penetrate tumors and better accumulate in tumor cells, but they are also toxic to healthy cells. Bigger NPs, in the range of $10 < \phi \leq 100$ nm, can only hardly be uptaken by healthy cells while they can easily penetrate tumor cells (48). Cells are equipped with mechanisms protecting them from ROS. The first defense line prevents ROS from forming and reacting with cell compounds. These are proteins (e.g., lactoferrin) that sequestrate metal ions (e.g., Cu, Fe). The second defense line is interruption of free radical chain reactions. It consists of a system of three enzymes: superoxide dismutase, catalase, and glutathione peroxidase, supported by low molecular weight antioxidants, such as glutathione (GSH), thioredoxin (Trx), or coenzyme Q_{10} (CoQ_{10}). The third defense line is repair enzymes responsible for nucleic acids damage e.g., DNA (91). When all mechanisms are depleted cell damage occurs. So, PDT-treatment of tumor cells, using the metal oxide nanoparticles, results in the cell death, as a consequence of irreversible DNA damage, plasma membrane disintegration, intracellular Ca^{2+} homeostasis disruption , strong reduction or complete exhaustion of the redox compound pool, essential in ATP synthesis (49).

There are two major ways of the NPs uptake into the cell: active uptake by endocytosis and passive uptake by diffusion. Due to their strong hydrophilic properties, TiO_2 NPs are uptaken into the cells by phagocytosis.

Zhang et al. (50) examined UV-excited TiO_2 NPs toxicity towards human hepatocarcinoma cells (SMMC-7721 line). After 30 min of UV exposure of 3,7mW/cm^2 intensity and TiO_2 concentration of more than 200μgr/ml, 88% of the cancer cells were killed. He advocated that the tumor cell damage occurred in two stages, initially damage of the cell membrane that leads to increased permeability, but does not produce a significant decrease in cell viability. Damage of intraocular contents follows at the second stage. The decrease in cell viability, eventually cell death, occurs as a result of leaking

intracellular components, or nano-TiO_2 trafficking into the damaged cells that directly attack nuclei and other intracellular components. It is also reported that molecular scavengers of both hydrogen peroxide and hydroxyl radicals, such as catalase and N-acetylcysteine could effectively diminish cell death when added to cell samples (51).

The conjugation of NPs of a precious metal with TiO_2 NPs increases the catalytic activity of this oxide. This phenomenon was confirmed in the studies of Abdulla-al-Mamun et al (52). They reported that Ag/TiO_2 nanocomposite showed an 100% effectiveness in eradication of human epithelial carcinoma (HeLa) cells compared to 20% of neat TiO_2 NPs under 5 min irradiation.

Lai Y.T in his study (53) showed that human cervical carcinoma cells (HeLa line) were efficiently killed by a mixed mechanism of apoptosis and necrosis when treated with folic acid modified TiO_2 and irradiated with UV light. 30 min exposure resulted in survival rate of less than 15%. In other studies, TiO_2 NPs were also conjugated with folic acid (FA) molecules. Feng et al (54) confirmed high biocompatibility of FA/TiO_2 nanocomposite and its incorporation into human nasopharyngeal carcinoma (KB) cells, nevertheless a photokilling effect of 38-43% was achieved that was strongly reversibly related to the concentration of folate. Kubota et al (55) performed in vitro and in vivo experiments with UVA activated TiO2 and T-24 human bladder cancer cells. Activated NPs killed 70% of T-24 cells in 5 minutes at a concentration of 100μgr/ml. In vivo it delayed the growth of tumor in mice for 30 days. Photoactivated TiO_2 NPs were also effective against lung adenocarcinoma cells. In his study Xu M et al (56) revealed that the possible mechanism of action of oxidative stress related to cell death and not to apoptosis. Cerium element doped TiO2 nanoparticles induced apoptosis under visible light illumination in human hepatoma (Bel 7402) cells. Wang L et al (57) observed apoptotic features in the cells after irradiation with visible light for 10 minutes. Apoptotic bodies appeared 4hours post irradiation. Human colonic mucinous adenocarcinoma cells (Ls-174-t line), human colon adenocarcinoma cells (LoVo line) have also been exposed to photoactivated titania with similar results (48).

Though the anticancer effect of TiO_2 is certain, the killing is devoid of specificity.

Hence, it is necessary to prepare improved TiO_2 nanoparticles, which can specifically identify and bind with the receptors of cancer cells, in order to increase the selective antitumor activity and reduce the non-selective cell death. Recently, monoclonal antibody proteins (CEA, pre-S1/S2, IL13a2R and EGFR) with high affinity and specificity have been immobilized on the surface of TiO_2 nanoparticles. These proteins are overexpressed on the surface of certain cancer cells, therefore the modified TiO_2 nanoparticles with a specific antibody are useful for directing the nanoparticles towards the specific cell population. The antibodies recognize cancer cells, bind to them and are eventually uptaken by means of phagocytosis. With selection of suitable antibodies, combined nanocomposites are able to precisely target and eradicate many types of cancer cells. Xu et al. (58) tried a simultaneous application of conjugates of TiO_2 NPs with monoclonal antibodies, along with reversible plasma membrane injury by means of electric field, so called electroporation. Using that method, it was possible to eradicate in vitro all human LoVo cancer cells that were subject to experiment, within merely 90 min of UVA irradiation while 39% of normal cells were killed. Phototoxicity was also confirmed against brain glioblastoma cells (59). When exposed to TiO_2 nanoparticles tethered to a cell specific antibody under visible light programmed cell death occurred. It was supported that the superoxide anion was the primary ROS for cell damage.

Lagopati et al (60) showed that the highly malignant MDA-MB-468 breast cancer cells are more susceptible to TiO_2 nanoparticles and UV-activated-TiO_2 nanoparticles-induced cell death compared to the MCF-7 cells. After UVA irradiation for 20 min the viability of MD-MB-468 cells dropped to 50% related mainly to apoptotic cell death. They concluded that TiO_2 nanoparticles possessed cell- specific toxicity, depending on the concentration of the particular particles. Similarly, in a study by Cascione M et al (61) the acute morphological and mechanical cytotoxic effects of TiO_2 exposure on MCF -7 breast carcinoma cells were assessed. The reduction of the cell viability induced by TiO_2NPs was dose and time dependent, and the same trend was observed after membrane damage evaluation; By using confocal and atomic force microscopy, they demonstrated that TiO_2NP exposure induced significant alterations in cellular membrane elasticity, due to actin proteins rearrangement in cytoskeleton.

In a study by Shang H. et al (62), Reduced Graphene Oxide -TiO_2 composites were successfully prepared by a simple hydrothermal reduction method. The composites expanded the absorption range from UV to visible region, and its photokilling efficiency was enhanced. Photocatalytic RGO-TiO_2 composites induced apoptosis of human hepatocellular cells (HepG2) cells by DNA oxidative damage, mitochondria disruption and elevation of intracellular Ca^{2+}concentrations leading in cell apoptosis. They suggested that RGO-TiO_2 composites could be an excellent candidate as a PDT photo- sensitizer for cancer treatment.

The potential of photocatalyzed TiO_2 nanoparticles to eradicate cancer cells became of particular interest against melanoma cells. Zeni PF et al (63) evaluated the cytotoxic effect of pure TiO_2 and nitrogen-doped TiO_2 nanoparticles, on a murine melanoma cell line (B16-F10) and fibroblasts (NIH 3T3) .The cells were subjected to PDT treatment and irradiated for 4 hours. PDT using UV radiation showed that TiO_2 and N–TiO_2 nanoparticles at 0.1mg/ml dosage had no effect on melanoma cells. At 0.5 mg/ml, survival rates with TiO_2 were 50%, whereas N–TiO_2 exhibited a higher photokilling effect, with only 7% cell viability. So the effect was concentration dependent. In fibroblasts, TiO_2 and N–TiO_2 presented a marked photokilling effect at 0.1 mg/ml, 60% and 80% respectively, under UV radiation. Gene expression analysis of this sample showed, under ultraviolet photoexcitation, an increase of pro-apoptotic BAX gene expression, suggesting cell death by apoptosis. The study concluded that this material is promising in the PDT treatment of melanoma. Seo JY et al (64) exposed melanoma cells (A-375) to TiO_2 at a concentration of 400 μg/mL for 30 min of UV irradiation. Only 14 % of the cancer cells treated with the nanoparticles survived. Cancer-cell survival also depended upon the amount of UV exposure; the longer the irradiation time, the greater the cell mortality.

Moosavi MA et al (65) showed the concentration-dependent capability of well-dispersed photo-activated N-TiO_2 NPs to induce terminal megakaryocyte differentiation or cell death in K562 leukemia cells. These cellular outcomes depend on intracellular ROS levels and are mediated by autophagy . In this situation, low PDT doses (10 μg/ml N-TiO_2, 12 J/cm^2) also increased ROS and autophagy levels in normal human peripheral blood lymphocytes, but it did not lead to any growth inhibitory or cytotoxic effects in this

human normal-cell model.

According to a study by Dorota F et al (66) Fe-dopped of TiO_2 nanotubes demonstrated superiority compared to undoped NTs in terms of PDT phototoxicity against cervical cancer cells, under near-visible light (2.30 mW cm^{-2}, ≈405 nm). Again they postulated that the mechanism involved was apoptosis. They also demonstrated that under dark conditions the nanoparticles did not exhibit cytotoxicity indicating their good biocompatibility.

Cancer has frequently been considered as a cancer stem cell disorder rather than only a disease of rapidly growing cells. Thus recognizing and targeting cancer stem cells is a great challenge. The monoclonal antibody (mAb) Nilo1, which can recognize the surface antigen in neural stem cells, has been successfully coupled to TiO_2 nanoparticles by G. Elvira et al (67). The Nilo1-TiO_2 complexes were verified to specifically deplete in vitro cancer stem cells upon UV-irradiation, which provided the basis for further applications of TiO_2 in cancer stem cells therapy.

5.7 TiO_2 Toxicity

TiO_2 fine particles (FPs) are believed to be chemically inert, poorly soluble and low toxicity. Therefore they have been used in many toxicological in vitro and in vivo studies as negative controls. However, when the particles become progressively smaller, their surface areas, in turn, become progressively larger, and their physicochemical properties dramatically change. Some of their properties can present a challenge to human health. Several studies have shown that TiO_2 NPs are more toxic than their fine size analogues and those effects are associated with their small size. Oberdorster et al reported that TiO_2 NPs (21 nm) caused a greater pulmonary inflammatory response at same mass burden compared to fine nanoparticles (FPs), with greater amounts of TiO_2 NPs entering the alveolar interstitium in the lungs. (68). Surface modification such as coating, influences the activity of TiO_2 NPs both in terms of cytotoxicity as well on biological response end points (68).

TiO_2 NPs has been abundantly used in a wide variety of applications including

stabilizing dyes, coatings, cosmetics, food products and pharmaceuticals, toothpastes, as well as a photocatalysers. As a consequence human exposure to TiO_2 is significant. The most common primary route of exposure is inhalation, but exposure can also occur with food products, dermal contact and intravenous or intraperitoneal injection. At the moment recommended exposure limits exist only for respirable dust. According to the United States National Institute for Occupational Safety and Health (NIOSH) the recommended exposure limit (REL) for TiO_2 NPs is at 0.3 mg/m^3, which was 10 times lower than the REL for TiO_2 FPs (69). Further research is needed in order to define safety exposure limits for other routes of exposure.

The level or concentration of TiO_2 NPs in the body system depends on the rate (or kinetic) of absorption, distribution, metabolism, and excretion of TiO_2 NPs (fig 7).

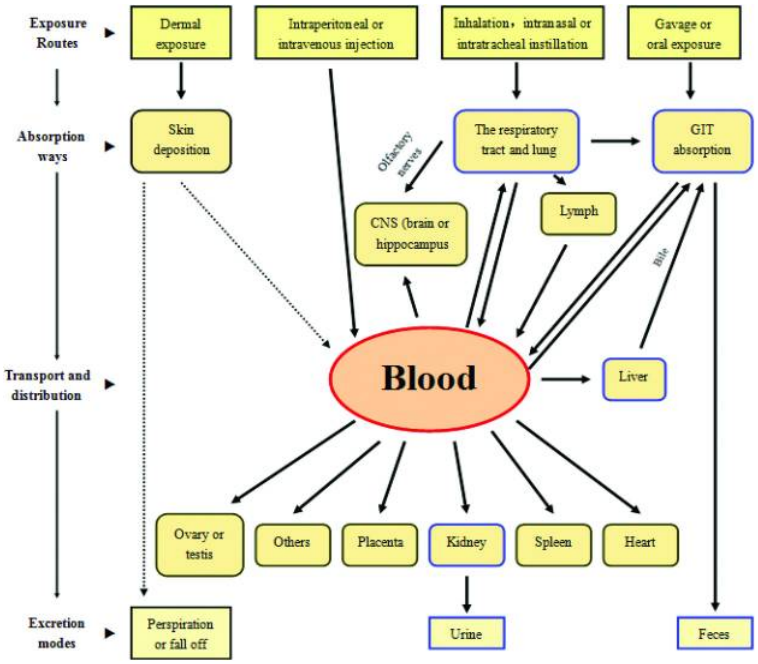

Fig 7. Toxokinetics of TiO_2 NPs in vivo Interrupted line indicate uncertainties.By Hong F et al 2017

5.7.a. TiO_2 absorption

Dermal absorption: The outer skin of human beings consists of a tough layer of stratum corneum that is difficult for inorganic particles to penetrate. Several studies have investigated dermal penetration by TiO_2 NPs. Some of them concluded that TiO_2 NPs did

not penetrate the intact human skin even though the particle size was less than 100 nm and the subcutaneous tissue was damaged. Further observation with scanning electron microscopy (SEM) showed that although some TiO_2 particles had lodged into vacant hair follicles, it did not penetrate the dermis or viable epidermis (70). Therefore, it can be concluded that TiO_2 NPs are not systemically available to a significant extent after dermal exposure.

Gastrointestinal absorption: it is proposed that the absorption through the GIT occurs via the lymphoid tissue and subsequently distributes to other organs (68).

Pulmonary absorption: The pulmonary system consists of the upper respiratory tract (nose and nasal passages, paranasal sinuses and pharynx) and the lower respiratory tract (larynx, trachea, bronchi and lungs). Inhalation exposure is one of the major routes for TiO_2 NPs to gain entry into the human body especially in occupational settings. Even though the inhalation, intratracheal instillation and intranasal studies in regards to pulmonary absorption are few and absent in humans, they suggest that TiO_2 NPs can translocate from the lung into the circulatory system to systemic tissues and from the nasal cavity into sensory olfactory nerves to the nervous system. In rats the majority of deposited TiO_2 NPs is transferred to the interstitial space following intratracheal instillation for 42 days with a small fraction of pulmonary NPs to gain entry into the blood circulation and reach extra-pulmonary tissues. Therefore that data suggest that the rate of NP migration to the circulatory system is low.

5.7.b Distribution

After the initial absorption of TiO_2 NPs, the systemic circulation can distribute the particles to all organs and tissues in the body. After TiO_2 NPs reach the systemic circulation, these particles potentially interact with plasma proteins (71) coagulation factors, platelets and red or white blood cells. The binding to plasma components may have a substantial effect on the distribution, metabolism, and excretion of the NPs. Binding to plasma components might neutralize or mask the adverse effects of TiO_2 NPs in the systemic circulation. Therefore the biokinetics of the engineered NPs is also dependent on the local corona environment. For both the hard and soft coronas, ROS will

oxidize adsorbed proteins, but this process, even with the exchange of proteins, likely does not deplete all ROS. Over longer times (24 h), it is possible that ROS concentration exceeds the protective effect of the protein corona and that low levels of ROS present throughout the 24 h period have a cumulative effect on lipid peroxidation (49).

TiO_2 NPs injected intravenously or intra-peritoneally were found in different organs, such as liver, spleen, kidneys, lung, lymph nodes, and brain. Intraperitoneal injection and intravenous injection are the most common methods of administration, by which NPs can then directly enter the blood circulation. Chen *et al* (73) reported that TiO_2 NPs deposited in the liver, kidney, spleen and lung. The order of the TiO_2 NP distribution (70/30 anatase/rutile, 20–30 nm) in the intravenously injected rats was liver > spleen > lung > kidney .TiO_2 NPs also have the potential to penetrate the blood–brain barriers (BBB) and blood- placenta barriers (74,75). However, these studies employed very high doses of TiO_2 NPs that may not be applicable to real human exposure.

There is insufficient data regarding nanoparticle metabolism. Excretion of TiO_2 NPs may be through kidneys/urine, and bile/feces. Those inhaled can also be captured by mucus and transported to larynx from where they can be excreted by sputum or enter GIT. However, there is evidence that not all NPs are removed by mucociliary clearance from the lung surface, resulting in prolonged TiO_2 NP retention. Approximately 25% of NPs can removed by mucociliary clearance within 24 h, while the rest can be retained for more than 48 h. Those can potentially further interact with the inner lung surface cells, especially macrophages, and dendritic and epithelial cells and enhance the probability of NPs traversing the epithelial barrier. So even though a large fraction of absorbed TiO_2 NPs could be excreted rapidly, it is possible that not all of these particles will be eliminated from the body. As a result, accumulation of TiO_2 NPs in some organs may take place in the human body after continuous exposure. A major site of accumulation seems to be the liver. However, there is a possibility that the accumulated TiO_2 NPs can be completely cleared from these sites if study time frame is increased .

5.7.c Types of Toxicity of TiO_2 NPs

The toxic effects of test substances are usually measured in terms of acute, sub-

acute, sub-chronic or chronic exposure conditions. Studies with a maximum of 2 weeks (14 days) study duration are normally referred to as acute toxicity studies. Sub-acute toxicity studies last for a maximum of 4 weeks (28 days), sub-chronic toxicity studies for a maximum of 13 weeks (90 days) and chronic toxicity studies last longer than 4 months. Acute and chronic exposure are of most importance.

C1) Acute toxicity

Acute toxicity information for TiO_2 NPs in humans is currently lacking. The acute toxicity of TiO_2 NPs has been frequently studied in rat and mouse models following exposure of different routes of administration. The majority of studies target the respiratory system. Studies exposing the pulmonary system to TiO_2 NPs produced both local and systemic symptoms and aggravate pre-existing symptoms. These inhalation studies showed that at sufficient lung burdens in both rats and mice TiO_2 NPs can cause pulmonary inflammation. TiO_2 NPs administered through the lung are more inflammatory than FPs of similar chemistry at equal mass concentrations. Liu et al (76) treated rats by intratracheal instillation with a single dose of TiO_2 NPs. Histopathological examinations of lung tissue after a week from exposure indicated that exposure to TiO_2 NPs induced dose-dependent inflammatory lesions . In addition, on an equal mass basis, pulmonary toxicity induced by 5 nm TiO_2 NPs was more severe than those induced by 21 and 50 nm TiO_2 particles.

The results from the other exposure routes cannot be ignored. Using acute dermal irritation studies in rabbits and the local lymph node assay in mice (CBA/JHsd mice), Warheit et al. (77) concluded that TiO_2 NPs were not a skin irritant or dermal sensitizer. Acute oral exposure studies showed biochemical changes, but systemic toxicity was not demonstrated. Intraperitoneal studies has shown that at the higher doses of an intraperitoneal exposure study done on mice, TiO_2 NPs (anatase, 5 nm; 5, 10, 50, 100, and 150 mg/kg BW; everyday for 14 days) caused serious damage to the liver, kidneys, and myocardium and disturbed the balance of blood sugar and lipid (78). A number of in vitro studies also show toxic effects of TiO_2 NPs on cells of the circulatory system.

Overall research evidence demonstrates that TiO_2 NPs can be absorbed through the lung or GIT into the systemic circulation and then distributed in different organs such as the liver, kidneys, spleen, or even the brain. Distribution and accumulation of TiO_2 NPs in the organs could induce organ injuries and inflammatory responses. However, most of the doses employed are too high to be realistic in occupational settings.

A study by Rafaiovich *et al.* generated a promising approach in order to solve the toxicity-related problems (79). Nanoparticle–polymer conjugates that he reported can be considered as significant therapeutic agents since they showed to be harmless to human cells and tissues. Prevention of penetration of metallic nanoparticles into normal human cells and target towards the insulting agents offers the most important step for treatment of infections and tumors with nanoparticle use.

C2) Chronic toxicity

Most chronic toxicity studies focus on the respiratory system. TiO_2 NPs exhibit moderate toxicity, inducing pulmonary inflammatory response and enhanced proliferation of pulmonary cells at relatively high doses. Studies on TiO_2 NPs have shown similar effects to TiO_2 FPs, such as increased incidences of pneumonia, squamous metaplasia, sustained pulmonary responses, enhanced proliferation of pulmonary cells, defects in macrophage function, alveolar epithelial metaplasia, progressive fibroproliferative lesions and accumulation of macrophages in interalveolar septa (77). Oberdorster et al. (68) investigated the correlation between particle size, in vivo particle persistence, and lung injury after a 12 weeks inhalation experiment in rats. Inflammation and lung injury were more exaggerated in case of NPs, compared to FPs. This effect could be explained by their larger specific surface area, the greater interstitial access, and their altered biopersistence, resulting in increased retention of NPs. In another study (81) intragastric long term exposure to TiO_2 NPs (5–6 nm) on ICR mice resulted in chronic spleen injury from alterations of inflammatory and apoptotic cytokines followed by reduced immunity.

In vivo and in vitro studies were conducted to investigate the genotoxicity of TiO_2 FPs and NPs, but results are conflicting. Some studies indicate that TiO_2 NPs are

genotoxic, whereas others give negative results. Even though the rationale for these conflicting results is not clear, use of different cell types, exposure metrics, crystalline structure, particle dispersion and NP sizes may be an explanation. Most of the cell lines, which show genotoxicity, are cells associated with the respiratory system and the circulatory system. Overall, the studies indicating that TiO_2 NPs are genotoxic outweigh the studies that state otherwise. Thus, TiO_2 NPs can be treated as potential hazards. Nevertheless more studies are needed to determine the conditions in which TiO_2 NPs genotoxicity occurs (77). The possible mechanisms for TiO_2 NP-induced genotoxicity involve DNA damage directly or indirectly via oxidative stress and/or inflammatory responses.

Similarly, limited in vivo and in vitro studies suggest that TiO_2 NPs exposures may exert certain reproductive and developmental toxicities. Similarly further studies are needed to clarify the mechanisms underlying these toxicity results (77).

Carginogenicity of TiO2 NPs has also been a concern for research. Experimental pulmonary animal studies support the carcinogenicity of TiO_2 NPs with both intratracheal exposure and inhalation. TiO_2 NPs can cause bronchoalveolar adenomas and cystic keratinizing squamous cell carcinomas at high doses (81). Bernard et al (82) conducted toxicological and carcinogenesis studies of dietary TiO_2-coated mica in rats fed with diets containing high concentrations of TiO_2-coated mica for up to 130 weeks. They found no evidence that TiO_2-coated mica produced either toxicological or carcinogenic effects. Several skin studies evaluated the carcinogenic effect of TiO_2 (83,84). They concluded that dermal exposure was not carcinogenic as the nanoparticles could not penetrate healthy skin and reach underlying skin structures.

Epidemiologic data on humans is currently lacking. So potential danger has to been extrapolated from carcinogenicity studies in animals. Those indicate that TiO_2 NPs can produce tumors when exposed through inhalation or intratracheal instillation and are more carcinogenic on an equal mass basis than TiO_2 FPs with adenomas and squamous cell carcinomas predominating. Based on the available studies, TiO_2 NPs were evaluated

by World Health Organization (WHO)/IARC as a Group 2B compound.

The exact mechanisms of TiO_2 NP-induced carcinogenesis are not clear. Recent evidence indicates that ROS formation, induction of inflammation and alterations in cell signal transduction induced by TiO_2 NPs may play an important role in the etiology of their carcinogenesis ability. ROS-induced signaling and activation of the IL family of cytokines, Bax, caspases 3 and 9, NF-κB, and p53, as well as phosphorylation of p38 and G_2M phase cell cycle arrest seem to be common findings (77). In regards to induction of inflammation leading to the production of ROS, inflammatory cytokines seem to play an influencing role. Elevated levels of ROS and down regulation of ROS scavengers and antioxidant enzymes are associated with various cancers. This depletion causes oxidative stress. Oxidative stress induces a cellular redox imbalance found in various cancer cells. ROS could induce non-selective DNA damage, which may result in genetic changes in active genes. Oxidative damage to cellular DNA can lead to mutations. The mutations in DNA may be involved in the initiation of various cancers. Therefore, oxidative stress induced by ROS generation may play an important role in the initiation and progression of multistage carcinogenesis of TiO_2 NPs.

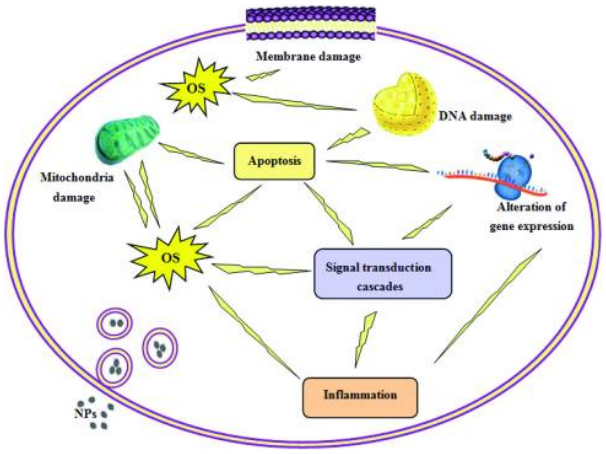

Fig 7. Mechanism of TiO_2 toxicity. By Fushi H. 2017

5.7.d. Specific organ toxicity

5.7.d.1.Lungs

As mentioned in the previous section, studies have shown that both acute and chronic toxicity can result from TiO_2 inhalation. In general, exposure to TiO_2 NPs may lead to histopathologic changes, and the release of ROS in the lung or pulmonary inflammation *in vivo*. In addition the expression of genes involved in the cell cycle, apoptosis, chemokines, and complement cascades are altered.

Chronic exposure to TiO_2 NPs caused significant accumulation of NPs in the lung, reactive oxygen species (ROS) production, increased lipid peroxidation, decreased antioxidant capacity, and enhanced expression of inflammatory cytokines in a dose dependent way. The innate immune activation of eosinophils, neutrophils, dendritic cells, and natural killer (NK) cells, followed by long-lasting activation of lymphocytes involved in adaptive immunity can be induced by TiO2 NPs (85).

5.7.d.2.Gastrointestinal Track (GIT)

Acute, subacute and chronic exposure to TiO_2 leads to liver changes according to various studies. NPs significantly accumulate in the liver, resulting in histopathological changes, hepatocyte apoptosis, inflammatory response, and liver dysfunction in mice. Moreover, TiO_2 NPs induced oxidative stress (OS) and alterations in the expression levels of genes involved in NP detoxification/metabolism regulation and radical scavenging action in the mouse liver. Vasantharaja et al (86) studied the serum biochemical changes in adult male Wistar rats. The changes between levels of total protein, glucose, aspartase transaminase, alanine transaminase and alkaline phosphatase indicated that TiO_2 NPs induced liver damage. Wang *et al.* (87) in his study showed in rats that daily TiO_2 NPs oral exposure for 30 days, liver edema and non-allergic mast cell activation in stomach tissues occured in young rats. In addition decreased intestinal permeability and molybdenum contents, and mild liver injury were observed in adult rats. Exposure to TiO_2 NPs, even at low doses, led to the accumulation of NPs , resulting in OS and ultimately induction of chronic gastritis.

5.7.d.3.Kidney

Zhao J F et al demonstrated that TiO_2 NPs induced OS, resulting in nephritis in mice(88). In this study, TiO_2 accumulation and histopathological changes were observed in the kidney, accompanied by an increase in ROS, and a decrease in antioxidase activity

and total antioxidant capacity as well as antioxidants. In addition, kidney function was disrupted, including an increase in creatinine, calcium and phosphonium, and a reduction in uric acid and blood urea nitrogen.

The same group showed (89) that when mice were intragastrically exposed to TiO_2 NPs for 90 consecutive days, similar kidney injury occcured , including peroxidation of lipids, proteins and DNA. Histopathological damage was characterized by a reduction in the number of renal glomeruli, fatty degeneration, disorganization of renal tubules, infiltration with inflammatory cells, tissue necrosis, GSH depletion, excessive production of ROS and nephrocyte apoptosis, necrosis, and dysfunction.These findings suggested that kidney injury induced by TiO_2 NPs in mice was associated with alterations in inflammatory cytokine expression and a reduction in the detoxification of NPs.

Intratracheal instillation of TiO_2 NPs in mice resulted in chronic renal damage as well in a dose-dependent manner. In an in vitro study by Meena R et al (90) human embryonic kidney cells (HEK- 293) were treated with titanium dioxide nanoparticles (TiO_2 anatase, <25 nm). Nano-TiO_2 inhibited the proliferation of HEK-293 cells by inducing cell apoptosis in a time- and dose-dependent manner. Moreover, nano-TiO_2 might induce oxidative stress-mediated DNA damage, which led to the activation of p53 gene and the up-regulation of Bax and caspase-3 (91).

5.7.d.4. Spleen

Li et al (92) investigated the toxicity of TiO_2 NPs in the mouse spleen. Mice were intraperitoneally injected with TiO_2 NPs at concentrations of 5–150 mg per kg BW daily for 45 days. They found that NPs accumulated in the spleen, resulting in pathological changes such as congestion and lymph nodule proliferation in spleen tissue and splenocyte apoptosis. In a molecular level, activation of caspase-3 and -9, inhibition of Bcl-2 expression, and alterations in the levels of Bax as well as cytochrome c were observed. With chronic exposure spleen histopathology significantly changed, correlating with decreased immunity.The toxicity effect of TiO_2 NPs and specifically TiO_2 deposition in the spleen, resulting in ROS overproduction, splenic inflammation and necrosis has a time depedent manner.

5.7.d.5.Heart and blood vessels

Several studies have revealed potential toxic effects of TiO_2 to the cardiovascular system. Sheng L et al (87) investigated the negative effects of TiO_2 NPs on the cardiovascular system in mice after chronic exposure . They suggested that NPs accumulated in the heart, leading to sparse cardiac muscle fibers, inflammation, cell necrosis, and cardiac biochemical dysfunction. TiO_2 NPs potentiated the production of ROS and lipids, protein and DNA peroxidation, and disturbed the antioxidant system in the mouse. In addition TiO_2 NP exposure resulted in arteriolar constrictions or impaired vasodilator responses.

Other studies have found atherosclerotic plaque progression in the aorta as well as cardiac conduction velocity and tissue excitability increase resulting in susceptability for arrythmias.

5.7.d.6.Ovary and testis

Zhao X et al (93) , in their study exposed female mice daily to TiO_2 NPs administered intragastrically for 90 days. The data showed that NPs accumulated in the ovary, resulting in significant reductions in the body weight, relative ovary weight and fertility. Altered hematological, serum parameters, and sex hormone levels, increased follicle atresia, and ovary inflammation and necrosis were also observed. Male Kunming mice were used to investigate the underlying effects of TiO_2 NP (10–250 mg per kg BW) exposure on testosterone (T) synthesis and spermatogenesis (94). The results showed that TiO_2 NPs increased spermatozoa abnormalities in the epididymis, decreased the layers of spermatogenic cells and vacuoles in seminiferous tubules, reduced the serum T levels. Testicular damage, sperm malformations, altered levels of serum sex hormones, changes in gene expression involved in spermatogenesis, and steroid and hormone metabolism following TiO_2 NP exposure in male mice were confirmed by other studies.

5.7.d.7. Brain

The brain is of utmost importance not only because of its functions but also due to the direct connection to the eye. The brain controls the processing of information, generation of movements and behavior (95). It is protected from harmful substances by the blood-brain barrier (BBB). As already mentioned in previous section there are three

main pathways for TiO_2 NPs to be transported into the brain namely the blood– brain barrier (BBB) pathway, the olfactory nerve translocation pathway, and the placental barrier pathway in fetus.

The BBB is mainly composed of endothelial cells, astrocytes, and pericytes. The endothelial cells are connected with each other through complicated tight junctions, while the connections are supported by the astrocytes and pericytes. Only specific substances with small size or low-molecular weight (MW) could be allowed to pass through the BBB by means of three main transport patterns (passive diffusion, active transport, and endocytosis). In another word, BBB is capable of protecting the healthy and functional CNS from being affected by harmful chemicals, toxins, and drugs in the circulatory system. NPs possess unique chemical–physical characteristics and tiny size, which makes them be similar to biomolecule. Therefore, they are able to pass through the BBB and enter into the brain. This fact was supported by a study by Li Y et al (75). After intratracheal installation of 3nm TiO_2 in a chronic way in mice, inflammatory cell aggregation and cell necrosis was presented in brain zones with consequent down regulation of body-brain index. The results also indicated upregulation of the amount of titanium oxide and accumulation resulting in damage of the tissue. Larger TiO_2 NPs of 200nm did not cause any significant damage in the brain as reported in another study (96), further supporting the fact that larger size nanoparticles may not be able to penetrate BBB. On the other hand, the permeability of BBB can be altered by NPs, which could assist in influx of exogenous substances into the brain. This was confirmed by a study using an in vitro model of BBB where authors reported damage of the BBB after acute and chronic exposure to TiO2 NPs. These findings meant that besides direct impairments, the indirect harmful impacts (inflammatory effects) of TiO_2 NPs on BBB integrity also occurred (97). The reasons of BBB breakdown are plenty, such as excessive ROS generation, inflammatory cytokines release and leukocyte adhesion, which are also the reasons of inflammation occurrence (98).

The olfactory nerve route is based on the retrograde transportation of TiO_2 nanoparticles from the nerve endings to the neurosome and consequently along the axons to the brain bypassing the BBB. This is the major way of NPs transportation after

inhalation. There are several studies confirming the transportation and effect of TiO_2 to the brain after intranasal instillation. Wang et al (99,100) discovered that when female mice were treated with TiO_2 NPs through intranasal instillation every other day, the titanium oxide was detected in the brain after 30 days. The higher concentration of TiO_2 was detected in the hippocampus, followed by the olfactory bulb, cerebellum, and the lowest at cerebral cortex. It appears that the hippocampus is the main target in the brain. The accumulation of nanoparticles resulted in oxidative stress leading to irregular arrangement and loss of neurons. In addition the metabolism of neurotransmitters such as Acetylcholine and Glutamate was affected. It is generally accepted that the hippocampus is mainly in charge of memory and learning. Therefore, impairments on it might probably induce neurodegenerative diseases, such as Alzheimer's diseases.

TiO_2 have been shown to cross blood placenta barrier. After pregnant mice/rats were exposed to nanoparticles, those substances could be detected in the brain of fetus, especially in the hippocampus area (101,102,103). They can disturb both the homeostasis of CNS, alter gene expression, increase the levels of dopamine and even induce neuronal death. Those harmful impacts on fetus brain have been demonstrated to be related with psychiatric disorders such as autism, schizophrenia and depression in their later life.

A novel way that metal-containing NPs can be absorbed into the brain could be through the eyes. It is inevitable that metal-containing NPs are blown into eyes or can be retained in the eye socket when bodies are exposed to NPs. Boyes et al (104) found that metal-containing NPs in workplace, after entering the eyes, could be conveyed into the nasal cavity through the nasolacrimal duct by drainage from the eye socket, which subsequently enter the CNS via the olfactory route.

Once the TiO_2 NPs are transported into the brain regions, major cells in the CNS, including the neurons and the glial cells, will be affected. Reactive oxygen species (ROS), apoptosis, and inflammation will be induced by TiO_2 NPs, which may lead to cell death and disturb the CNS functions or even induce neurodegenerative disease. Molecular mechanisms underlying the neurotoxicity of TiO_2 NPs might mainly include oxidative stress, apoptosis, inflammation, and disturbance of ATPases or neurotransmitters. Similarly, these sorts of mechanisms could be present in other types of nanomaterials.

Those results have been suggested by both in vitro and in vivo studies (95).

Little is known regarding elimination of the nanoparticles. The excretion of TiO_2 NPs from the brain is limited and possibly even at negligible dosage of TiO_2 NPs they can accumulate in the brain with minimal elimination, so chronic or long- term exposure might be potentially risky for the brain health.

There are several parameters affecting the transportation and neurotoxicity of the titanium oxide NPs into the brain.

a) Crystal type

TiO_2 NPs two crystal phases possess subtle different physicochemical characteristics, which lead to different toxicities. It was reported that the toxicity of anatase form was higher than that of rutile (75). That was not confirmed though in all studies. So, concerning the TiO_2 NPs, how different crystal forms of TiO_2 NPs affect neurotoxicity is still unclear.

b) Size

Dimension of nanomaterial is another vital factor which can influence the nanotoxicity. When it comes to the effects of TiO_2 NPs on the CNS, little is known about how different sizes affect the neurotoxicity of TiO_2 NPs. Larger nanoparticles and fine particles do not seem to cross BBB, transport and accumulate in the brain (96).

c) Administration route

The olfactory route seems to be the most effective route for TiO_2 transportation to the brain possibly due to the retrograde axonal transport.

d) Shape and Surface modification

The morphology of NPs is crucial to their toxicity. However, how different shapes (mainly including nanobelts, nanorods, nanotubes, and nanospheres) of TiO_2 NPs modulate their transportation to the brain, how they get excreted from the CNS, and how they exert

toxic effects on neurons or glial cells is still largely unknown. Moreover, the surface coating can regulate the physicochemical properties of TiO_2 NPs, which might influence their toxicity (105). Whether TiO_2 NPs coated with inorganic or organic materials could alleviate or exacerbate the harmful impacts on the CNS is unclear as well.

e) Concentration

The exposure concentration is an important factor that determines the toxicity of TiO_2 NPs. Most *in vitro* studies present declining trends in the cell viability with an increase in concentration of TiO_2-NPs, which suggest an explicit dose-effect relationship. In a study by Liu S et al (106) PC12 cell viability was greatly reduced in a concentration- dependent and time-dependent manner. The results indicated that the nano-TiO_2 decreased the cell viability and induced an apoptosis of the cells by enhancement of intracellular ROS generation.

NPs generally do not show any toxicity until the exposure concentration reaches a certain threshold. Nevertheless, the dose tolerance of different cells to TiO_2-NPs is dissimilar. The dose tolerance of TiO_2-NPs is much more complicated as it could also be correlated with the exposure way. Generally, animals are able to tolerate a higher exposure concentration through the intravenous injection than the respiratory tract (98). It is probably because intravenously injected NPs would be processed by liver before entering into the CNS and liver is the well-known detoxification organ. Apart from those mentioned above, there are several factors not yet in available studies that need to be considered. Other physicochemical properties of NPs, except of size, such as surface chemistry, charge and surface area, could manipulate the interaction with circulatory and cellular proteins (protein corona), which consequently disturb and impair normal biological functions. NPs tend to form aggregates and agglomerates that can also influence their transportation toxicity. Exposure time would affect the toxicity levels of the same NPs as well. In most cases, the longer exposure time is usually with severer toxic effects.

5.7.d.7.a. Mechanism of brain cell damage

Titanium oxide can enter the glial cells TiO_2 NPs very fast. This quick internalization suggests that the NPs are predominantly taken up by phagocytosis and pinocytosis that are two main types of endocytosis. In the cellular level, the endocytosis absorbing NPs could affect the cytomembrane integrity. In addition they can passively diffuse across the cell membrane (98).

The activation of microglias or astrocytes (glial cells) is considered as the first indicator of neuroinflammation in most toxicological studies. There are many biological end points demonstrating the glial cell activation. The two main that are commonly used in neurotoxicological studies are morphological changes, and the biological marker, protein IBA-1 which is increased. The activated microglia are able to uptake NPs more rapidly and to a greater extent than normal healthy control microglia and then produce proinflammatory mediators, while the normal migration and surveillance functions are damaged. Astrocytes activation can be evidenced by increase of the biological marker glial fibrillary acidic protein expression. It is also related to decreased acethylcholinesterase (AChE) activity, which results in the impairment of brain cognitive functions.

However, in most cases, microglias and astrocytes respond differently to NPs even though they share common innate immune responses. Microglias prefer to increase the release of proinflammatory cytokines, while astrocytes prefer to enhance the production of anti-inflammatory. Researchers observed TiO_2-NPs transferred into the nucleus of neural cells after passing through the cell membrane due to their small size, which provided the possibility of NPs altering the expressions of key genes of signaling pathways controlling immune response, like the release of proinflammation cytokines (98).

Apart from glial cells, neurons in the CNS are also a main target of metal-containing NPs. TiO_2-NPs exposure damaged neurons through affecting, to a certain extent, the production of neurotrophin growing factors, such as ciliary neurotrophic factor and brain-derived neurotrophic factor. Both factors play important roles in the survival of existing neurons and the growth and differentiation of new neurons and synapses (107). In general, astrocytes and microglias were more susceptible to metal-containing NPs than neurons, probably because of their strong cell uptake capability.

According to a large number of toxicological studies on the NPs, most researchers agree to consider the oxidative stress damage as the prime mechanism of NPs causing various toxic effects. NP-cell interaction can produce free radicals to promote the occurrence of oxidative stress, and then induce lipid peroxidation, DNA damage and structure disruption of protein. However, the relationship between oxidative stress and inflammatory process is close but complex. On the one side, some researchers suggested that oxidative stress activates the inflammation (85). Much worse, when there is already an inflammation happening in the CNS, metal-containing NPs could cause severe and long-lasting adverse effects. TiO_2-NPs exposure promoted exaggerated neuroinflammatory responses by persistent microglial activation in the preinflamed brain, eventually leading to more damage in the brain, such as neural death and brain disorder (86). It is likely that exposure to NPs can aggravate neuroinflammation in patients with one of several brain diseases with underlying microglia activation such as Parkinson's disease, Alzheimer's disease and multiple sclerosis. Márquez- Ramírez et al (108) reported the uptake and internalization of TiO_2 NPs by glial cells, induced an inhibition in their proliferation. Strong morphological changes were found, which were associated with depolymerization of F-actin and apoptotic cell death. This result suggested that the exposure of brain cells to TiO_2 NPs could cause brain injury and contribute to the development of neurodegenerative diseases.

5.8 Ocular Effects of titanium dioxide NPs

Due to their small size, the toxicity effects of nanomaterials on ocular surface have their own characteristics. The small size of nanoparticles ensures the close contact with ocular surfaces, anchorage to the cornea for longer residence time, penetration of the barriers of ocular surface, and entrance in posterior segments of the eye. Nanoparticles can potentially cross all the barriers of the eye, such as the cornea, conjunctiva, the sclera and especially, blood-retinal barriers (BRBs) (109). The periocular route is also a promising path for therapeutic NPs, because the sclera has a large surface area and relatively high permeability, so that NPs could penetrate into the outer BRBs (110).

Once contacting and entering the eyes, nanoparticles may subsequently induce cellular toxicity as well as systematic immune response not only at ocular surface, but also to

lens, retina, or even optic nerve and macula. As already mentioned, NPs after entering the tear film, could be conveyed into the nasal cavity through the nasolacrimal duct by drainage from the eye socket, which subsequently enter the central nervous system via the eye–nose–brain route. Nanoparticles tend to have different behaviors regarding dispersion or agglomeration state in different solvents, which in turn influence their size as well as toxicity profile. Besides, it is also well accepted that nanoparticles display different sizes or agglomeration state in biological media because of the formation of protein corona (111). Accordingly, their corresponding toxicity may vary when applied to various intracular compartments.

When eyes are exposed to TiO_2 nanoparticles, the nanoparticles act as foreign bodies and can induce cellular damage to the ocular surface. External stimuli that injure the ocular surface can cause conjunctival goblet cells to secrete mucins that normally protect the ocular surface. Eom Y et al (112) in an animal study found that exposure to TiO_2 nanoparticles 10μg/ml caused ocular surface damage. Repetitive exposure induced goblet cell exhaustion, which reduced the ocular surface protection provided by the MUC5AC against TiO_2 nanoparticles. On the contrary single exposure resulted in increase in mucin production. Exposed eyes also demonstrated increased tears LDH levels as compared to controls. LDH release assays are appropriate ways to measure cellular damage. Furthermore, the TiO_2 groups had higher grades of ocular surface staining using rose bengal dye that indicates devitalized surface epithelium cells. From these results, it was determined that exposure to TiO_2 nanoparticles could induce ocular surface cell damage and poor protection of the ocular surface epithelium by the tear film.

Another study by Eom Y et al (113) reported that after whole-body exposure of rats to airborne TiO_2 nanoparticles, induction of ocular surface immune system was observed, where the Type 2 helper T-cell pathway played a key role accompanied by an increase of interferon-c (IFN-c), IL-4, and IL-17 levels in anterior segment of eyeball.

Han JU et al (114) directly applied TiO_2 NPs to the ocular surface of both healthy and Dry Eye (DE) animal model eyes. Corneal clarity score, inflammatory cell infiltration, and LDH level in tears all increased after TiO_2 challenge, the effect being larger in the DE model than in the normal model. These results suggest that exposure to TiO_2

nanoparticles damages the ocular surface in both normal and DE models. Furthermore, the extent of surface damage was more prominent in the DE model than in the normal model. In agreement with the other studies the MUC5AC level in the tear samples increased after TiO_2 challenge in the normal model. However, a lower level of MUC5AC in the DE model compared with that of the normal model suggests that the protective function of mucin on the ocular surface is reduced when ocular surface is damaged. Therefore after TiO_2 challenge dry eyes could be more vulnerable to particulate matter exposure.

Sun D et al (111) in his study has shown that nanoparticle exposure (<40nm) could cause destruction to corneal epithelium cells and corneal toxicity through different mechanisms such as cell membrane damage, mitochondrial dysfunction, or cell death[-]
It is also reported that the particulate matters could also increase the risk of allergic conjunctivitis.

In an vitro study by Lee H (115), Statens Seruminstitut Rabbit Cornea (SIRC) cells were used to test the cytotoxicity of five nanoparticles including zinc, silver, ceria, silica, titanium . For TiO_2 toxicity was not observed in low concentrations ,which means more than 100 µg/mL of TiO2 was needed to produce a toxic effect . Generation of reactive oxygen species was observed, and expression of apoptosis related biomarkers including Bax and Bcl-2 were changed after treatment of zinc oxide nanoparticles, while no other significant toxicity related changes were observed in cornea cells treated with Ag, CeO_2, SiO_2 and TiO_2 nanoparticles. Therefore based on various studies the *Opinion on nanoform* TiO_2, of the EU Commission's Scientific Committee on Consumer Safety (SCCS) came to the conclusion that the eye irritation potential of TiO_2 NPs appears to be low.

A human lens epithelial cell line (HLE B-3, ATCC) was used in another study by Wu Q et al (116). UVB irradiation along with application of TiO_2 NPs induced excessive cellular ROS generation and elevated intracellular Ca^{2+}, and eventually exertion inhibitory effect on HLE B-3 cell proliferation.

The retina can be the target of neuronal toxicity of nanoparticles, in common with the central and peripheral nervous system. Furthermore, the ability of nanoparticles to

pass through the BRBs might increase the possibility of toxicity, simultaneously promoting distribution in the retinal layers (110). Sanders et al exposed human ARPE-19 retinal pigment epithelial cells to 0.3-100 μg/ml of differently sized anatase, rutile and anatase/rutile TiO_2 NMs under 4hrs of UVA irradiation. He reported that the smaller TiO_2 NMs <31nm elicited the most pronounced phototoxicity (LC_{50} < 5 μg/ml), whereas the largest TiO_2 NMs (size:142 nm and 214 nm; TEM) were the least toxic. Nevertheless insufficient data exists regarding in vivo retina TiO_2 toxicity. Other studies showed that TiO_2 nanoparticles of 20–50 nm did not induce noticeable toxicity to retinal constituent cells as well as retina of C57BL/5 mice and zebrafish (117).

Finally, because of their ability to induce an inflammatory response, cytokine production and oxidative stress it is possible that NPs can induce iritis or changes of the lens (118).

6. Application of TiO_2 to the ocular surface. Is there a perspective?

Overall, there are few studies on TiO_2 effect on the ocular tissues. Short term exposure of rabbit corneas to TiO_2 NPs resulted in ocular irritation with increase or decrease after few days of mucin secretion, increased ocular surface staining and conjunctival irritation. (119). This effect can further be exaggerated in dry eyes. It is expected that application of TiO_2 on the eye surface will potentially have an adverse effect on healthy cells. Nevertheless no significant toxicity related effect has been shown after treatment of Statens Seruminstitut Rabbit Cornea (SIRC) cells with increasing concentrations of TiO_2 up to 100μm/ml for 24 hours without UVA exposure. (115). It is therefore possible that if only the diseased area of ocular surface is exposed the rest of the surface cells may not suffer irreversible damage.

Another important issue is the fate of TiO_2 nanoparticles applied on the ocular surface. Intraocular penetration of nanoparticles as well as distribution and accumulation in the posterior segment and the brain, can be a major problem. As mentioned there are two main routes by which drugs can be absorbed from the ocular surface to reach the posterior segment: through the cornea and through the conjunctiva. High molecular mass drugs of up to 150 kDa do not generally cross the corneal epithelium, but may reach the

posterior segment directly by crossing the conjunctiva, sclera, choroid, choriocapillaris, and retinal pigment epithelium, or indirectly by traveling through the retrobulbar space to the optic nerve. These findings indicate that the increase of a molecule in the retina may derive from a direct passage of this through the conjunctiva and sclera and less through the tight corneal junctions. Intraocularly TiO_2 can have a toxic effect on human lens cells (116) and retina pigment epithelium cells even in the absence of UV light.

The precise mechanism through which a substance reaches the brain from the eye surface is not fully explored at the moment. In a study by Lambiase (120), application of neurotrophic growth factor (NFG) on the ocular surface resulted in detection of the molecule in various parts of the brain, retina and optic nerve. His study provides evidence that molecules applied on the ocular surface can actually appear in the brain. Similar results have been reported by Koevary et al (121). After topical instillation of insulin, the molecule has been detected in the cerebrospinal fluid (CSF) and brain of rats. Based on the available neuroanatomical studies, one reasonable hypothesis is that from the eye surface the substance reaches the CSF with the meninges surrounding the optic nerve acting as a gateway. It subsequently diffuses in the CSF and finally disseminates throughout the brain. A second feasible alternative pathway from eye to brain is represented by optic nerve, although this is a very slow route. However, contrasting data exists regarding the presence of molecule anterograde transport along the optic nerve axons. As a third route nanoparticles applied on the conjunctiva get absorbed in the vascular circulation and subsequently cross the blood brain barrier. Knowledge of potential routes of absorption of a substance from the ocular surface will help finding methods of application that can potentially eliminate intraocular and systemic dissemination and as a consequence eliminate toxicity.

Although the bibliography on ocular applications of titanium oxide nanoparticles is sparse based on the current evidence we can postulate that UVA activated NPs can be used as therapeutic agents in treating ocular surface pathology. Nevertheless both dosing and duration of treatment need to be evaluated. For example it has been reported (122) that UVA irradiation of human Tenon's fibroblasts results in their death when irradiated with 2,5 j/cm^2 at a concentration of 150μgr/ml. Concentrations higher than that and

more intensive radiation may have an adverse effect on ocular surface structures.

A)Corneal infections

Based on current evidence the microbiocidal spectrum of UVA activated titanium includes all common pathogens of corneal ulcers such as Gram positive and negative bacteria and to a less extend fungi and acanthamoeba species. All severe corneal infections most likely will require patient admission to hospital for intensive topical treatment in order to disinfect the ulcer and avoid severe complications such as corneal penetration and eventually visual loss. Due to wide use of antibiotics multidrug resistant bacteria have started to appear and alternative treatments are sought for. The photocatalytic and microbiocidal potential of TiO_2 can help in reducing the microbial load, acting alone or synergistically with applied antibiotics and thus shorten the course of the disease. As there is no evidence that photodynamic therapy with titanium dioxide could induce resistance , the treatment can be repeated to enhance the effect.

Effect of photocatalysis appear to be time, dose and intensity of radiation dependent as found in many studies but methods and experimental conditions have not been standardized. Therefore there is no specific protocol for TiO_2 NPs antibacterial use . During corneal crosslinking the cornea sustains an energy dose of 5,4J/cm^2 . In some studies energy used was up to 15J/cm^2 with no corneal complications (123). Duration of irradiation used varied in different studies . Tsuang et al (124) reported the bactericidal effect of TiO2 nanoparticles occurred in 60 min under irradiation of 8mW/cm^2 . Infection severity as measures by CFUs may also play a role. The optimum concentration of NPs has to be defined that can ideally be applied in every case.

B) Ocular surface neoplasias

Titanium oxide nanoparticles have shown to be active against various tumor cells as mentioned previously , including melanoma cells and oral squamous carcinoma cells (125,63). Those are histologically similar to the ocular surface counterparts. Melanoma cells when irradiated in vitro with N-dopped TiO2 NPs for four hours they demonstrated

a survival rate of 7% when high concentration of 0,5mg/ml was used. Plain TiO2 NPs caused a 50% survival rate of carcinoma cells. Therefore PDT can become a promising treatment for ocular surface melanomas.

NPs can attack the tumor either with passive (EPR effect), or active targeting. The latter involves specific ligands to the cell. Nanoparticles can enter the tumor cell by diffusion or more likely endocytosis due to their hydrophilicity. We can thus assume that nanoparticle solution or gel applied on the ocular surface can both act at the tumor's surface as well as be absorbed in deeper tumor tissue. This hypothesis need to be proved in order to assure that topically applied nanoparticles, can penetrate into tumors sufficiently for the PDT treatment to be effective . So far topical therapy of ocular tumors with antimetabolites and interferon has been successfully used as these substances are absorbed by tumor cells. Similarly we can postulate that titania NPs will become absorbed to tumor cells , even in deeper layers. Those superficial neoplasias are readily accessible to UVA irradiation.

Irradiation with UVA for an appropriate time can lead to destruction of tumor cells via oxidation and induced apoptosis and cell death. This fate can be governed by the intracellular location of the NPs. Irradiation time varied in different reports from 20 minutes to several hours. In terms of in vivo treatments application of UVA for a very long period of time may not be feasible, so fragmentation of treatment may be necessary. It remains to be tested if the effect of continuous vs fragmented treatment is the same. In all cases survival rate of tumor cells decreased with longer treatment (62). Various concentrations have been tried but sensitivity of specific eye related tumor cell lines need to be assessed.

Nanoparticle size is one of the key properties. The smaller the diameter size the bigger the surface area/ volume ratio, allowing a higher number of atoms around them thus enhancing their reactivity. Nanoparticles less than 10nm can easily penetrate tumor cells buy at the same time are toxic to healthy cells. Those with diameter size >10nm can more hardily enter the healthy cells but still maintain their ability to penetrate tumour

cells. Nanoparticle size is also very important factor concerning their toxicity, especially their neurotoxicity. Although there is no clear evidence it seems that microparticles are less or non toxic compared to nanoparticles. There are several reports confirming that nanoparticles smaller than 100nm easily penetrate the blood-brain as well as the blood-placenta barrier, as well as accumulating in various organs. Size affects the cellular uptake and intracellular accumulation of nanoparticles and NPs of 50 nm or smaller are incorporated in the cells more easily via receptor mediated endocytosis (126).Generally the smaller size nanoparticles are the more active.

In conclusion it is necessary for further experiments to be conducted in order to determine the right treatment parameters for ocular use.

6.1 Minimizing healthy tissue exposure and systemic adsorption

Photoactivation of TiO2 nanoparticles can be achieved with UVA emitting devices already used in ophthalmic practice, that allow both pattern, duration and intensity manipulation of UVA radiation of both diffuse and localized lesions. Thus, focal radiation can result not only in targeted lesion treatment but also sparing of nearby healthy tissue.

Another important issue concerns the method of introduction of nanoparticles to the area to be treated. Although direct application of a solution on the lesion could be an option , especially for flat diffuse or multifocal lesions, absorption from conjunctiva, sclera and further penetration beyond the treated area can result in rapid elimination of the agent. Therefore TiO_2 can be used as a surface coating or impregnate other materials that can be adapted to match the treated area. Such materials could be for example contact lenses or collagen cross linked shields (127) that can placed on the irradiated lesion . Collagen shields cross linked with TiO_2 can also be used for combined release of antibiotics and metal ions. Doping of contact lenses with titanium oxide nanoparticles is possible and has been attempted in order to improve the optical properties of the CLs (128). Punctual plugs can be used to minimize absorption through the nasal mucosa and access to the olfactory route.

Localized targeted treatment can also reduce the side effect and the potential risks of systemic accumulation of TiO_2. Even then, inadvertently , nanoparticles will penetrate through compromised ocular barriers , such as an infected cornea, and locate in the posterior segment of the eye . From there evidence exists that molecules will translocate to the retina and the optic nerve and also found in CNS. This can happen either via the optic nerve and anterograde transportation or from adsorption of NPs in the retrobulbar space and through there to the cerebrospinal fluid leading to diffusion to the brain. Depending on the treatment protocols potential toxicity is a downside of titanium oxide use. Further studies on activated titanium oxide toxicity on ocular cell lines are needed. So far, there is enough evidence to support that production of oxidative species lead to cell apoptosis and death. Although this is the intended effect on tumor cells and microbes, it can affect healthy tissues that need to be protected from exposure, so minimizing absorption is paramount.

In several studies TiO_2 has been coupled with other substances in order to enhance the effect and minimize toxicity. For ocular use, Titanium oxide nanoparticles can potentially be bound to other molecules with known effect on cornea tissue. Riboflavin is a photosensitizer that has been successfully adsorbed on titania surface through phenols (63,129). The photocatalytic action of both compounds is enhanced. In addition UVA radiation of riboflavin induces collagen lamellae cross linking in the cornea, thus enhancing of the rigidity of the cornea which can be severely compromised by the infection process. Binding of Titanium NPs to higher molecular weight molecules could possibly delay their intraocular penetration and reducing their potential toxicity.

7.Conclusion

TiO_2 nanoparticles have been extensively used in several applications due to their biocompatibility, low cost, stability, and photocatalytic properties. The photocatalytic properties of titanium after irradiation with UVA light are the principle of their use as an antimicrobial and antitumor agent. The ocular surface has the advantage of being directly

accessible to treatment both in terms of topical application of therapeutic agents as well as irradiaton with UV. Therefore ocular surface pathologies, such as corneal infections and neoplasias could be good candidates for photodynamic therapy with TiO_2 NPs. Photocatalysis has been an effective treatment for a wide range of microorganisms that are causative of corneal ulcers . Those often require intensive treatment, and can become resistant to antibiotics. Photocatalytic action of TiO_2 can be an adjunctive treatment modality to help eliminate the microbial load, reduce drug resistance and shorten the disease course. Ocular surface neoplasias can also be treated with photodynamic therapy in the same way, either for reduction of size or complete eradication. Previous reports suggest susceptibility of melanoma cells to PDT with TiO_2. In all treatment modalities parameters such as concentration of TiO2, irradiation time and energy have to been established, although we are far for suggesting treatment protocols.

Nevertheless the major issue of toxicity remains. Titanium nanoparticles can penetrate ocular tissues and enter the eye where potentially they can damage retina and lens cells. It can also translocate to the brain either through absorption from the olfactory tracks , via the periocular tissues and possibly through the unexplored intraocular route of anterograde transportation in the optic nerve. It is thus important to minimize TiO_2 absorption as well as inadvertent exposure of healthy tissues. The ocular surface is offered for localized targeted treatment as the agent can be directly applied on the area concerned.

More research is needed in this promising field and standardization of environmental conditions as well as treatment parameters for a treatment protocol to be established that is both effective and safe.

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