Article:
Yu, H, Guo, Z, Wang, S et al. (8 more authors) (2019) Fabrication of Hybrid Materials from Titanium Dioxide and Natural Phenols for Efficient Radical Scavenging against Oxidative Stress. ACS Biomaterials Science and Engineering, 5 (6). pp. 2778-2785. ISSN 2373-9878

https://doi.org/10.1021/acsbiomaterials.9b00535

Characterization, Synthesis, and Modifications

Fabrication of hybrid materials from titanium dioxide and natural phenols for efficient radical scavenging against oxidative stress

Huayang Yu, Zhili Guo, Shuqi Wang, Algy Kazlauciunas, Simran Channa, Ganwarige Sumali N. Fernando, David P Martin, Sergey A Krasnikov, Alexander N. Kulak, Christine Boesch, and Natalia N. Sergeeva

ACS Biomater. Sci. Eng., **Just Accepted Manuscript** • DOI: 10.1021/acsbiomaterials.9b00535 • Publication Date (Web): 03 May 2019

Fabrication of hybrid materials from titanium dioxide and natural phenols for efficient radical scavenging against oxidative stress

*Huayang Yu,† Zhili Guo,‡ Shuqi Wang,† Ganwarige Sumali N. Fernando,‡ Simran Channa,† Algy Kazlauciunas,† David P. Martin,† Sergey A. Krasnikov,† Alexander Kulak,† Christine Boesch‡ and Natalia N. Sergeeva†**

†Department of Colour Science, School of Chemistry, University of Leeds, Woodhouse Lane, Leeds LS2 9JT, U.K.

‡School of Food Science and Nutrition, University of Leeds, Woodhouse Lane, Leeds LS2 9JT, U.K.

Corresponding Author

* Email: n.sergeeva@leeds.ac.uk

KEYWORDS. Oxidative stress, titanium dioxide, polyphenols, surface modification, antioxidants, LPO and MTT assay, cytotoxicity

ABSTRACT. Oxidative stress caused by free radicals is one of the great threats to inflict intracellular damage. Here, we report a conventional approach to synthesis, characterization and evaluation of radical activity of titanium based composites. We have investigated the potential of natural antioxidants (curcumin, quercetin, catechin and vitamin E) as radical scavengers and stabilizers. The titanium oxide composites were prepared via three steps including sol-gel synthesis, carboxylation and esterification. A degree of the functionalization was assessed by TGA and shown to be between 20% to 30%. The characterization of the titanium-phenol composites was carried out by FTIR, XRD, UV-vis, LD and SEM methods. The radical scavenging ability of the novel materials was evaluated using DPPH and in vitro assay using isolated rat liver mitochondria. The novel materials exhibit both higher stability and an antioxidant activity compared to bare TiO_2. It was found that curcumin and quercetin based composites show highest antioxidant efficiency among the composites under study followed by catechin and vitamin E based materials. The results from MTT assay carried on Caco-2 cell line, indicates that the composites do not contribute to the cytotoxicity *in vitro*. This study demonstrates that a combination of powerful antioxidants with titanium dioxide can change its functional properties and provides a convenient strategy against oxidative stress.

Introduction

Reactive species (ROS/ RNS) can be considered as a double-edge sword: playing an important role in regulating vital cellular functions, but at the same time, they can also trigger oxidative stress, therefore contributing to the acceleration of aging, diabetes, development of neurodegenerative and cardiovascular diseases as well as initiation and progression of cancer. [1-9] Oxidative stress has a detrimental impact on cell life cycle by inducing extensive damage to DNA, lipids and proteins.[1,10-14] Antioxidative defence mechanisms involve both enzymes and non-enzymatic pathways.[15,16] Common antioxidants include the vitamins A, C and E, the superoxide and glutathione enzymes; other examples cover mixed carotenoids, flavonoids, antioxidant, minerals and co-factors.[16,17] Polyphenolic compounds such as flavonoids show scavenging activity for various reactive species including superoxide[18-19], peroxyl radicals[20,21] and peroxynitrite[22]. Their structure and ability to interact and to penetrate the membrane of a phospholipid bilayer are thought to be the major factors of flavonoid antioxidant activity.[23,24] Flavonoids such as quercetin and catechin are common and found in many edible plants consumed daily in a variety of products e.g. chocolate, red wine and tea.[25]

Titanium dioxide (TiO_2) is a naturally occurring mineral and is being used as a white pigment. The global market of TiO_2 was

estimated to be USD 15.76 billion in 2018 and is expected to increase until at least 2025. Its production accounts for ca. 70% of the total volume of pigments manufactured worldwide with a large proportion used in food-drug-cosmetics (FDC) sector. Many applications of titania are related to human health and include personal care products, pharmaceuticals and food products (E171 in the EU) to name a few.[26] However, recent studies suggest that nanoparticulate TiO_2 can pose a risk to the human health as it can initiate the production of strong oxidants e.g. free radicals leading to the formation of hydrogen peroxide, when exposed to TiO_2 containing products.[26-36] However, even a food grade TiO_2 (E171) contains at least 36% of nano-TiO_2, with the particle size less than 100 nm. [28] While some sectors cannot avoid using nano-TiO_2, some such as FDC sector could benefit from alternative approaches. TiO_2 is often associated with its surface morphology.[37] Electronic properties of TiO_2 vary significantly depending on the physical properties such as the particle size and crystallinity, and photochemical activity. Therefore, it is possible to alter these properties through surface functionalisation. Many natural phenols and polyphenols are biocompatible and economically viable; and some can have demonstrated to provide protection from oxidative stress.[38-42] Their use to manipulate the surface properties of substrates by coating, precipitation and encapsulation strategies has become very attractive.[43-49]

In this study, we demonstrate that plant-derived natural phenols such as quercetin (capers), curcumin (curry), α-tocopherol (vitamin E) and catechin (green tea) can be used to covalently modify the surface of titanium dioxide, leading to a significant increase in the radical scavenging capability of the hybrid material. A consequence of intracellular ROS accumulation is the lipid peroxidation (LPO) of mitochondria membrane and/or an increase in the permeability of the late. Thus, this study aims to assess the effects of the materials on iron-induced LPO and explores the impact of these composites on hydrogen peroxide production in isolated mitochondria. The final objective is to investigate the effect of polyphenols-TiO_2 materials on mitochondrial oxidative stress production and cytotoxicity.

Materials and methods

The materials were characterised by Attenuated Total Reflection (ATR-PLATINUM) and Fourier Transform Infrared Spectroscopy (FTIR, BRUKER ALPHA). The particle size distribution of the products was measured by Dynamic Light Scattering (DLS, Malvern ZETASIZER NANO ZSP). X-Ray Diffraction (XRD, D2 PHASER BRUKER and Diffrac Commander Software) was used to identify the amorphous or crystalline properties of the products. Scanning Electron Microscopy (SEM, Quorum Q150RS, Coating unit-13nm of gold) was used to analyse the topography of the particle surface.

Synthesis of titanium dioxide microspheres via sol-gel assisted method

1-Hexadecylamine (98%, 5 g) was dissolved in 825 ml of absolute EtOH. The aqueous KCl solution (3 mL) was added into the mixture followed by the quick addition of titanium (IV) tetraisopropoxide (17.6 mL) under vigorous stirring at room temperature. The reaction mixture was kept static for 18 h to allow TiO_2 suspension to be formed. The suspension of TiO_2 was filtered, washed with EtOH and dried to yield TiO_2 microspheres as a white powder.

Carboxylation of titanium dioxide surface

The TiO_2 microspheres (8 g) were dispersed in 450 mL of doubly distilled water and stirred at room temperature for 30 min. Then, the mixture was ultrasonicated for 30 minutes and monochloroacetic acid (5 g) was added slowly under vigorous stirring. The reaction mixture was refluxed at 100°C for 12 hours. The reaction was cooled to room temperature and the supernatant was decanted. The residue was washed with deionised water to reach a neutral pH. Finally, the microspheres were collected by vacuum filtration and dried under vacuum yielding carboxylated TiO_2 as a white powder.

Synthesis of titanium dioxide composites: [TiO_2-QR], [TiO_2-CT], [TiO_2-αTC] and [TiO_2-CR]

Carboxylated TiO_2 (0.7 g) was dispersed in 95 mL of isopropyl alcohol and stirred under reflux at 80°C for 30 minutes. Then 0.05 mL of con. H_2SO_4 was added to the reaction. Typically, 0.25 g of an appropriate natural phenol was dissolved in 2.5 ml of isopropyl alcohol and added to the mixture. The resulted reaction mixture was refluxed at 80°C for 3 hours. The reaction was cooled to room temperature and the suspension was filtered. The esterified TiO_2 composites were washed with absolute ethanol and deionised water and dried under the vacuum and then in the oven at 80°C for 24 hours.

Determination of radical scavenging activity by DPPH assay

Antioxidant ability was measured using DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) assay.[50] In brief, 0.1 mM DPPH stock solution in 60% ethanol and 0.5 mg/mL stock solutions of TiO_2 and its composites in DMSO were prepared. A concentration range from 0.00625-10 mg/mL was mixed with DPPH radical stock solutions and incubated for 30 min in the dark. Absorption spectra of the samples were recorded. The A_0 and A_n values were measured as intensities of the absorption peak at 517 nm in UV-vis spectra of each sample after background subtraction. Radical scavenging activity was calculated using following equation 1:

$$\%\text{Activity} = \frac{A_0 - A_n}{A_0} \times 100\%$$

Equation 1. A_0-absorbance of DPPH solution; A_n-absorbance of DPPH with the corresponding concentration of TiO_2 material.

Isolation of mitochondria fractions from rat liver tissue

Mitochondria fractions were isolated from rat liver (obtained from the Animal Unit, University of Leeds) according to literature[51] Briefly, fresh liver tissue was minced in ice-cold buffer (0.01 M Tris/MOPS, pH 7.4; 0.2 M sucrose; 0.1 mM EGTA) in a tissue to buffer ratio of 1:5-1:10. The homogenate was centrifuged at 600 x g for 10 min at 4°C to remove cell debris. The resulting supernatant was centrifuged at 5,000 x g for 20 min at 4°C and the supernatant discarded. The pellet was washed with isolation buffer and centrifuged again (5,000 x g, 20 min, 4°C). The pellet containing mitochondria was carefully re-suspended and frozen in aliquots at -80°C. Mitochondria protein was quantified using BCA assay (Pierce, Fisher Scientific, Loughborough, UK) with bovine serum albumin as standard.

Inhibition of mitochondria oxidative stress

The test compound dependent inhibition of iron sulphate induced lipid peroxidation was assayed as thiobarbituric acid reactive

substances in mitochondria as recently described[52]. Briefly, mitochondria samples (1 mg protein per mL) were pre-incubated with test compounds (0.025, 0.05, 0.1, 0.2, 0.4 mg/mL) for 30 min at 37°C, centrifuged to remove non-associated compounds and subsequently challenged with 5 mM iron (II) sulphate. Samples were incubated for 20 min at 90 ℃ after protein precipitation and addition of TBA. The resulting dye was extracted with butanol and absorbance was measured at 540 nm using a plate reader.

Cytotoxicity assessment by MTT assay

Human intestinal Caco-2 cells (obtained from ECACC) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) non-essential amino acids and 1% (v/v) penicillin/streptomycin mix. Cells were seeded at 8.2 × 10^4 cells/ cm^2 in 24-well plates and after 48 h (≥ 90% confluence) incubated with TiO_2-composites at concentrations ranging from 0, 0.25 - 2.5 µg/mL for 24 h. Then, the medium was replaced by DMEM containing MTT (0.5 mg/mL) and the formazan dye solubilized with DMSO following incubation. Absorbance was measured at 570 nm using a plate reader and cell viability calculated as % change compared to control cells.

Statistical analysis

The data is expressed as means ± SD, results were considered statistically significant, when p value was < 0.05. All experiments were independent and conducted in triplicate or more.

Results and discussion

Surface functionalisation of titanium dioxide with natural phenols

Figure 1A summarises a modification of titanium dioxide [TiO_2] with natural phenols such as quercetin (QR), catechin (CT), curcumin (CR) and α-tocopherol (TC) as the most biologically active form of vitamin E. The hybrid materials have been prepared in three steps: (1) sol-gel synthesis of TiO_2, (2) carboxylation of the TiO_2 surface and (3) surface esterification with polyphenols. The sol gel approach allows to control the structure, size and shape of TiO_2 particles and thus has been used in this study. The titanium dioxide microspheres [TiO_2] were produced from $Ti(OPr^i)_4$ *via* surfactant assisted sol-gel method.[53-55] This is an efficient synthetic strategy based on the hydrolysis of titanium (IV) alkoxides. A rate of the hydrolysis and consequently a desired particle size has been controlled by the amount of aqueous KCl used in the reaction. It has been shown that the low calcination temperatures (<300°C) lead to a relatively low photoactivity due to a low crystallinity of the TiO_2 sample.[37] To avoid an increase in the photoactivity, the synthesised amorphous TiO_2 was used

without hydrothermal treatment. Functionalisation of the TiO_2 surface with carboxylic groups was achieved using chloroacetic acid in water. A surface of carboxylated TiO_2 microspheres [TiO_2-CO_2H] was esterified with the natural phenols to produce covalently modified titanium dioxide composites: from quercetin [TiO_2-QR], catechin [TiO_2-CT], α-tocopherol [TiO_2-αTC] and curcumin [TiO_2-CR].

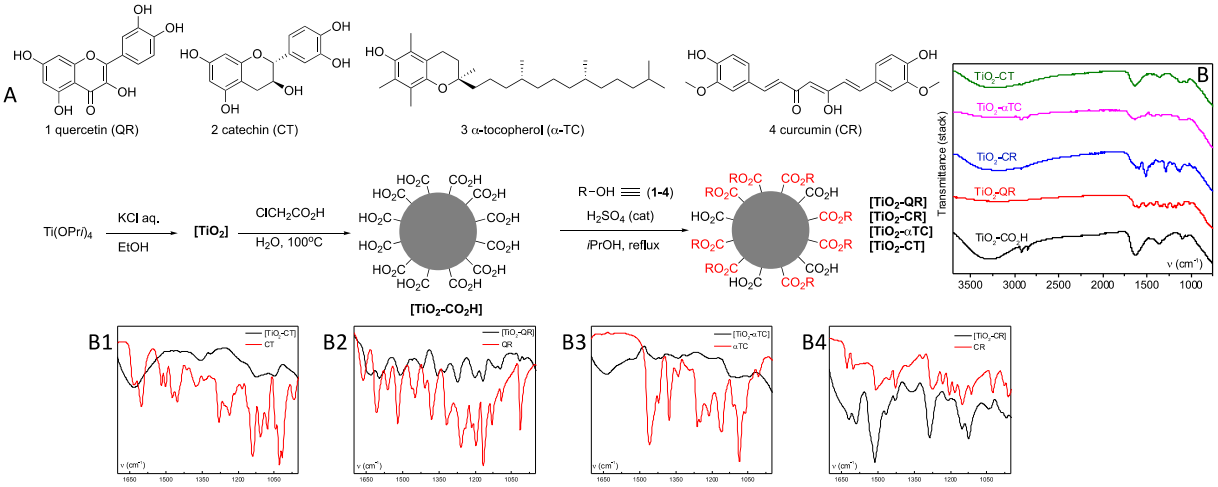


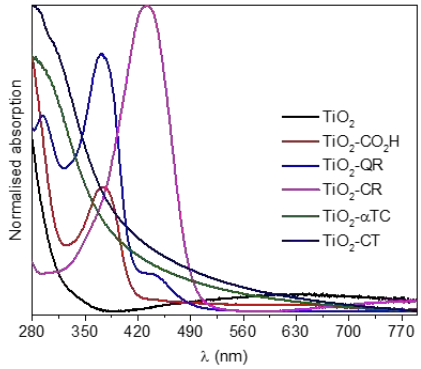
Figure 1. A) Preparation of [TiO_2], [TiO_2-CO_2H], [TiO_2-QR], [TiO_2-CT], [TiO_2-αTC] and [TiO_2-CR]. B) FTIR spectra of the modified TiO_2 composites (stack) and B1-B4) the detailed IR region (1700-950 cm^{-1}) showing the spectra of the pure polyphenol and the respective TiO_2 modified material.

The reaction was carried out in isopropanol in the presence of sulphuric acid as a catalyst. The progress of the esterification has been followed by FTIR, the strong peak at 3273 cm^{-1} associated

with O-H stretching band of [TiO_2-CO_2H] decreased over time with typical reaction times of 90 minutes. (Fig. 1B)FTIR was also used as a primary tool to confirm a success of the functionalisation of the carboxylated titanium dioxide with the natural phenols. (Fig. 1B) FTIR spectrum of the starting [TiO_2-CO_2H] features the strong bands at 1630 cm^{-1} (C=O) and 1356 cm^{-1} (C-O), and a large band starting from 900 cm^{-1} associated with Ti-O-Ti stretching. All esterified TiO_2 based composites (Fig.1 B1-B4) show a strong change of C=O signal accompanied by the characteristic bands of the attached natural phenol moieties, confirming the successful esterification. For instance, a flavonoid based material [TiO_2-QR] shows the strong peaks at 1635 cm^{-1} (C=O), 1592 (C=C), 1510 (B-ring) and 1270 cm^{-1} (C-O), which are common features of quercetin. Similarly, [TiO_2-CT] displays the strong bands at 1629 cm^{-1} (C=O), 1463-1440 cm^{-1} (C-H), 1179-1127 cm^{-1} (C-H), and 1049 cm^{-1} (C-O-C). FTIR spectra of the lipophilic polyphenol based materials feature [TiO_2-CR]: 1617-1588 cm^{-1} (C=O), 1289-1123 cm^{-1} (C-C, C-O and C-O-C) and [TiO_2-αTC]: 1630 cm^{-1} (C=O), 1289-1123 cm^{-1} (C-C, C-O, C-O-C).

Optical properties, crystallinity, morphology and stability

Optical properties of the TiO_2 materials were analysed using UV-vis absorption spectroscopy. It is evident from Fig. 2 that the light absorption ability of the synthesised composites has been



efficiently enhanced compared to that of the bare [TiO_2] (λ_{max} <260 nm).

Figure 2. Normalised UV-vis absorption spectra of the TiO_2 materials.

The covalent functionalisation with polyphenols leads to the drastic changes in blocking of UVA (320-400 nm) and visible light radiation as the synthesised composites absorb between 320-600 nm. UV-vis spectrum of the carboxylated [TiO_2] exhibits a bathochromic shift of the TiO_2 band (λ_{max} 265 nm) and a new signal at 345-422 nm (λ_{max} 375 nm). Significant broadening in the absorption spectra is observed for [TiO_2-αTC] and [TiO_2-CT] composites suggesting that the spectra are represented by a few overlapping absorption signals with an increased contribution of long-wavelength bands with the maxima of absorption at 277 nm and at 264 nm, respectively. Since

the absorbance of both wavelengths increased, the ability of UV blocking also has improved. The TiO_2 materials modified with the quercetin and curcumin, which have a large π-π system, show distinct colour change from the original TiO_2 and carboxylated TiO_2 samples. Highly substituted [TiO_2-QR] exhibits a near-blue absorption (λ_{max} 372 nm) with a shoulder (λ_{max} 440 nm) extending into the visible region of up to 500 nm. Absorption spectrum of the [TiO_2-CR] indicates a significant contribution from curcumin fragment as the material has a large band (λ_{max} 433 nm) in the visible region with a cut off around 500 nm.

Powder XRD (PXRD) was used to identify the amorphous or crystalline nature of the titanium dioxide based materials. PXRD analysis shown in Fig. 3, confirms the structure of the TiO_2 microspheres to be amorphous. While an annealing step of synthesis of bare TiO_2 was omitted, PXRD of [TiO_2-CO_2H] shows the mixed-phase pattern of two titania polymorphs. The surface modified composites show a similar arrangement. Co-existence of anatase (A) and rutile (R) can be clearly identified from characteristic diffraction peaks: anatase (101) peak at 2-theta of 25.6° and rutile (110) at 2-theta of 27.5°.

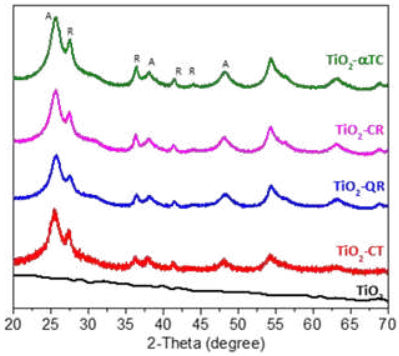


Figure 3. Powder XRD patterns of TiO_2 and modified TiO_2 composites: [TiO_2-CT], [TiO_2-QR], [TiO_2-CR] and [TiO_2-αTC].

The surface morphologies of TiO_2 composites have been analysed using scanning electron microscopy (SEM). SEM images (Fig. 4) show a smooth surface of bare TiO_2 particles with a particle size of around 1.7 µm. A carboxylated TiO_2 sample shows particles of a similar size with etched surfaces. A similar effect on the surface morphology is observed in the titania-polyphenol materials with crystal clusters clearly visible on the TiO_2 surface. In the series, the particle sizes are comparable with an average dimeter of 1.5 µm, 1.3 µm, 1.6 µm and 1.3 µm for [TiO_2-QR], [TiO_2-CT], [TiO_2-CR] and [TiO_2-αTC], respectively.

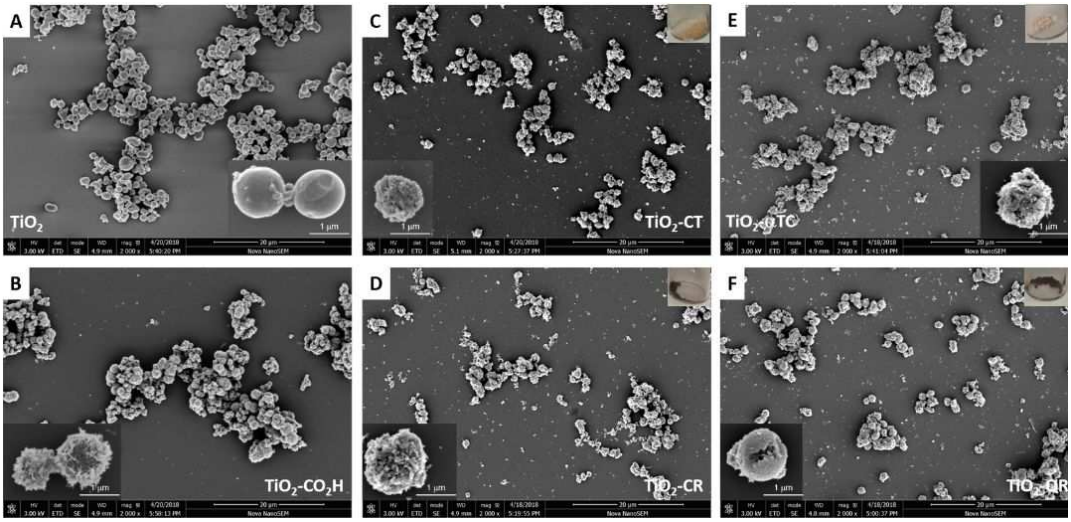


Figure 4. Surface morphology of titanium dioxide and the composites acquired by SEM imaging. A) [TiO_2], B) [TiO_2-CO_2H], C) [TiO_2-CT], D) [TiO_2-CR], E) [TiO_2-αTC] and F) [TiO_2-QR].

Generally, polyphenols show a low stability; thus, zeta potential analysis has been carried out to assess the stability of the TiO_2 composites and the effect of esterification. Water was used as a dispersion medium, and while a surface charge remains negative for all materials, the analysis reveals that the surface modification with polyphenols results in an improved stability of the materials. Compared to other samples, [TiO_2-QR] and [TiO_2-αTC] composites are the most stable with the zeta potential of -31.1 mV and -30.4 mV, respectively. Lower zeta values have been recorded for other samples: [TiO_2-CO_2H] -27.9 mV, [TiO_2-CT] -27.5 mV and [TiO_2-CR] -28.6 mV, with bare TiO_2 (-24.9 mV) being the least stable. Interestingly, overall stability has been improved with attachment of antioxidants.

Antioxidant properties and *in vitro* activity

Initial radical scavenging efficacy of the TiO_2 composites has been evaluated using DPPH radical assay. DPPH (1,1-diphenyl-2-picryl-hydrazyl) is a stable free radical, which can be neutralised by antioxidants.[50] It produces a deep-violet solution with an absorption maximum at 517 nm. In this study, a final DPPH concentration of 50 µM was used, which falls within a range of accuracy for spectrophotometric measurements corresponding to a transmittance between 20-60%.[56] A stock solution (0.5 mg per mL) of the corresponding TiO_2 material was prepared in DMSO; and a concentration of TiO_2 and its composites in the range of 62.5-250 µg per mL was used. Aliquots (50-200µL) of the corresponding materials were taken and the volume was made to 200 µL with 60% ethanol. The radical scavenging reaction was started by the addition of 200 µl of 0.1 mM DPPH stock solution. Figure 5a summarises the antioxidant activity of TiO_2 and its composites. Bare TiO_2 is inefficient in radical scavenging showing a low activity even at high concentrations. Moreover, at a higher concentration of TiO_2, an increase of DPPH signal is observed. In contrast, the radical scavenging activity of the polyphenol-TiO_2 hybrids has been increased showing that the antioxidant activity of free polyphenols has been successfully transferred to TiO_2-hybrids upon covalent functionalisation. All composites enhance the radical scavenging even at a very low concentration. [TiO_2-CR]

shows the highest efficiency of 93% compared to other polyphenols-TiO_2 conjugates followed by [TiO_2-QR] (67%), while [TiO_2-CT] and [TiO_2-αTC] exhibit much lower activity reaching only 30% and 20%, respectively.

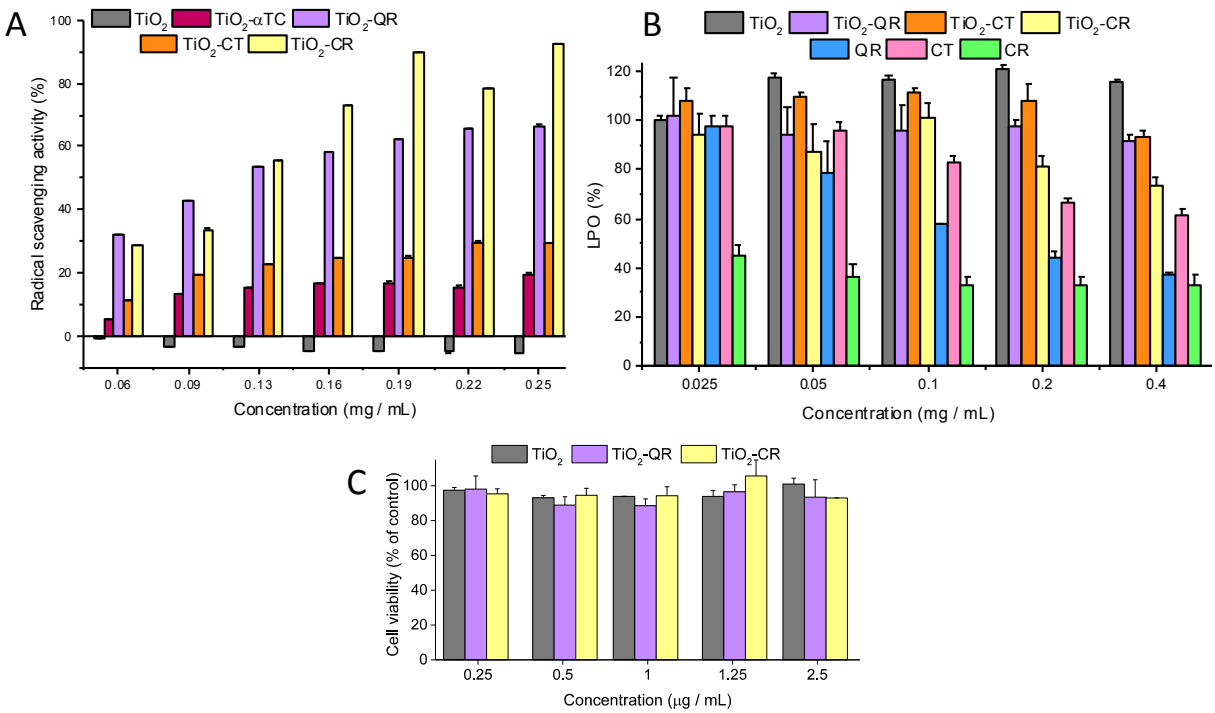


Figure 5. (a) Antioxidant activity of bare TiO_2 and the composites: [TiO_2-QR], [TiO_2-CT], [TiO_2-αTC] and [TiO_2-CR] assessed by DPPH radical scavenging assay. The A_0 and A_n values have been measured as intensities of the absorption peak at 517 nm from the control (final DPPH conc. 0.05 mM) and the samples (the final concentrations of the composites: 0.06, 0.09, 0.13, 0.16, 0.19, 0.22, 0.25 mg/mL) incubated in the dark at 25°C for 30 min. (b) Effects of polyphenols and polyphenol-TiO_2 composites: [TiO_2-QR], [TiO_2-CT], [TiO_2-αTC] and [TiO_2-CR] on iron(II) sulphate induced

lipid peroxidation (LPO) in rat liver mitochondria. (c) Effect of polyphenol-TiO_2 composites on Caco-2 cells was tested by MTT assay. Cells were cultured with [TiO2-QR] and [TiO2-CR] at various concentrations (0, 0.25, 0.5, 1.0, 1.25, 2.5 µg/mL) for 24 hr. Untreated control was considered as 100%, and data are expressed as the percentage of untreated control. ($p < 0.05$)

To study the effects of the composites on antioxidant activity *in vitro*, mitochondrial lipid oxidation (LPO) and MTT cytotoxicity tests have been carried out. The effects of polyphenols and their conjugates on inhibition of lipid peroxidation have been studied in isolated mitochondria, a model that has been employed in previous research[25, 56] and is linked to the properties of polyphenols to associate with cellular membranes.[25,41] Mitochondria membrane association of quercetin has been demonstrated recently[58] and affects mitochondria function[23,42]. The LPO assay determines the activity of a membrane-associated compound as an excess of the material is removed and therefore only test compounds integrated or associated with the membrane fraction remain to react in the assay. To understand a trend in antioxidant activity, LPO assay has also been carried out on free polyphenols used for modifications. As shown in Figure 5b, among free polyphenols, curcumin and quercetin are far more effective than catechin. They show similar effects on LPO reducing it to 33% and 37%,

respectively, at the highest concentration used, while the presence of catechin reduces it to 62% only. At the lowest concentration (0.05 mg/mL), only curcumin is inhibiting LPO. Due to a low DPPH activity, [TiO_2-αTC] has not been tested. A similar trend was observed among TiO_2-polyphenol conjugates: again, the highest potent inhibition in mitochondrial LPO was achieved by [TiO_2-CR] followed by [TiO_2-QR]. Both hybrids show an increase in LPO inhibition with higher concentrations. The curcumin conjugate [TiO_2-CT], that slightly increases the mitochondrial LPO at lower concentrations, is demonstrating inhibition compared to the bare TiO_2, which displays an approx. 20% increase in LPO at all concentrations investigated. The photocatalytic properties of titanium dioxide are likely to have contributed to the increase in lipid peroxidation[59,60].

We have carried out toxicology assessment of TiO_2 and the most efficient [TiO_2-CR] and [TiO_2-QR] composites using MTT assay. In this experiment, the Caco-2 intestinal cell line has been used, a model commonly employed for *in vitro* toxicology studies to assess the cell-toxic effects of micro- and macronutrients.[61-63] To choose an appropriate concentration range, we have considered the cytotoxicity of food grade TiO_2 (E171, which typically contains 30% of the nanoparticles). Since, a daily intake of TiO_2 from food is in the range of 15-37.5 mg per day for an average body weight of 75 kg, an adult would ingest between 6-15 ng of TiO_2 per cm^2 of

intestine (250 m^2) or 1.8-4.5 ng/cm^2 if TiO_2 is nanoscaled.[64] In addition, the concentration should be below 50 µg/mL to avoid interactions between MTT-dye and TiO_2, which can affect the assay results.[65] Moreover, a high concentration of the pigments can interfere with MTT reading due to a residual absorption of the pigments even after washing. DMSO has been used to prepare the stock solutions of the materials, the final concentration of DMSO has been kept in the range of 0 - 0.5% to minimise its cytotoxic effect.[66] Thus, Caco-2 cells were cultured with various concentrations (2.5, 1.25, 1, 0.5, 0.25 and 0 µg/mL) of TiO_2, [TiO_2-QR] and [TiO_2-CR] in the dark for 24 h before carrying out the MTT assay as described in Materials and methods. The results of the MTT assay have been summarised in Figure 5c. The data indicates that the cell viability in the presence of bare TiO_2 does not drop below 5% even at the highest concentration of 2.5 µg/mL, which is 400-fold (1.35 µg/cm^2) as high as the daily intake of an adult. MTT results for the [TiO_2-QR] and [TiO_2-CR] materials show a small decrease in the cell viability similar to that observed in the TiO_2 sample. The results indicate that the composites do not contribute to the cytotoxicity *in vitro*. Moreover, as the nanosized TiO_2 content has been decreased through our preparation method, this is in accordance with the published data on the cytotoxicity driven by particle size.

Conclusions

In this study, we have shown a versatile method to fabricate polyphenol-titanium dioxide materials using a combination of sol-gel synthesis and surface modification approach. Further analysis such as XRD and SEM reveals that the surface modification introduces a degree of crystallinity into amorphous TiO_2, while the particle size stays almost unaffected at about 1.4 μm. Importantly, the covalent functionalisation leads to a desirable tuning of their optical properties resulting in a bathochromic shift and an increased efficiency to absorb UV-vis light. Antioxidant activity of the composites has been evaluated using DPPH radical scavenging and *in vitro* LPO assays. DPPH results indicate that TiO_2-hybrid materials possess an antioxidant activity in contrast to bare TiO_2. It was found that curcumin and quercetin based composites show highest antioxidant efficiency followed by catechin and vitamin E based materials. A similar trend has been observed *in vitro* with the studied antioxidants exhibiting inhibitory potency on mitochondrial lipid peroxidation. MTT assay has been carried on Caco-2 cell line for [TiO_2-QR] and [TiO_2-CR] and bare TiO_2 samples. The materials did not induce any significant cytotoxicity even at the concentrations as high as 400-fold of daily intake. This study shows that the combination of powerful antioxidants with titanium dioxide can improve its antioxidant activity.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. All authors contributed equally.

ACKNOWLEDGMENT

This work was supported through the Clothworker's Scholarship, the British Skin Foundation and the University of Leeds.

REFERENCES

1. Finkel, T.; Holbrook, N. J., Oxidants, oxidative stress and the biology of ageing. *Nature* **2000**, *408* (6809), 239-247. DOI: 10.1038/35041687.

2. Lin, M. T.; Beal, M. F., Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. *Nature* **2006**, *443* (7113), 787-795. DOI: 10.1038/nature05292.

3. Gorrini, C.; Harris, I. S.; Mak, T. W., Modulation of oxidative stress as an anticancer strategy. *Nat. Rev. Drug Discov.* **2013**, *12* (12), 931-947. DOI: 10.1038/nrd4002.

4. Andersen, J. K., Oxidative stress in neurodegeneration: cause or consequence? *Nat. Med.* **2004**, *10* (7), S18-S25. DOI: 10.1038/nm1434.

5. Lavie, L., Oxidative stress in obstructive sleep apnea and intermittent hypoxia - Revisited - The bad ugly and good: Implications to the heart and brain. *Sleep Med. Rev.* **2015**, *20*, 27-45. DOI: 10.1016/j.smtv.2014.07.003.

6. Hybertson, B. M.; Gao, B.; Bose, S. K.; McCord, J. M., Oxidative stress in health and disease: the therapeutic potential of Nrf2 activation. *Mol. Aspects Med.* **2011**, *32*, 234-246. DOI: 10.1016/j.mam.2011.10.006.

7. Uttara, B.; Singh, A. V.; Zamboni, P.; Mahajan, R. T., Oxidative stress and neurodegenerative diseases: a review of upstream and downstream antioxidant therapeutic options. *Curr. Neuropharmacol.* **2009**, 7, 65-74. DOI: 10.2174/157015909787602823.

8. Rinnerthaler, M.; Bischof, J.; Streubel, M. K.; Trost, A.; Richter, K., Oxidative stress in aging human skin. *Biomolecules* **2005**, *5*, 549-589. DOI: 10.3390/biom5020356.

9. Markesbery, W. R., Oxidative stress hypothesis in Alzheimer's disease. *Free Radic. Biol. Med.* **1997**, *23*, 134-147. DOI: 10.1016/s0891-5849(96)00629-6.

10. Avery, S. V., Molecular targets of oxidative stress. *Biochem. J.* **2011**, *434*, 201-210. DOI: 10.1042/bj20101695.

11. Davies, M. J., Protein oxidation and peroxidation. *Biochem. J.* **2016**, *473*, 805-825. DOI: 10.1042/bj20151227.

12. Stadtman, E. R.; Levine, R. L., Protein oxidation. *Ann. N. Y. Acad. Sci.* **2000**, *899*, 191-208. DOI: 10.1111/j.1749-6632.2000.tb06187.x.

13. Berlett, B. S.; Stadtman, E. R., Protein oxidation in aging, disease, and oxidative stress. *J. Biol. Chem.* **1997**, *272*, 20313-20316. DOI: 10.1074/jbc.272.33.20313.

14. Vassallo, N., Polyphenols and health: new and recent advances. New York: Nova Biomed. Books. **2008**.

15. Das, K.; Roychoudhury, A., Reactive oxygen species (ROS) and response of antioxidants as ROS-scavengers during environmental stress in plants. *Front. Environ. Sci.* **2014**, *2*, A53. DOI: 10.3389/fenvs.2014.00053.

16. Maritim, A. C.; Sanders, R. A.; Watkins, J. B., 3rd, Diabetes, oxidative stress, and antioxidants: a review. *J. Biochem. Mol. Toxicol.* **2003**, *17*, 24-38. DOI: 10.1002/jbt.10058.

17. Sheng, Y.; Abreu, I. A.; Cabelli, D. E.; Maroney, M. J.; Miller, A.-F.; Teixeira, M.; Valentine, J. S., Superoxide Dismutases and Superoxide Reductases. *Chem. Rev.* **2014**, *114*, 3854-3918. DOI: 10.1021/cr4005296.

18. Chun, O. K.; Kim, D.-O.; Lee, C. Y., Superoxide Radical Scavenging Activity of the Major Polyphenols in Fresh Plums. *J. Agric. Food Chem.* **2003**, *51*, 8067-8072. DOI: 10.1021/jf034740d.

19. Jovanovic, S. V.; Simic, M. G., Antioxidants in nutrition. *Ann. N. Y. Acad. Sci.* **2000**, *899*, 326-334.

20. Nakao, M.; Takio, S.; Ono, K., Alkyl peroxyl radical-scavenging activity of catechins. *Phytochem.* **1998**, *49*, 2379-2382. DOI: 10.1016/S0031-9422(98)00333-1.

21. Boadi, W. Y.; Iyere, P. A.; Adunyah, S. E., In vitro exposure to quercetin and genistein alters lipid peroxides and prevents the loss of glutathione in human progenitor mononuclear (U937) cells. *J. Appl. Toxicol.* **2005**, *25*, 82-88. DOI: 10.1002/jat.1049.

22. Pollard, S. E.; Kuhnle, G. G.; Vauzour, D.; Vafeiadou, K.; Tzounis, X.; Whiteman, M.; Rice-Evans, C.; Spencer, J. P., The reaction of flavonoid metabolites with peroxynitrite. *Biochem. Biophys. Res. Commun.* **2006**, *350*, 960-968. DOI: 10.1016/j.bbrc.2006.09.131.

23. Santos, A. C.; Uyemura, S. A.; Lopes, J. L.; Bazon, J. N.; Mingatto, F. E.; Curti, C., Effect of naturally occurring flavonoids on lipid peroxidation and membrane permeability transition in mitochondria. *Free Radic. Biol. Med.* **1998**, *24*, 1455-1461. DOI: 10.1016/S0891-5849(98)00003-3.

24. Oyewole, A. O.; Birch-Machin, M. A., Mitochondria-targeted antioxidants. *FASEB* **2015**, *29*, 4766-4771. DOI: 10.1096/fj.15-275404.

25. Hendrich, A. B., Flavonoid-membrane interactions: possible consequences for biological effects of some polyphenolic compounds. *Acta Pharmacol. Sinica* **2006**, *27*, 27. DOI: 10.1111/j.1745-7254.2006.00238.x.

26. Weir, A.; Westerhoff, P.; Fabricius, L.; Hristovski, K.; von Goetz, N., Titanium dioxide nanoparticles in food and personal care products, *Environ. Sci. Technol.* **2012**, *46*, 2242–2250. DOI: 10.1021/ES204168D.

27. Grande, F.; Tucci, P., Titanium Dioxide Nanoparticles: a Risk for Human Health? *Mini Rev. Med. Chem.* **2016**, *16*, 762-769. DOI: 10.2174/1389557516666160321114341.

28. Shi, H.; Magaye, R.; Castranova, V.; Zhao, J., Titanium dioxide nanoparticles: a review of current toxicological data. *Part. Fibre Toxicol.* **2013**, *10*, 15. DOI: 10.1186/1743-8977-10-15.

29. Warheit, D. B.; Donner, E. M., Risk assessment strategies for nanoscale and fine-sized titanium dioxide particles: Recognizing hazard and exposure issues. *Food Chem. Toxicol.* **2015**, *85*, 138-147. DOI: 10.1016/j.fct.2015.07.001.

30. Jayaram, D. T.; Runa, S.; Kemp, M. L.; Payne, C. K., Nanoparticle-induced oxidation of corona proteins initiates an oxidative stress response in cells. *Nanoscale* **2017**, *9*, 7595-7601. DOI: 10.1039/C6NR09500C.

31. De Angelis, I.; Barone, F.; Zijno, A.; Bizzarri, L.; Russo, M. T.; Pozzi, R.; Franchini, F.; Giudetti, G.; Uboldi, C.; Ponti, J.; Rossi, F.; De Berardis, B., Comparative study of ZnO and TiO_2 nanoparticles: physicochemical characterisation and toxicological effects on human colon carcinoma cells. *Nanotoxicol.* **2013**, *7*, 1361-1372. DOI: 10.3109/17435390.2012.741724.

32. Runa, S.; Lakadamyali, M.; Kemp, M. L.; Payne, C. K., TiO_2 Nanoparticle-induced oxidation of the plasma membrane: importance of the protein corona. *J. Phys. Chem. B* **2017**, *121*, 8619-8625. DOI: 10.1021/acs.jpcb.7b04208.

33. Gurr, J. R.; Wang, A. S.; Chen, C. H.; Jan, K. Y., Ultrafine titanium dioxide particles in the absence of photoactivation can induce oxidative damage to human bronchial epithelial cells. *Toxicol.* **2005**, *213*, 66-73. DOI: 10.1016/j.tox.2005.05.007.

34. Hussain, S. M.; Hess, K. L.; Gearhart, J. M.; Geiss, K. T.; Schlager, J. J., In vitro toxicity of nanoparticles in BRL 3A rat liver cells. *Toxicol. In Vitro* **2005**, *19*, 975-983. DOI: 10.1016/j.tiv.2005.06.034.

35. Maynard, A. D.; Warheit, D. B.; Philbert, M. A., The new toxicology of sophisticated materials: nanotoxicology and beyond. *Toxicol. Sci.* **2011**, *120*, S109-S129. DOI: 10.1093/toxsci/kfq372.

36. Jugan, M. L.; Barillet, S.; Simon-Deckers, A.; Herlin-Boime, N.; Sauvaigo, S.; Douki, T.; Carriere, M., Titanium dioxide nanoparticles exhibit genotoxicity and impair DNA repair activity in A549 cells. *Nanotoxicol.* **2012**, *6*, 501-513. DOI: 10.3109/17435390.2011.587903.

37. Schneider, J.; Matsuoka, M.; Takeuchi, M.; Zhang, J.; Horiuchi, Y.; Anpo, M.; Bahnemann, D. W., Understanding TiO_2 photocatalysis: mechanisms and materials. *Chem. Rev.* **2014**, *114*, 9919-9986. DOI: 10.1021/cr5001892.

38. Akhlaghi, M.; Bandy, B., Mechanisms of flavonoid protection against myocardial ischemia–reperfusion injury. *J. Mol. Cell. Card.* **2009**, *46*, 309-317. DOI: 10.1016/j.yjmcc.2008.12.003.

39. Bjorklund, G.; Dadar, M.; Chirumbolo, S.; Lysiuk, R., Flavonoids as detoxifying and pro-survival agents: What's new? *Food Chem. Toxicol.* **2017**, *110*, 240-250. DOI: 10.1016/j.fct.2017.10.039.

40. Lagoa, R.; Graziani, I.; Lopez-Sanchez, C.; Garcia-Martinez, V.; Gutierrez-Merino, C., Complex I and cytochrome c are molecular targets of flavonoids that inhibit hydrogen peroxide production by mitochondria. *Biochim. Biophys. Acta - Bioenerg.* **2011**, *1807*, 1562-1572. DOI: 10.1016/j.bbabio.2011.09.022.

41. Sandoval-Acuña, C.; Ferreira, J.; Speisky, H., Polyphenols and mitochondria: an update on their increasingly emerging ROS-scavenging independent actions. *Arch. Biochem. Biophys.* **2014**, *559*, 75-90. DOI: 10.1016/j.abb.2014.05.017.

42. Steffen, Y.; Gruber, C.; Schewe, T.; Sies, H., Mono-O-methylated flavanols and other flavonoids as inhibitors of endothelial NADPH oxidase. *Arch. Biochem. Biophys.* **2008**, *469* (2), 209-219. DOI: 10.1016/j.abb.2007.10.012.

43. Lee, H.; Dellatore, S. M.; Miller, W. M.; Messersmith, P. B., Mussel-inspired surface chemistry for multifunctional coatings. *Science* **2007**, *318*, 426-430. DOI: 10.1126/science.1147241.

44. Kang, S. M.; Rho, J.; Choi, I. S.; Messersmith, P. B.; Lee, H., Norepinephrine: material-independent, multifunctional surface modification reagent. *J. Am. Chem. Soc.* **2009**, *131*, 13224-13225. DOI: 10.1021/ja905183k.

45. Ye, Q.; Zhou, F.; Liu, W., Bioinspired catecholic chemistry for surface modification. *Chem. Soc. Rev.* **2011**, *40*, 4244-4258. DOI: 10.1039/c1cs15026j.

46. Hong, S.; Kim, J.; Na, Y. S.; Park, J.; Kim, S.; Singha, K.; Im, G. I.; Han, D. K.; Kim, W. J.; Lee, H., Poly(norepinephrine): ultrasmooth material-independent surface chemistry and nanodepot

for nitric oxide. *Angew. Chem., Int. Ed.* **2013**, *52*, 9187-9191. DOI: 10.1002/anie.201301646.

47. Cordoba, A.; Monjo, M.; Hierro-Oliva, M.; Gonzalez-Martin, M. L.; Ramis, J. M., Bioinspired quercitrin nanocoatings: a fluorescence-based method for their surface quantification, and their effect on stem cell adhesion and differentiation to the osteoblastic lineage. *ACS Appl. Mater. Interf.* **2015**, *7*, 16857-16864. DOI: 10.1021/acsami.5b05044.

48. Cordoba, A.; Satue, M.; Gomez-Florit, M.; Hierro-Oliva, M.; Petzold, C.; Lyngstadaas, S. P.; Gonzalez-Martin, M. L.; Monjo, M.; Ramis, J. M., Flavonoid-modified surfaces: multifunctional bioactive biomaterials with osteopromotive, anti-inflammatory, and anti-fibrotic potential. *Adv. Healthcare Mater.* **2015**, *4*, 540-549. DOI: 10.1002/adhm.201400587.

49. Lee, J. S.; Lee, J. S.; Lee, M. S.; An, S.; Yang, K.; Lee, K.; Yang, H. S.; Lee, H.; Cho, S. W., Plant flavonoid-mediated multifunctional surface modification chemistry: catechin coating for enhanced osteogenesis of human stem cells. *Chem. Mater.* **2017**, *29*, 4375-4384. DOI: 10.1021/acs.chemmater.7b00802

50. Sharma, O. P.; Bhat, T. K., DPPH antioxidant assay revisited. *Food Chem.* **2009**, *113* (4), 1202-1205. DOI: 10.1016/j.foodchem.2008.08.008.

51. Frezza, C.; Cipolat, S.; Scorrano, L., Organelle isolation: functional mitochondria from mouse liver, muscle and cultured fibroblasts. *Nat. Protoc.* **2007**, *2*, 287-295. DOI: 10.1038/nprot.2006.478.

52. Boesch-Saadatmandi, C.; Wagner, A. E.; Wolffram, S.; Rimbach, G., Effect of quercetin on inflammatory gene expression in mice liver in vivo - role of redox factor 1, miRNA-122 and miRNA-125b. *Pharmacol. Res.* **2012**, *65*, 523-530. DOI: 10.1016/j.phrs.2012.02.007.

53. Brinker, C. J. a. S., G.W. (1990) Solgel Science: The Physics and Chemistry of Solgel Processing. Academic Press Incorporation, San Diego.

54. Wang, C.-C.; Ying, J. Y., Sol–gel synthesis and hydrothermal processing of anatase and rutile titania nanocrystals. *Chem. Mater.* **1999**, *11*, 3113-3120. DOI: 10.1021/cm990180f.

55. Liu, H.; Yang, W.; Ma, Y.; Cao, Y.; Yao, J.; Zhang, J.; Hu, T., Synthesis and characterization of titania prepared by using a photoassisted sol–gel method. *Langmuir* **2003**, *19*, 3001-3005. DOI: 10.1021/la026600o.

56. Ayres, G. H., Evaluation of accuracy in photometric analysis. *Anal. Chem.* **1949**, *21*, 652-657. DOI: 10.1021/ac60030a002.

57. Dorta, D. J.; Pigoso, A. A.; Mingatto, F. E.; Rodrigues, T.; Pestana, C. R.; Uyemura, S. A.; Santos, A. C.; Curti, C., Antioxidant activity of flavonoids in isolated mitochondria. *Phytother. Res.* **2008**, *22*, 1213-1218. DOI: 10.1002/ptr.2441.

58. Fiorani, M.; Guidarelli, A.; Blasa, M.; Azzolini, C.; Candiracci, M.; Piatti, E.; Cantoni, O., Mitochondria accumulate large amounts of quercetin: prevention of mitochondrial damage and release upon oxidation of the extramitochondrial fraction of the flavonoid. *J. Nutr. Biochem.* **2010**, *21*, 397-404. DOI: 10.1016/j.jnutbio.2009.01.014.

59. Brezová, V.; Gabcová, S.; Dvoranová, D.; Stasko, A., Reactive oxygen species produced upon photoexcitation of sunscreens containing titanium dioxide (an EPR study). *J. Photochem. Photobiol. B* **2005**, *79*, 121-134. DOI: 10.1016/j.jphotobiol.2004.12.006.

60. Kiwi, J.; Nadtochenko, V., New Evidence for TiO_2 photocatalysis during bilayer lipid peroxidation. *J. Phys. Chem. B* **2004**, *108*, 17675-17684. DOI: 10.1021/jp048281a.

61. Alvarez-Hernandez, X.; Nichols, G. M.; Glass, J., Caco-2 cell line: a system for studying intestinal iron transport across epithelial cell monolayers. *Biochim. Biophys. Acta* **1991**, *18*, 205-208. DOI: 10.1016/0005-2736(91)90165-5.

62. Artursson, P.; Palm, K.; Luthman, K., Caco-2 monolayers in experimental and theoretical predictions of drug transport. *Adv. Drug Deliv. Rev.* **2001**, *46*, 27-43. DOI: 10.1016/S0169-409X(00)00128-9.

63. Sambuy, Y.; de Angelis, Y.; Ranaldi, G.; Scarino, M. L.; Stammati, A.; Zucco, F., The Caco-2 cell line as a model of the intestinal barrier: influence of cell and culture-related factors on Caco-2 cell functional characteristics. *Cell Biol. Toxicol.* **2005**, *21*, 1-26. DOI: 10.1007/s10565-005-0085-6.

64. Brun, E.; Barreau, F.; Veronesi, G.; Fayard, B.; Sorieu, S.; Chanéac, C.; Carapito, C.; Rabilloud, T.; Mabondzo, A.; Herlin-Boime, N.; Carrière, M., Titanium dioxide nanoparticle impact and translocation through ex vivo, in vivo and in vitro gut epithelia, *Part. Fibre Toxicol.* **2014**, *11:13*, (pp. 16). DOI: 10.1186/1743-8977-11-13.

65. Lupua, A. R.; Popescu, T., The noncellular reduction of MTT tetrazolium salt by TiO_2 nanoparticles and its implications for cytotoxicity assays, *Toxicol. In Vitro* **2013**, *27*, 1445-1450. DOI: 10.1016/j.tiv.2013.03.006.

66. Timm, M.; Saaby, L.; Moesby, L.; Hansen, E. W., Considerations regarding use of solvents in in vitro cell based

assays, *Cytotechnol.* **2013**, *65*, 887-894. DOI: 10.1007/s10616-012-9530-6.

For Table of Contents Use Only

Fabrication of hybrid materials from titanium dioxide and natural phenols for efficient radical scavenging against oxidative stress

Huayang Yu, Zhili Guo, Shuqi Wang, Ganwarige Sumali N. Fernando, Simran Channa, Algy Kazlauciunas, David P. Martin, Sergey A. Krasnikov, Alexander Kulak, Christine Boesch and Natalia N. Sergeeva*