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Title Evaluation of Specific Migration According to EU Normative of Titanium Oxide Particles from Chitosan/Metal Complexes Films into different Food Simulants. A Comparative Study of the Nano-Sized vs Micro-Sized Particles

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Abstract

ABSTRACT Although, the current market of food packaging produced with engineered nanoparticles is increasing, concern over toxicity due to their possible migration into foodstuff affects consumer acceptance which has been recognized as a key factor to successfully negotiate market opportunities. Data of science have shown the possibility of engineered nanoparticles (i.e., metal(oxide)) migration from packaging into foodstuff, nevertheless in most of the studies, the migration was in compliance with overall migration limits, and specific migration limits imposed by EU normative and U.S. Food and Drug Administration (FDA). TiO₂ is authorized as food color additive (E 171) by the U.S. FDA and European Commission (EFSA) with the stipulation that the additive should not to exceed 1% w/w, and without the need to include it on the ingredient label. More than that the grade used in food does not have any particle size specifications. In this peculiar case, data of science revealed that it may contain particles in the nanoscale, and nano-form of TiO₂ is not approved as additive for food. In the light of the above mentioned, TiO₂ remain a critical challenges in food industry. In April 2019, French Government announced the suspension of the placing on the market of foodstuffs containing titanium dioxide (TiO₂, E 171) starting from January 2020 to December 2020. The French ban does not apply to non-food products such as medications, cosmetics and toothpastes. TiO₂ remains authorized in other Member States of the EU. In this context, the aim of this research was that to evaluate thin films based on the α -chitosan (CS)/titanium dioxide (TiO₂) food grade nano- and micro- sized in terms of (i) migration according to European Normative 1130-1:2004, (ii) cytotoxicity and (iii) antioxidant activity by means of DPPH tests to define if they can be used as active films for foodstuff. As far as we know, this is the first research evaluating and quantifying the migration behavior of α -chitosan/titanium dioxide food grade micro- and nano- sized complexes. The resulting bio-inorganic material exhibited a high free-radical scavenging against 1,1-Diphenyl-2-picrylhydrazyl (DPPH). The average percentage inhibition of the CS/Metal complex of nano-sized revealed a maximum value of 41.51 % as compared to that of the CS/Metal complex micro-sized, which achieved a value of 27.13%. This shows that, chitosan complexed with titanium dioxide nano-sized is a better radical scavenger than the chitosan complexed with titanium dioxide micro-sized, which it's explained by the decrease of particle size and resulting increasing surface area. With regard to the specific migration tests, which were quantified using acid digestion followed by inductively coupled plasma-mass spectrometry (ICP-MS) detection of titanium, the results revealed that titanium can migrated from chitosan matrix after incubation in different food simulants over 10 days at 40 °C or 10 days at 5 °C. The amount of titanium migration is observed as increasing with particle size. The smaller the particle size, the higher the titanium migration. Cytotoxicity analysis using Caco-2 cells, clone HTB-37™, from human colon carcinoma, and Resazurin and CCK-8 methods demonstrated that the CS/Metal complexes films had no cell toxicity if the samples are purified with ethanol and neutralized with 0.1 M NaOH.

Keywords α -Chitosan TiO₂ nanoparticles TiO₂ microparticles Food simulants Antioxidant Migration Cytotoxicity Food packaging

Corresponding Author DANIELA ENESCU

Corresponding Author's Institution INTERNATIONAL IBERIAN NANOTECHNOLOGY LABORATORY

Order of Authors DANIELA ENESCU

Suggested reviewers Jaime Grunlan, Orietta Monticelli, Seonghyuk Ko, David Julian McClements

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“Evaluation of Specific Migration According to EU Normative of Titanium Oxide Particles from Chitosan/Metal Complexes Films into different Food Simulants. A Comparative Study of the Nano-Sized vs Micro-Sized Particles”

”

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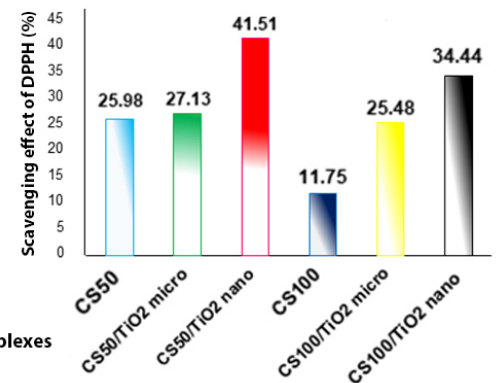
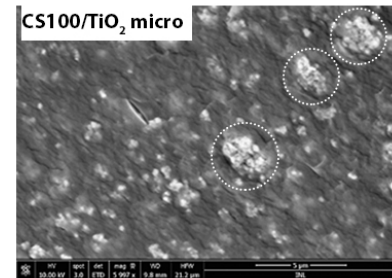
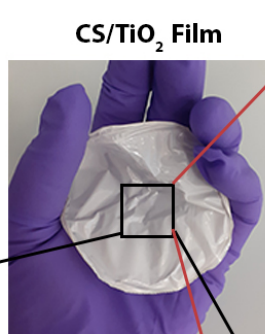
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HIGHLIGHTS

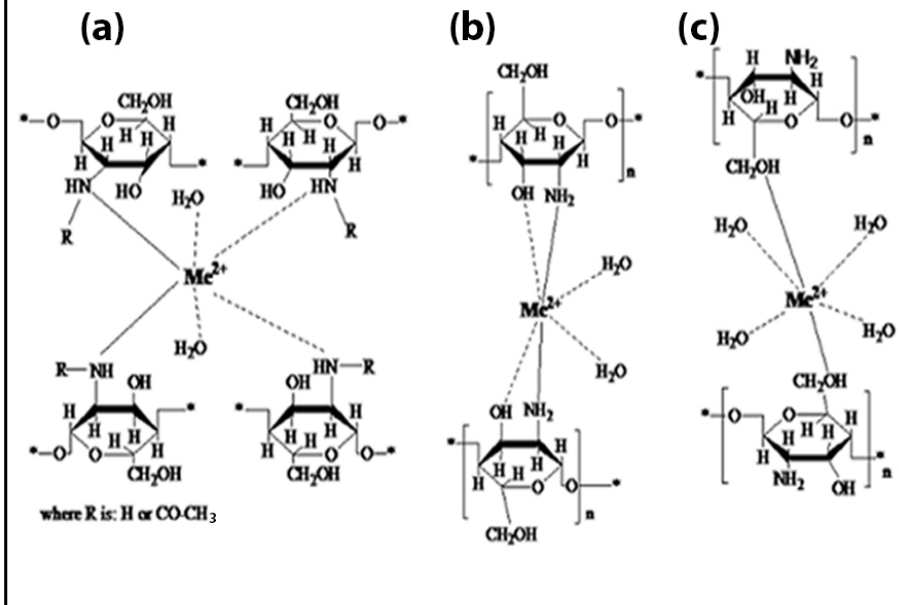
- Comparative migration study of nano- vs micro- sized TiO_2 food grade particles from α -chitosan films were investigated for the first time.
- The work highlights the importance of verification of raw materials impurities: i.e. metal oxides (commercial food grade).
- The new bio-inorganic material although revealed a good antioxidant activity, there is possibility of titanium migration, but in a negligible percent, meaning all the titanium is still in the polymeric matrix following the test.
- Cytotoxicity analysis using Caco-2 cells, clone HTB-37TM, from human colon carcinoma, and Resazurin and CCK-8 method demonstrated that the Chitosan/Metal complexes films had no cell toxicity if the samples are purified with ethanol and further neutralized with 0.1 M NaOH.



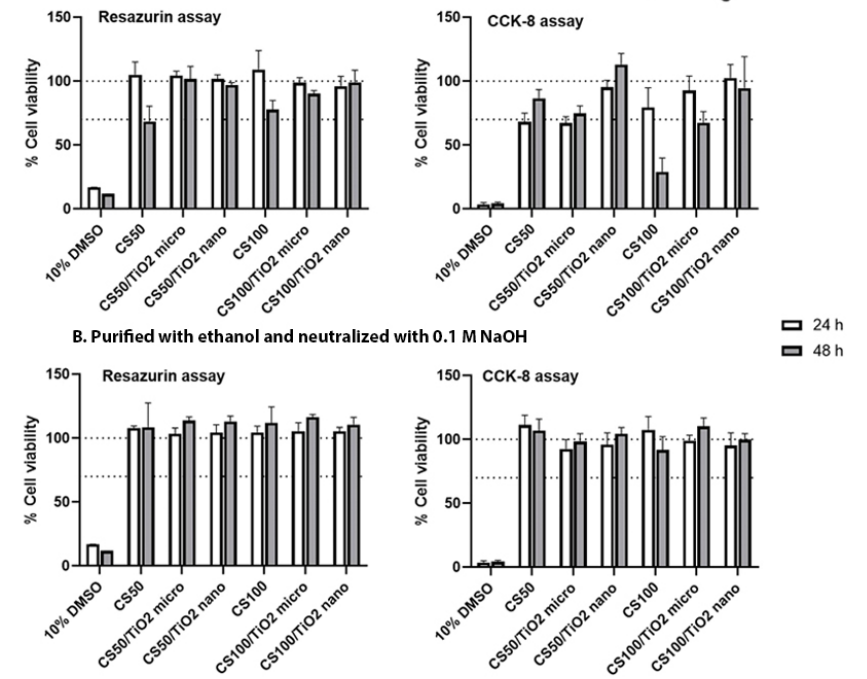
Evaporation



Possible structures of CS/TiO₂ Film



Cytotoxicity assay - CS/Metal complexes



Evaluation of Specific Migration According to EU Normative of Titanium Oxide Particles from Chitosan/Metal Complexes Films into different Food Simulants. A Comparative Study of the Nano-Sized vs Micro-Sized Particles

D. Enescu^{a*}, A. Dehelean^{b**}, C. Goncalves^{a***}, M. A. Cerqueira^a, D. A. Magdas^b, P. Fucinos^a, L. M. Pastrana^a

^a International Iberian Nanotechnology Laboratory (INL), Department Life Sciences, Research Unit: Nano4Food/Food Processing, Av. Mestre Jose Veiga s/n, 4715-330 Braga, Portugal

^b National Institute for Research and Development of Isotopic and Molecular Technologies, 400293 Cluj-Napoca, Romania

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*Corresponding author. International Iberian Nanotechnology Laboratory (INL), Department Life Sciences, Research Unit: Nano4Food/Food Processing, Braga, Portugal; National Institute for Research and Development of Isotopic and Molecular Technologies, Cluj-Napoca, Romania

E-mail addresses: daniela.enescu@inl.int (D. Enescu), adriana.dehelean@itim-cj.ro (A. Dehelean), catarina.goncalves@inl.int (C. Goncaves).

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Keywords:

α -Chitosan

TiO₂ nanoparticles

TiO₂ microparticles

Food simulants

Antioxidant

Migration

Cytotoxicity

Food packaging

1. Introduction

Competition in food & beverage packaging sector is of continuing concern, therefore innovation is crucial. Over the past decade *active*¹ and *intelligent*² food packaging become a real answer to longer shelf life (Mohammadi et al., 2019; Kaewklin et al., 2018) and long distance transportation (Galstyan et al, 2018; Park et al., 2015; Koutsoumanis et al., 2015; Yam et al., 2005; Pavelková, 2013; Manthou, & Vlachopoulou, 2001; Nopwinyuwong, Trevanich, & Suppakul, 2010; Smits et al., 2012; O’Grady, & Kerry, 2008) vs traditional food packaging.

Active packaging includes, e.g., the usage of nanoparticles³ (metal (oxides)). Hence, the application of nanoparticles (metal (oxides) to plastic and bio-based materials, in addition to improving their physical properties (gas/water/aroma barriers; mechanical/thermal properties, etc.); reducing the weight of food packaging materials; etc., it may open new possibilities by adding other functions, such as antimicrobial / antioxidant properties (Sorrentino, Gorrasi, & Vittoria, 2007).

Polysaccharides have been widely studied in the last decade as biobased packaging, and chitosan (CS)-a linear polysaccharide, composed of β -(1,4)-linked 2-acetamido-2-deoxyglucopyranose and 2-amino-2-

¹ Unlike traditional food packaging, which must be totally inert, *active* food packaging is designed to extend the shelf life or to maintain or improve the conditions of packaged food. They are designed to deliberately incorporate components that would release or absorb substances into or from the packaged food or the environment surrounding the food (European Commission Regulation No. 450/2009, 2009).

² Intelligent packaging denotes features that deliver information about brand protection or information for consummator on safety and quality of the food products like smart labels, for e.g., Tempix time/temperature indicator[®] (Sweden); RF Sensor tag from Ready-to-eat[®] (Romania); RF Sensor tag from Boglar Champ S.R.L[®] (Romania); “Fresh Meter” time-temperature indicator from Bizerba’s OnVu[®] (US); To-Genkyo[®] time temperature indicator (Japan); Timestrip[®] from TimestrioPlc; Check Point[®] from Vitsab; Fresh-Check[®] from LifeLines; Cook-Che[®] from Keep-it Technology; Colour-Therm[®] from Colour-Terms; eO[®] from 3MTM, Minnesota; TopCroTM from TRACEO (<http://cryolog.com>); etc (Enescu et al., 2019a).

³ Nanoparticles due to their small size have considerable greater surface area to mass ratio and consequently more surface atoms than their microscale counterparts, which favor, a better interaction with the polymer matrix, and the performace of the resulting material.

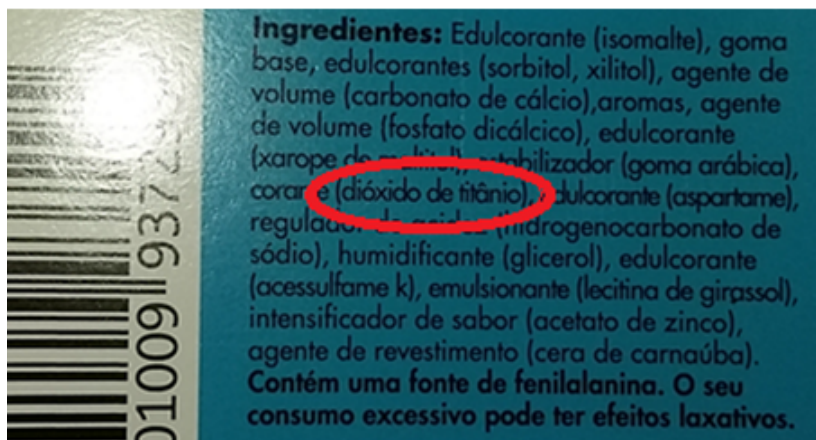
deoxyglucopyranose- has been particularly studied, especially due to its film-forming properties. Chitosan is the partly deacetylated derivative of chitin (poly(N-acetyl-D-glucosamine)), which is the second most abundant polysaccharide in nature after cellulose with an annual production of about 10 billion tons (Abdou, Elsabee, & Elkholy, 2011). Chitosan is non-toxic, biodegradable, biofunctional, and biocompatible, which makes it suitable for various applications (Enescu et al., 2009b; Enescu et al., 2009c; Enescu et al., 2019d; El Tahlawy, 2008; Fabra et al., 2016; Campos et al., 2018; Cazon, & Vazquez, 2019; Rinaudo, 2006; Yoshida et al., 2014). Other advantages derive from the antibacterial and antifungal properties of chitosan. Even if not completely understood, the antimicrobial activity comes from chitosan positive charges that notably interfere with the negatively charged cell surface of microorganisms, modifying the membrane permeability (Begin, & Van Calsteren, 1999). Due to its ability to form active edible matrices, chitosan coating can be expected to limit contamination of food surface (Campaniello et al., 2008; Durango, Soares, & Andrade, 2006). Nevertheless, this marine polysaccharide is moisture sensitivity and has lower barrier and mechanical properties compared with synthetic polymers, properties which can be remarkable improved by the addition of metal oxide fillers such as TiO₂ micro- and nano- sized (Mazin et al., 2015; Yun, Yun, Yoon, & Byun, 2016; Amin, & Panhuis, 2012). Therefore, there is no doubt that polysaccharide-metallic (oxide) based materials have made significant progress in many areas (Archana, Singh, Dutta, & Dutta, 2013; AbdElhady, 2012; Zemskova et al., 2018), active food packaging being one of the most promising area (Youssef et al., 2015; Al-Naamani, Dobretsov, & Dutta, 2016). Kaewklin et al., 2018 reported promising active food packaging based on chitosan-TiO₂ nanocomposites for prolonging storage life of tomato fruit. TiO₂ have higher UV-blocking properties, which can be advantageous for food storage (Yemmireddy, & Hung, 2015), as well as prevents microbial growth (Ge, Schimel, & Holden, 2012). Chawengkijwanich et al., 2008 reported that TiO₂-coated packaging film considerably reduce *Escherichia coli* contamination of food surfaces.

Although, polysaccharides are attractive due to their low-toxic property and apparent lack of side effects (Xie, et al., 2016) it is still necessary to evaluate the potential risks and toxicity of polysaccharide-metallic (oxide) based materials in detail. In addition, limited information is available on metal (oxide) migration from food contact materials into foodstuff, which subsequently it can induce toxicity through oral ingestion (Jovanović, 2015; Frohlich, & Frohlich, 2016). TiO₂ is approved as a food additive (E 171) by FDA and European Commission (EC, 1994; FDA, 2002; FDA, 2015) and it has been used for decades (Enescu et al., 2019a and the references cited within) as a pigment for food colouring (i.e. to provide characteristic optical properties such as increased brightness

and/or whitening) or added to granular and powdered foods as anti-caking agents (Enescu et al., 2019a and the references cited within). The *nano-form of TiO₂ is not an approved additive for food*, however the grade used in food does not have any particle size specifications. Studies have shown it may contain up to approximately 36 % of particles in the nanoscale (Weir et al., 2012). Hence, the presence of ‘nano-TiO₂’ in food is not new. The estimated dietary exposure of humans to TiO₂ nanoparticles has been reported to be up to 1.1 and 2.2 mg/kg body weight/day in the UK and US, respectively (Weir et al., 2012). It is noteworthy that the amount of TiO₂ nanoparticles consumed was 2–4 times higher for children than for adults, which may be due to the fact that products heavily consumed by children had some of the highest levels of TiO₂ nanoparticles, such as gums⁴, candies, desserts, and beverages. Weir et al., 2012 have quantified the amount of titanium in common food products and reported that candies, sweets and chewing gums contain the highest amount of TiO₂ in the scale of < 100 nm. Chen et al., 2013 reported that chewing one piece of chewing gum can result in an intake of 1.5–5.1 mg of TiO₂ nanoparticles.

In the light of the above mentioned the potential risks of TiO₂ nano-sized is that it can migrate from food packaging into foodstuff and the main concern are that it is not normally eaten and metabolized. Furthermore, nanoparticles are able to enter the cells, tissues and organs of our bodies much more easily than larger particles.

⁴ The image displays the presence of titanium dioxide in the chewing gum.



[EFSA \(2016\)](#) re-evaluated titanium dioxide, and the Panel on Food Additives and Nutrient Sources added to Food concluded that the absorption of orally administered TiO_2 is extremely low and the low bioavailability of TiO_2 appears to be independent of particle size. The Panel conclude that the use of TiO_2 as a food additive does not raise a genotoxic concern. However, the Panel did not establish an acceptable daily intake (ADI). The Panel concluded that once definitive and reliable data on the reproductive toxicity of E 171 were available, the full dataset would enable the Panel to establish a health-based guidance value (ADI) ([EFSA, 2016](#)).

French Government, in April 2019, ([The National Law Review, 2019](#)) announced the suspension of the placing on the market of foodstuffs containing titanium dioxide (TiO_2 , E 171) starting from January 2020 to December 2020. TiO_2 remains authorized in other Member States of the EU. The European Commission should discuss whether the suspension of TiO_2 complies with EU Food Legislation and decide eventually whether it should be extended at EU level or not.

In this context, the current research has evaluated the migration of TiO_2 (commercial food grade) from marine polysaccharide polymeric matrix into different food simulants. A particular focus of the migration studies in forced conditions according to the European Standard EN 13130-1:2004 and Plastics Regulation (EU) No 10/2011 ([EN 13130-1, 2004](#); [Commission Regulation \(EC\) No. 10/2011, 2011](#)) was center on the particle size, micro vs nano. A wide range of analytical techniques are required for detection and characterization of micro- and nano- sized particles, because no single technique can provide all relevant information ([Artiaga, Ramos, Camara, & Gomez-Gomez, 2015](#)). Hence, the amount of TiO_2 micro- and nano- sized particles in the obtaining food simulating solutions were evaluated by Inductive Coupled Plasma-Mass Spectrometry (ICP-MS). In addition, Scanning Electron Microscopy - Energy Dispersive X-ray (SEM-EDS) and Transmission Electron Microscopy (TEM) were performed for qualitative information. The molecular/supramolecular structures of the resulted ligand-metal complexes were investigated by means of Ultrahigh-vacuum micro-Attenuated Total Reflection/Fourier Transform Infrared Spectroscopy (UHV micro-ATR/FTIR), Wide-Angle X-ray diffraction (WAXD), and Inductive Coupled Plasma-Mass Spectrometry (ICP-MS). The antioxidant activity of TiO_2 nano- and micro- sized on marine polysaccharide polymeric matrix was studied by UV-vis spectroscopy. The cytotoxic effects were all assessed through quantification of loss in cell viability.

2. Experimental

2.1. Materials

All employed chemicals and reagents were of analytical grade. α -Chitosan with viscosity 50 mPas, deacetylation degree 95% (CS50) and with viscosity 100 mPas, deacetylation degree 95 % (CS100), obtained from Alpha-chitin, were supplied by Heppe® Medical Chitosan GmbH (Germany). Glacial acetic acid (99%) was provided by Fisher Chemical® (Germany) and was used without any purification. Water (Milli Q) was obtained using a Millipore purification system (resistivity: 18.2 M Ω .cm at 25 °C). Ethanol absolute (≥ 99.8 % vol.) (EtOH) was supplied by Sigma-Aldrich® (Germany). 100 % extra virgin olive oil (OO) imported from La Espanola® (Spain). 1,1-Diphenyl-2-picrylhydrazyl (DPPH) and 6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox®) was supplied by Sigma-Aldrich® (Germany). Methanol (CHROMASOLV™, 99.9%vol.) was supplied by Honeywell® (Germany). Nitric acid (60 % vol.) and hydrofluoric acid (47-51 % vol.) were provided by Merck®(Germany). Titanium standard for calibration of ICP (TraceCERT®); 1000 mgL⁻¹ Titanium metal in 2 % nitric acid and 0.05 % hydrofluoric acid supplied by Sigma-Aldrich® (Germany). Nano-sized TiO₂ food grade, anatase crystalline form, AMS: 10-25 nm, purity 99.9 % - according to TDS (supplier, not given). Micro-sized TiO₂ food grade, anatase crystalline form, AMS: 40-50 μ m, purity 99 % - according to TDS (supplier, not given), and micro-sized TiO₂ food grade, anatase crystalline form, AMS: 0.5 μ m, purity 99% - according to TDS, supplied by Foodchem® (China). Minimum essential media (MEM) was purchased from Milipore® (Berlin, Germany). Trypsin-EDTA (0.25% trypsin-0.1% EDTA), penicillin/streptomycin 100x and fetal bovine serum (FBS), were bought from Merck Millipore® (Burlington, MA, USA). Sodium pyruvate solution 100 mM, resazurin sodium salt and cell counting kit were obtained from Sigma-Aldrich® (St. Louis, MO, USA).

2.2. Materials Preparation

2.2.1. Production of the CS/Metal complexes films

Chitosan was dissolved in 0.5 M⁵ acetic acid aqueous solution to attain a concentration of 2 % (w/v). The solution was stirred at room temperature (RT) for 24 h, and then ultrasonicated for 10 minutes at 37 Hz, 100 W (Ultrasonic bath: Elmasonic P®, Elma Schmidbauer GmbH).

TiO₂ nano- and micro- sized aqueous suspensions (0.5% w/v) were vigorously stirred in a flask equipped with a magnetic bar for 1h at 900 rpm at RT and then, the suspension was ultrasonicated for 10 minutes at 50% amplitude (Branson Digital Sonifier® 450).

CS/Metal complexes films were prepared by mixing acidified aqueous solutions of CS and aqueous dispersion of TiO₂ nano- and micro-sized. In a typical procedure, CS/Metal complexes were prepared by adding dropwise, under stirring (900 rpm) at RT, an aqueous solution of 0.5% by weight of TiO₂ over the acidified aqueous solution of CS (amine/Ti = 2/1 molar ratio). Finally, after 3 h stirring at 900 rpm at RT the mixture was poured into Petri dish and kept at room temperature for film formation until constant weight was reached (48 h). Afterwards, the films were washed with ethanol (analytical grade) to eliminate free metal ions from the surface of the complex and maintained at 50% RH and RT before analyses.

2.3. Materials Characterization

The film thickness was measured with a digital micrometer (Filetta® - electronic outside micrometer, Shut Geometrical Metrology®). For each sample, the values obtained at ten different locations were averaged (thickness: 24 µm ± 0.9). The microstructural and elemental analyses of the cross-sectional CS/Metal complexes films as well as of TiO₂ micro- and nano- sized commercial food grade powders were done with a scanning electron microscope (Quanta 650 FEG) with an energy dispersive X-ray probe (INCA Energy Oxford, UK) attachment. The cross-sectional CS/Metal complexes films were performed by cryogenic fracture in liquid nitrogen and then sputter-coated with gold for 63s at a working pressure of 1.4 E⁻³ mbar before the scanning electron microscopy - energy-dispersive X-ray spectroscopy (SEM-EDS) measurements. Whereas, the SEM-EDS measurements in the case of TiO₂ micro- and nano-sized commercial food grade powders, due to their semiconductive nature, were carried out without sputter-coated with gold. The powders were fixed on aluminum stubs with a double-stick conductive carbon

⁵ The acetic acid was chosen at the concentration of 0.5 M corresponding to maximum of chitosan solubility (Rinaudo, Pavlov, & Desbrieres, 1999).

substrate. X-ray patterns of TiO₂ micro- and nano- sized commercial food grade powders, neat CS50 fibers, neat CS100 fibers, neat CS50 thin films, neat CS100 thin films, and CS/Metal complex thin films were carried out by a PANalytical X'pert MPD-PRO (WAXD, PANalytical, Model: X PERT PRO MRD) Bragg-Brentano θ - θ geometry diffractometer using CuK α radiation at 45kV and 40 mA. The 2θ scan range was 5° - 80° with a step size of 0.01° and a time/step of 0.5 s. FT-IR spectra of TiO₂ micro- and nano- sized commercial food grade powders were recorded on a Bruker-Vertex 80 v spectrometer (range 4000-400 cm⁻¹, 32 scans, resolution 4 cm⁻¹) under ultrahigh vacuum conditions, thus the absorption of atmospheric moisture and other gas species (CO₂) is avoided. Specimens prepared as KBr pellets were used. Dried, powdery TiO₂ was mixed thoroughly with KBr and then pressed using a hydraulic press (Specac®, UK) to homogeneous disc with a thickness of 0.3 mm. The TiO₂ concentration in the samples was 0.1%, calculated with respect to KBr. Ultrahigh-vacuum ATR-Fourier Transform Infrared spectra (ATR-FTIR) of neat CS50 thin films, neat CS100 thin films, and CS/Metal complex thin films were recorded on a Bruker-Vertex 80 v spectrometer (range 4000-400 cm⁻¹, 32 scans, resolution 4 cm⁻¹) under ultrahigh-vacuum conditions.

2.4. DPPH•-Free Radical Scavenging Ability Assay

The antioxidant activity of TiO₂ nano- and micro- sized incorporated into chitosan films was evaluated using 1-Diphenyl-2-picrylhydrazyl (DPPH•) stable radical scavenging assay according to a procedure described by [Dumitru, Mitchell, Davis, & Vasile, 2017](#). 20 mg of CS/Metal complex films were placed in a flask containing 2 mL of methanol and was stirred at 900 rpm for 3 h at room temperature in Eppendorf mixer device (ThermoMixer™ C, Fisher Scientific®). The supernatant solution obtained was analyzed for DPPH• radical scavenging activity. 2 mL of methanolic solution of DPPH• (0.06 mM) was mixed with 500 μ L of supernatant solution. The reaction mixture was vortexed vigorously and incubated for 30 min at room temperature in the dark. The remaining DPPH• was determined by the absorbance at 517 nm using UV-vis spectrophotometer (quartz cell length: 1 cm; solvent: methanol; spectrophotometer: UV-vis 2250, Shimadzu®).

The radical scavenging activity (RSA) of the CS/Metal complex was calculated as the percentage of DPPH• radical inhibition according to Equation (1):

$$RSA (\%) = \left(1 - \frac{A_{sample}}{A_{control}}\right) \times 100 \quad \text{Equation (1)}$$

where: A_{sample} represents the absorbance of the sample solution and $A_{control}$ represents the absorbance of DPPH[•] methanolic solution without the addition of the CS/Metal complex films. Trolox[®] was used as a common standard for the calibration of the method, which makes the comparison of the measured values easier. The values were expressed in μM Trolox equivalent/ gram dry sample.

2.5. Specific migration tests of CS/Metal complexes films into food simulant media and analyzed by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS)

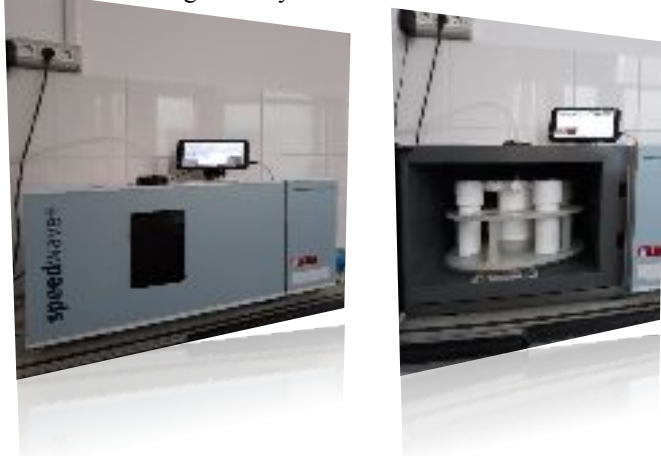
Migration of TiO₂ micro- and nano- sized particles from chitosan matrix has been tested by full immersion of CS/Metal complexes films (size: 0.6 dm² and weighted accurately) in two food simulants and sealed in clean wide-mouth jars at certain conditions of temperature and contact time. Ethanol (EtOH): 100 ml of 95 % v/v at 40 °C for 10 days, and olive oil (OO): 100 g of 100% wt. at 5 °C for 10 days. These specific test conditions were established by the European normative EN 13130-1:2004. For each formulation, the specific migration tests were carried out in triplicate.

As procedural blanks, the food simulant was filled into sealed jars and stored under the same conditions, as the CS-Metal complexes films samples, to check for contamination. All results were blank subtracted.

After the incubation period, the CS-Metal complexes films were removed and

- (i) the simulant (EtOH: 95 % v/v) was evaporated to dryness to avoid instabilities of the nebulizer and resuspended in 5 mL of nitric acid and sonicated 10 minutes, followed by addition of 3 mL of hydrofluoric acid, and subsequently digested using a microwave digestion system (Speedwave ENTRY by Berghof^{®6}).

⁶ Microwave digestion system



(ii) the simulant (OO⁷) was sonicated for 10 minutes and 50 mL of nitric acid were added. The sample was mixed at 40 °C using a magnetic stirrer for 30 min. The mixture was transferred into a separatory funnel to achieve the oil: acid phase separation. After phase's separation, the acidic phase was placed into PTFE (polytetrafluoroethylene) digestion vessels, followed by addition of 3 mL of hydrofluoric acid and microwave treatment.

The microwave digestion (Speedware ENTRY by Berghof[®]) was set to ramp from room temperature to 145 °C in 3 min, held for 5 min; from 145 °C to 170 °C in 5 min, held for 10 min; after from this temperature to 190 °C in 2 min, held for 15 min; and then from 190 °C to 75 °C in 1 min, held for 10 min. The degradation was performed under 1000 w magnetron power and 40 bar.

After microwave treatment, the solution was transferred to 50 mL vial and diluted with ultrapure water before being introduced for metal quantification by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) using ELAN DRC-e mass spectrometer (PerkinElmer SCIEX[®], USA) equipped with a Meinhardt nebulizer. The instrumental parameters employed for ICP-MS analysis were: nebulizer gas flow (0.96 Lmin⁻¹Ar), auxiliary gas flow (1.20 Lmin⁻¹Ar), plasma gas flow (15 Lmin⁻¹Ar), lens voltage (10.5 V), ICP RF power (1100 W), CeO/Ce=0.003, Ba⁺⁺/Ba⁺=0.014. The standard solutions were established in a range of 1-20 µg L⁻¹ titanium standard. The linearity of the calibration curve was considered acceptable (the correlation coefficient R²>0.999).

2.6. Determination of Titanium content into CS/Metal complexes films

Titanium content into CS/Metal complexes films was established by Inductively Coupled Plasma-Mass Spectrometry using different digestion methods (a) microwave digestion method (Speedwave ENTRY by Berghof[®]) as follows: the investigated films were placed in a digestion tube, and 2 mL nitric acid, 2 mL hydrofluoric acid, and 3 mL hydrogen peroxide were added. The microwave digestion was set to ramp from room temperature to 145 °C in 3 min, held for 5 min; from 145 °C to 170 °C in 5 min, kept for 10 min; then to 190 °C in 2 min and held for 15 min. After the digestions were completed, the solutions were cooled and transferred to 50 mL with 5 % (v/v) nitric acid aqueous solution and then diluted (1 mL of solution with 99 mL of ultrapure water) before ICP-MS testing. The

⁷ The extraction procedure of the metal from olive oil was carried out according to [Szycczewski et al., 2015](#) and [Yasar, Baran, & Alkan, 2012](#) with some modifications.

microwave digestion method allowed complete digestion of the films and clear solutions without residuals were obtained. The concentration of Ti was then measured using titanium standard solution with concentrations in a range of 150-2000 $\mu\text{g/L}$, with regression coefficient of 0.9986; (b) dry ashing digestion method, the films were pyrolyzed at 500 °C for 3 h in a electric furnace. After cooling, 1 ml of nitric acid was added, dried in the oven and after that, the samples were transferred in a electric furnace at 500°C for 1 h (Chen, Dong, Zhang, & Ding, 2019; Sahrawat, Ravi Kumar, & Rao, 2002). Afterwards, the sample volume was adjusted to 50 ml with 5 % (v/v) nitric acid, and then diluted before the ICP-MS measurements. The concentration of Ti was then measured using titanium standard solution with concentrations in a range of 150-2000 $\mu\text{g/L}$, with regression coefficient of 0.9986.

2.7. *Qualitative determination of titanium migrated from chitosan films into food simulants*

Qualitative analysis to identify the presences of titanium migrated into food simulants was performed by Scanning Electron Microscopy (SEM)-Energy-Dispersive X-ray spectroscopy (EDS): Sample preparation was performed by drop casting of the migration food simulant solutions onto 0.5 x 0.5 cm silicon wafer (previously cleaned with acetone and isopropanol to remove any organic substances) and dried in air. SEM images were collected on a Quanta 650 FEG. An energy dispersive X-ray probe (INCA Energy Oxford, UK) was used to characterize the element composition of the individual particles. Transmission Electron Microscopy (TEM)- Energy-Dispersive X-ray spectroscopy (EDS) was also used. Observations were carried out with voltage acceleration of 200 kV at α_3 , spot 1 and magnification: 30KX-100KX (JEOL JEM 2100, Izasa Scientific®, Portugal).

2.8. *Cell viability cytotoxicity assay*

The cytotoxicity assay was carry out on the dried neat CS films and CS/Metal complexes films purified with ethanol (analytical grade). In addition, to check if the toxicity of neat CS films and CS/Metal complexes films is acetic acid trace-dependent the test films after purification with ethanol were further neutralized in 0.1 M NaOH.

Hence, the cytotoxicity assay was carried out as follow: Caco-2 cells, clone HTB-37™, from human colon carcinoma, were obtained from the American Type Culture Collection (ATCC®). Caco-2 cells (passage 25-40) were cultured in minimum essential medium (MEM), supplemented with 20% fetal bovine serum (FBS), 1% sodium pyruvate and 1% penicillin/streptomycin. The cells were kept at 37 °C and 5% CO₂ in 75 cm² flasks. For the cytotoxicity assessment, confluent cells were detached using 0.25% trypsin-EDTA solution, then precipitated by

centrifugation at 1080 rpm for 5 min and resuspended in fresh medium MEM at a concentration of 1×10^5 cells.mL⁻¹. Cells were seeded onto 96-wells plates at a density of 1×10^4 cells (100 μ L of cellular suspension) per well and left adhering overnight in a humidified atmosphere of 5% CO₂ in air at 37 °C.

The cytotoxicity of the both types of films ((i) *purified* and (ii) *purified & neutralized*) was determined indirectly by the (a) resazurin and (b) CCK-8 assays. After adhesion, the culture medium was removed and replaced by 200 μ L of culture medium. Films were placed on the top of the culture medium and incubated. At each time-point (24 or 48h), samples were removed and replaced by the biomarker.

(a) Resazurin Assay

100 μ L of resazurin, diluted in culture medium (10% v/v) at a final concentration of 0.01 mg.mL⁻¹, were added to each well. The fluorescence intensity that is proportional to the number of viable cells was measured, after 3 h of incubation, using a Microplate Fluorescence Reader (Synergy, BioteK H 1, USA) at an excitation wavelength of 560 nm and an emission wavelength of 590 nm. The % cell viability was expressed as fluorescence of treated cells compared to the fluorescence of cells growing in the culture medium.

(b) CCK-8 Assay

100 μ L of CCK-8, diluted at 5% (v/v) in culture medium, were added to each well. After 3h of incubation, the absorbance was measured at 425 nm using a Microplate Reader (Synergy, BioteK H1, USA). The cell viability was expressed in percentage of absorbance in treated cells in relation to the absorbance of cells growing in the cell culture medium (MEM).

A negative control was performed using cells growing in culture medium (MEM), considered as 100% cell viability. A positive control was done using 10% DMSO.

2.9. Statistical analysis

For all analyses, determination were made in triplicate as independent experiments and displayed as mean \pm standard deviation. To assess the impact of formulation variables on the results, statistical analysis was performed using one-way analysis of variance (ANOVA) and the post-hoc Tukey's test (SigmaStat 4.0®) at a 95 % confidence level. Differences were considered significant at $p \leq 0.05$.

3. Results and discussion

3.1. Raw materials analysis to identify the presence of the impurity

Considering that the main purpose of this study is to analyze the migration of metal oxides from the chitosan polymeric matrix, therefore, before the synthesis of chitosan-metal complexes, all the raw materials (i.e., metal oxides) were analyzed from the point of view of impurities. Hence, the purity, particle size and crystalline phase of all types of TiO₂, micro- and nano- sized particles, commercial food grade were analyzed by Scanning Electron Microscopy-Energy-Dispersive X-ray spectroscopy (SEM-EDS), Wide Angle X-ray Diffraction (WAXD), and Fourier Transform Infrared spectroscopy (FT-IR) (*see Fig. 1*).

Fig 1

Fig 1 displays morphological verification in terms of impurities, particle size, and crystalline form of both types of TiO₂, micro- and nano- sized, commercial food grade. SEM-EDS, UHV FT-IR and WAXD analyses revealed that, in terms of purity; particle size as well as crystalline form, some of the metal oxides purchased were not in agreement with their technical data sheets. It was revealed impurities such as Si and Al, but in a negligible quantity (EDX) in the TiO₂ nano- sized, and in high quantity in one of TiO₂ micro- sized. Furthermore, WAXD revealed in some of them the presence of both crystalline forms (anatase as well as rutile). Therefore, the metal oxide in which was discovered high impurity amount and different particle size, as confirmed by SEM-EDS, WAXD, and UHV FT-IR analyses, was not further used in this work.

3.2. Chitosan-Metal complexation

3.2.1. Ultrahigh-vacuum micro-ATR/FTIR spectroscopy

The potential interactions between components, chitosan and titanium dioxide were evaluated by UHV micro-ATR/FTIR spectroscopy. The spectra of the neat CS50 and CS100 films and the CS/Metal complexes films (a) CS50/TiO₂ micro- and nano- sized and (b) CS100/TiO₂ micro- and nano- sized are presented in Fig. 2. In the neat CS50 film spectrum, the broad band centred at 3358 cm⁻¹ corresponding to the stretching vibrations of the hydroxyl and amino groups ($\nu_{\text{O-H}}$, $\nu_{\text{as N-H}}$ and $\nu_{\text{s N-H}}$) (Park, & Kang, 2005) shifted at about 3150 cm⁻¹ in CS50/TiO₂ micro sized complex, and at about 3122 cm⁻¹ in CS50/TiO₂ nano sized complex, indicating the involvement of both amino and hydroxyl functional groups of chitosan into complexation of metal cation. A shift can confirm the creation of a new bio-inorganic material regarding its possible structure presented in Scheme 1. The next change suggesting the creation of bio-inorganic material is the shift of the absorption band at 1541 cm⁻¹ assigned to the in-plane

deformation of the protonated amino group ($\delta_{\text{NH}_3^+}$) of chitosan (Darder, Colilla, & Ruiz-Hitzky, 2003) at about 1535 cm^{-1} in CS50/TiO₂ micro sized complex, and at about 1529 cm^{-1} in CS50/TiO₂ nano sized complex. Moreover, UHV micro-ATR-IR spectra are in agreement with XRD observations (see XRD section). Similar trends were revealed also by the CS100/TiO₂ micro- and nano- sized complexes. A broad band due to the axial hydroxyl and amino groups stretching vibrations ($\nu_{\text{O-H}}$, $\nu_{\text{as N-H}}$ and $\nu_{\text{s N-H}}$) centred at 3346 cm^{-1} is detectable in CS and which was shifted in CS100/TiO₂ micro- and nano- sized complexes to 3226 cm^{-1} and 3211 cm^{-1} respectively, indicating the involvement of both amino and hydroxyl functional groups of chitosan into complexation of metal cation. The absorption bands at 1541 cm^{-1} assigned to the in-plane deformation of the protonated amino group ($\delta_{\text{NH}_3^+}$) (Yuan, et al., 2011) shifted at 1535 cm^{-1} in CS100/TiO₂ micro-sized complex and at 1533 cm^{-1} in CS100/TiO₂ nano-sized complex suggesting the interaction of the amine groups with the Ti cation.

Fig 2

3.2.2. Wide-Angle X-Ray Diffraction

The Wide-Angle X-ray diffraction was applied to study the extent of metal binding by polymer ligand where the key functional groups are extensively utilized in binding, thereby disrupting the original polymer structure and the consequent changes in the WAXRD spectra.

The Wide-Angle X-ray diffraction pattern of neat “as-received” chitosan fibers vs neat chitosan films are displayed in Figs. 3a and 3b. Neat “as-received” CS50 fibers exhibits characteristic peaks at $2\theta = 8.66^\circ$ and 20.39° , respectively and neat “as-received” CS100 fibers exhibits characteristic peaks at $2\theta = 8.65^\circ$ and 20.11° respectively, whereas both types of neat CS films exhibit characteristic peaks at $2\theta = 8.65^\circ$ and 18.53° , respectively (Yuan, et al., 2011; Hsu et al., 2015). In addition, according to Webster et al., 2007 these characteristic diffraction peaks come from amorphous region, whereas the characteristic crystalline peak usually appears at $2\theta = 23^\circ$. The peak in the range $8^\circ - 13^\circ$ refers to the disordered portions of the polymer in the C-2 amino group (Webster et al., 2007). The second broad region from $2\theta = 10^\circ - 23^\circ$ are attributed to the hydrophilic pockets in the polymer, i.e. a hydroxyl alcohols from both C6 and the glucosamine monomeric ring structure and amide groups on the backbone of chitosan. Moreover, according to reported results (Yuan, et al., 2011; Yin et al., 2004; Schmuhl, Kreig, & Kiezer, 2001) the degree of crystallinity depends on the deacetylation degree (DDA) and molecular weight of the chitosan: higher degree of deacetylation

lower molecular weight arises to higher degree of crystallinity. This may be attributed to the fact that chains of chitosan with higher DDA are more flexible and have fewer large acetyl side groups. In our study the crystallinity of both types of chitosan was the same taking into account that they have the same DDA (i.e. 95 %), with the exception that in case of chitosan with higher molecular weight, the peak intensity at $2\theta = 18.53^\circ$ was lower than that of chitosan with lower molecular weight. Comparing the Wide-Angle X-ray diffraction pattern of neat “as received” chitosan fibers with neat chitosan films can be observed that the diffraction peaks at $2\theta = 20.39^\circ$ and $2\theta = 20.11^\circ$, respectively shifted at $2\theta = 18.53^\circ$ as well their intensity decreased after processing chitosan fibers into films, leading to an amorphous state of the films. Similar tendency was observed in the work reported by [Cervera et al., 2004](#).

Figs. 3c and 3d show the WAXRD patterns of neat CS films and their complexes. As mentioned above, diffractographs of neat CS50 film and neat CS100 film exhibits characteristic peaks at $2\theta = 8.65^\circ$ and 18.53° , respectively ([Yuan, et al., 2011](#); [Hsu et. al., 2015](#)). It was found that after the interaction with TiO_2 , the signal at $2\theta = 18.53^\circ$ in WAXRD pattern of chitosan films is appeared indicating that metal ions were complexed with NH_2 and OH groups which form bridge coordination model (Scheme 1). This indicates a great deal of these titanium ions favors interaction with the amorphous region of chitosan to form a bridge coordination between NH_2 and OH groups or absorbed in the amorphous pockets. There are two coordination models proposed for chitosan-metal complexes: a *bridge model* in which it is supposed that metal ions binds various nitrogen atoms from within the same chain or from adjacent chain ([Schlick, 1986](#)) and a *pendant model* refers to a coordination as a one-to-one pendant-like bond of metal to an amino group, confirmed by means of WAXRD studies ([Ogawa, Oka, & Yui, 1993](#)).

It was reported that bridge model is more favorable than pendant model ([Qu et al., 2011](#); [Lu, Cao, & Shen, 2008](#)). Indeed, this was confirmed in a comparative study on interaction between copper (II) and chitin/chitosan and not titanium (II), and the tendency of ligands to coordinate with Cu^{2+} was $-\text{NH}_2 \square \text{C}_3 -\text{OH} \square \text{H}_2\text{O} \square -\text{NHCOCH}_3$, suggesting that amine groups ($-\text{NH}_2$) on chitosan prefer to bind with Cu^{2+} and acetamide groups ($-\text{NHCOCH}_3$), on chitin lose their coordination with Cu^{2+} in aqueous medium ([Lu, Cao, & Shen, 2008](#)). The peaks at 25.5° , 37.2° , 38.1° , 38.8° , 48.3° , 54.4° , 55.3° , 62.8° , 69.0° , 70.5° and 72.2° correspond to TiO_2 micro- sized (crystalline) and 25.4° , 27.5° , 36.2° , 37.9° , 41.4° , 48.1° , 54.4° , 62.9° , and 69.1° correspond to TiO_2 nano- sized (crystalline).

Figs 3

Based on ATR-FTIR and WDXRD results, the coordination structure shown in Scheme 1 might be considered for both CS/Metal complexes. The metal ion is located like a bridge, connected one or more chains of chitosan through OH and $R:NH_2$ or $NH-CO-CH_3$ groups (Lu, Cao, & Shen, 2008; Reicha, Shebl, Badria, & EL-Asmy, 2012).

Scheme 1

3.3.3. Antioxidant Activity. DPPH[•]- Free Radical Scavenging Ability Assay

DPPH[•] tests were conducted to see whether the TiO₂ nano- and micro- sized retained their antioxidant potential when complexed in chitosan films. For this reason, chitosan films incorporating as an antioxidant different sized TiO₂ particles from 10 nm to 0.5 μ m, respectively were prepared. Moreover, SEM-EDS analyses were performed on

⁸ DPPH[•]: 1-Diphenyl-2-picrylhydrazyl, commercially available stable free radical (Fig. 4), compound that consists of a nitrogen free radical which shows strong absorption at 517 nm because of its single electron (Reicha, Shebl, Badria, & EL-Asmy, 2012), gets reduced by gaining a hydrogen or electron (Oliveira, & Rodriques, 2017; Ruiz-Navajas et al., 2013).

Fig. 4

Hence, DPPH[•] is a useful reagent for investigating the free radical-scavenging activity of compounds. This method is based on the reduction of alcoholic DPPH[•] solution in the presence of an antioxidant into non-radical DPPH-H (Prior, Wu, & Schaick, 2005), and the reduction in color is monitored over time (Shon, Kim, & Sung, 2003); (The solutions lose the characteristic of deep purple color to yellow with the absorption of (i) hydrogen moiety or (ii) single electron from the antioxidant. In other words, the UV absorption at 517 nm decreases when the single electron is quenched by (i) single electron transfer (Oliveira, & Rodriques, 2017) or (ii) by the proton radical scavenger of the hydrogen-donating antioxidant and is transformed into a nonradical form (Ruiz-Navajas et al., 2013)).

different areas of the films to see if the antioxidant was dispersed and distributed homogenously (*see* SEM-EDS micrographs).

The scavenging properties of neat CS films and the CS/Metal complexes films against DPPH[•] radical are displayed in Table 1. The scavenging effect increased for the chitosan with the low molecular weight. The neat chitosan films showed a RSA of 11.7% for CS100 and of 25.9% for CS50. The results are similar with those reported by Hormis et al., 2015a; Hromis et al., 2015b for chitosan with 80 % DD. Chitosan used in our study was from crab shells. Yen et al., 2008 noted that crab chitosan has good antioxidant properties, scavenging ability on hydroxyl radicals. According to Yen et al., 2008, the scavenging mechanism of chitosan is related to the fact that free radical can react with the residual free amino (NH₂) groups to form stable macromolecule radicals, and the NH₂ groups can form ammonium (NH₃⁺) groups by absorbing a hydrogen ion from the solution. Whereas, Xie et al., 2001 stated that the antioxidant activity of chitooligomers and its derivatives is related to the amount and activity of the hydroxyl group at C6 and the amine group at C2 of the chitosan molecule (Hromis et al., 2015a). Song et al., 2016 reported that the active hydroxyl in the chitosan backbone plays a more important role in DPPH[•] scavenging than the amino (Song et al., 2016). Park et al., 2003 previously reported higher a radical scavenging effect of DPPH[•] radical of low molecular weight chitosan than of a higher one.

The DPPH[•] activity of the CS/Metal complexes films was found to increase in a molecular weight and particle size-depend manner. The change in color (Fig. 5) in the test sample after incubation indicates the nature of the CS/Metal complexes films to be antioxidant. A similar aspect was reported by Kalyanasundharam, & Prakash, 2015.

CS50/TiO₂ nano- sized complex exhibited remarkable improvement on DPPH[•] - radical scavenging activity. The RSA was 41.5%, whereas the RSA of CS100/TiO₂ nano- sized complex was 34.4%. Similar results in the DPPH assay were reported by Kalyanasundharam, & Prakash, 2015. The authors reported that TiO₂ nanoparticles obtained either chemically or by biological method have effective free radical inhibition (37% and 52%, respectively). The radical scavenging potential is dependent of various factors such as particle size, morphology, defects, etc. Thus, the difference of antioxidant activity results between micro- and nano- sized TiO₂ could be attributed also to the metal oxide dispersion/distribution into film (*see* SEM-EDS).

Table 1

Fig 5

3.3.3.1. SEM-EDS

The morphology of the neat CS50 film, neat CS100 film, and the CS/Metal complexes films were studied by SEM-EDS (**Figs. 6**). SEM micrographs of the neat CS film exhibited a smooth surface, whereas SEM-EDS micrographs of the CS/Metal complexes films revealed the existence of metal aggregates being distributed all over the matrix (some of them having up to 5.4 micron in diameter). Nevertheless, it was found that, due to the different size of initial particle, a better dispersion and distribution was obtained for the complexes containing the nano-sized TiO₂. SEM micrographs display a composition contrast, the bright area reflect the particles with high atomic number which correspond in this case to the titanium atoms, whereas the dark area reflect the particles with low atomic number which correspond to carbon atoms. Further, the EDS mapping confirmed the presence of titanium aggregates.

Fig 6

3.3.4. Migration tests

3.3.4.1. Migration of titanium from chitosan matrix into food-simulating solutions analysed by ICP-MS

Ironically, although the active food packaging-associated engineered nanoparticles offer new opportunities, e.g., the extension of the shelf-life of foodstuff, might also have detrimental effects on consumer' health. Hence, one of the necessary factors to assess the applicability of Chitosan/Metal complexes films is the release behaviour of the metal (oxide) in the application process (Au, Pham, Vu, & Park, 2012). Currently there is not a “specific migration limit⁹ (SML)” for TiO₂ micro-sized.

⁹ “The specific migration limit” (SML) is defined as the maximum permitted level of a named substance migrating from the final material or article into food or food simulants.

In this context, Table 2 display a comparative specific migration study of nano- vs *micro*- sizedTiO₂ from CS/Metal complexes films into different food simulants investigated by means of Inductively Coupled Plasma-Mass Spectrometry. Results show that the migration of titanium is higher as the particle size decrease; i.e. the smaller the particle size is, the higher the titanium migration.

In 95 % aqueous ethanol it was observed that titanium migrated from CS50/TiO₂micro-sized complex was 17.4±0.9 ng·dm⁻²; from CS50/TiO₂nano-sized complex was 34.4±2.0 ng·dm⁻²; from CS100/TiO₂ micro-sized complex was 15.6 ±1.6 ng·dm⁻² and from CS100/TiO₂nano-sized complex was 39.8±1.0 ng·dm⁻². Thus, 1.97x10⁻⁴% to 4.46x10⁻⁴% of Ti migrated into the EtOH food simulant. This indicated that, substantially, all the titanium is still in the polymer matrix following the migration test.

In olive oil, it was revealed that titanium migrated from CS50/TiO₂ micro-sized complex was 13.0±1.4 ng·dm⁻²; from CS50/TiO₂nano-sized complex was 29.0±1.9 ng·dm⁻²; from CS100/TiO₂ micro-sized complex was 14.3±1.6 ng·dm⁻² and from CS100/TiO₂nano-sized complex was 48.5±3.0 ng·dm⁻². This represent approximately only 1.47x10⁻⁴% to 5.44x10⁻⁴% of the total titanium in the chitosan matrix, meaning essentially all the titanium is still in the polymer matrix following the test. The lower migration might be explained by different solubility of Ti in the food simulants used for this test. Thus, the olive oil food simulant let to a lowest migration which might be due to low solubility of Ti in fatty simulants. Extremely low migration content in fatty simulant was reported by Bott, Stormer, & Franz, 2014 in titanium¹⁰-food contact materials, 0.09-0.1 µg/kg. In addition, the authors stated that due to the usual size, shape and aggregation of nanoparticles in plastic nanocomposites nanomaterials are immobilized in food contact plastics and therefore exposure of the consumer to nanomaterials via migration from food contact plastics cannot be expected.

To the best of our knowledge there a limited literature addressing TiO₂ release from food packaging (Lin et al., 2014). The literature stated that migration of nanoparticles is a competitive process that depends on the compatibility of the nanoparticles to the solid (film) and liquid (food simulant) phase during swelling of the solid phase surface as it comes into contact with the liquid phase (Lin et al., 2014). In the research of Lin et al., 2014 was stated that titanium migrated from nano-TiO₂-polyethylene composite due to: (1) dissolution from the surface of film and (2)

¹⁰ Titanium nitride: the only plastic additive that is approved in *nanofom* and listed under FCM no.807 with some specifications and restriction in use in the positive list of the EU Plastic Regulation, such as to be used only in PET bottles at concentration up to 20 mg/kg.

dissolution from the cut edges of the solid phase (film) into the liquid phase (food simulant); and (3) diffusion from film, but this is not likely occurred as the nanoparticles are too big to diffusion in polymer matrix (30 nm and 100 nm).

Taking into account the above mentioned, can be supposed also that the low migration observed in this work is a physical mechanism that affect mass transfer such as dissolution from the cut edges of the solid phase (film), but the diffusion from film is not likely occurred, since titanium is not physical embedding into chitosan matrix, but it is coordinated by the functional groups of chitosan matrix which might slow down the migration process.

Table 2

3.3.4.2. Qualitative determination of TiO_2 migration into food simulants by SEM-EDS and TEM-EDS

SEM-EDS and TEM-EDS microscopies were unable to identify the presence of titanium migrated into food simulant. In Fig. 7 can be observed only the presence of Si from the silicon wafer used for SEM-EDS analysis. TEM-EDS data not shown.

Fig 7

3.3.5. Quantitative determination of TiO_2 complexed with chitosan by ICP-MS

TiO_2 content in the Chitosan/Metal complexes films was evaluated by using two different digestion methods such as microwave digestion and dry ashing digestion in order to get the most effective quantification method (see Table 3). The results revealed that the microwave digestion method is more appropriate for quantify the amount of TiO_2 complexed with chitosan as compared with dry ashing digestion method. With microwave digestion method, the sample was completed digested, and the loss was small owing to shorter digestion, whereas with dry ashing digestion method, the titanium content found was lower which might be explained by the large loss of the sample during the pyrolysis.

Table 3

3.3.5. Cytotoxicity

The cytotoxicity assay aims to detect the potential of a material to produce lethal or sub lethal effects in biological systems at the cell level. In this context, the cytotoxicity of both types of CS/Metal complexes films ((i) purified with ethanol and (ii) purified with ethanol and neutralized with 0.1 M NaOH) was evaluated with Caco-2 cell line measuring the cellular viability after 24 or 48 h of incubation. The cellular viability was evaluated through the resazurin or CCK-8 assays, measuring fluorescence or absorbance, respectively.

Figs. 8

The cells cultured directly in presence of (i) purified films showed cell viability below 70% which is considered toxic according to [ISO 10993-5:2009, 2009](#). But, the cells cultured directly in presence of (ii) neutralized films after 24 h or 48 h of contact demonstrated cellular compatibility, i.e. more than 70% of cell viability, in both assays (Figs. 8C and 8D).

In the light of the above findings, the low viability is due to the presence of acetic acid traces and not of the presence of the metal oxide (micro/nano sized).

4. Conclusions

This research evaluated the migration of TiO₂ nano- vs micro- sized from chitosan films based on the [European Normative 13301-1:2004](#). Results revealed that titanium can migrate from chitosan matrix after incubation in different food simulants over 10 days at 40 °C or 10 days at 5 °C. Apart the migration of TiO₂ nano- vs micro- sized, it was studied also the antioxidant potential (DPPH). The results obtained in the DPPH assay showed effective free radical inhibition by micro- and nano- sized TiO₂. The average percentage inhibition of CS/Metal complexes nano-sized was showed the maximum value (41.51%) as compared to that of CS/Metal complexes micro-sized (27.13%). This indicates that CS/Metal complexes nano-sized is good radical scavenger than micro-sized due to decrease particle size and increase surface area.

In the light of above findings, although the addition of TiO₂ brings good performance for marine polysaccharide polymeric matrix, at the same time security risks might exist. Hence, a *compromise* must be made between the level of migration and antioxidant activity, taking into account that currently for TiO₂ *micro sized there is not a specific*

limit migration (SML) established by European Normative or U.S. FDA. Whereas, the *nano-form* of TiO₂ is *not yet an approved additive for food*, however the grade used in food does not have any particle size specifications. In the current research, the amount of titanium migration is observed as increasing with particle size. The smaller the particle size, the higher the titanium migration.

Cytotoxicity tests showed that the neat CS films and CS/Metal complexes films presented low cell viability without neutralization. But, after neutralization in 0.1 M NaOH cell viability increased, showing this approach was suitable as a final preparation step of these films.

Based on migration and cytotoxicity assays, as a future perspective, the resulted CS/Metal complexes films might be applied as food packaging. But due to their sensitivity to moisture, they can be promoted for fat-based/dry food.

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Fig. 1 (a). SEM-EDS spectra of TiO₂ micro- and nano- sized raw materials commercial food grade; **(b)** WAXD spectra of TiO₂ micro- and nano- sized raw materials commercial food grade; **(c)** UHV FT-IR spectra of TiO₂ micro- and nano- sized raw materials commercial food grade.

Fig. 2. ATR-FTIR spectra of **(a)** neat CS50 film and CS50/TiO₂ micro- and nano- complexes films and **(b)** neat CS100 film and CS100/TiO₂ micro- and nano- complexes films.

Figs. 3. WAXRD patterns of **(a)** neat “as-received” CS fibers, **(b)** neat CS films, and CS-Metal complexes films **(c)** CS50/TiO₂ micro- and nano- sized and **(d)** CS100/TiO₂ micro- and nano- sized.

Scheme 1. Possible structures of CS-Metal complexes (the structures were designing by means of ChemDrawPro8[®] software).

Fig. 4. Chemical structure of DPPH[•].

Table 1

RSA of CS/Metal complexes films.

Fig. 5. Change in color of CS-Metal complexes after incubation.

Figs. 6. SEM micrographs and elemental distribution from EDS mapping of the neat CS films and the CS-Metal complexes films.

Table 2

Determination by ICP-MS of the amount of titanium migrated from CS-Metal complexes films into different food simulant after incubation period of 10 days at 40 °C or 10 days at 5 °C, conditions established by European Normative EN 13130-1:2004.

Fig. 7. SEM-EDS image of migrates from CS-Metal complexes films migration content in 95% ethanol food simulant after incubation for 10 days at 40 °C on a silicon wafer as holder.

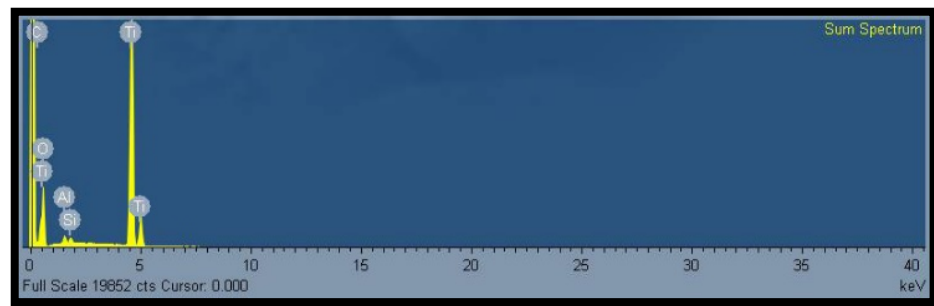
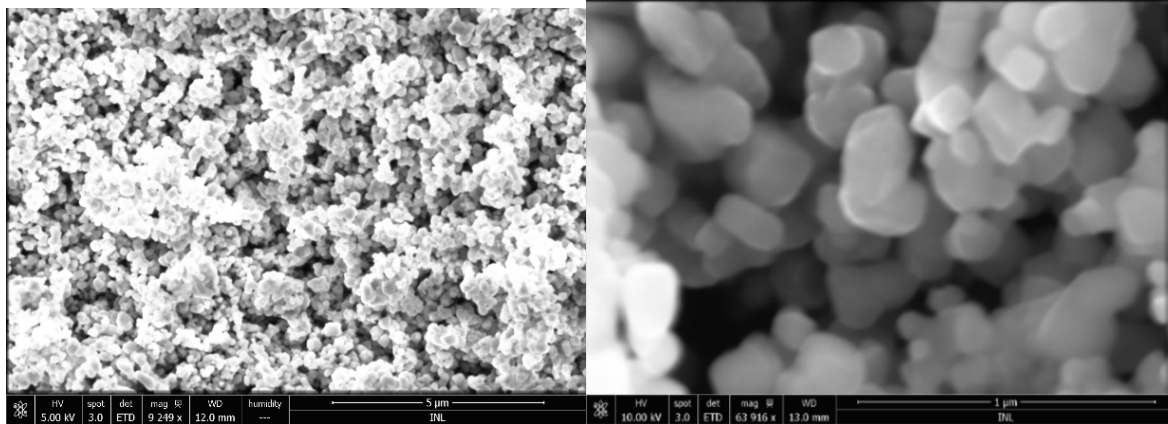
Table 3

Determination of titanium content in CS-Metal complexes films by ICP-MS using different digestion methods (i.e., microwave digestion and dry ashing digestion).

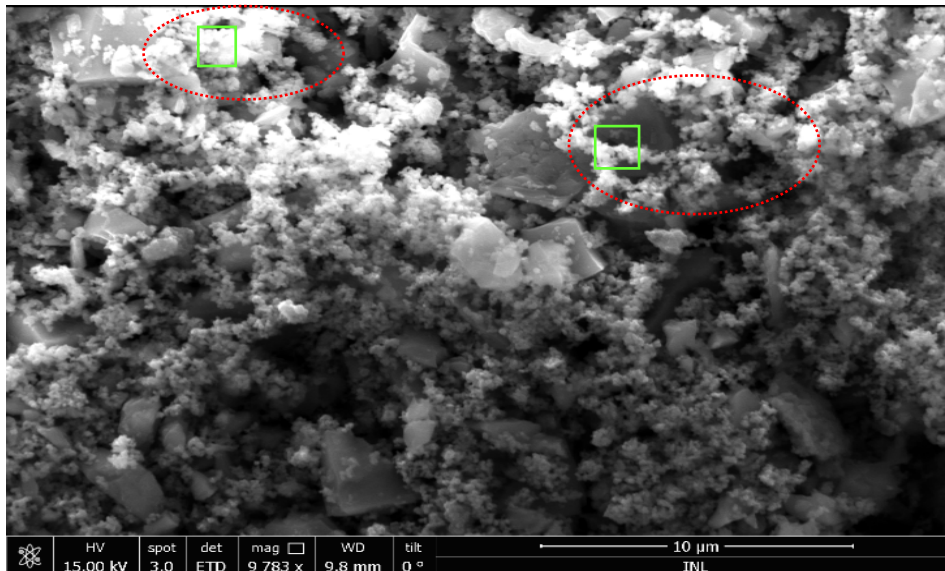
Figs. 8. Effect of CS/Metal complexes films purified with ethanol (**A** and **B**) and CS/Metal complexes films purified with ethanol and neutralized with 0.1 M NaOH (**C** and **D**) on the viability of Caco-2 cells after incubation for 24h or 48h at 37 °C. The cell viability was determined by measuring fluorescence (**A** and **C**) using the resazurin assay (excitation 560 nm/emission 590 nm). The cell viability was also determined by measuring absorbance (**B** and **D**) using the CCK-8 assay (absorbance 425 nm). Culture medium was used as negative control (100% cell viability) and 10% DMSO as positive control. Values show Mean \pm SD (N=4).

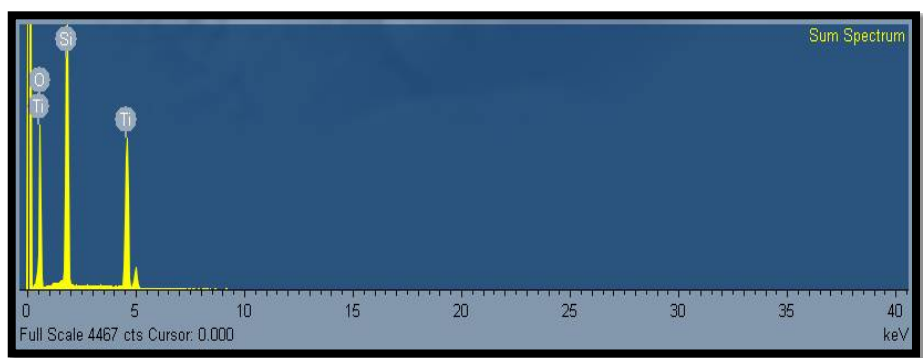
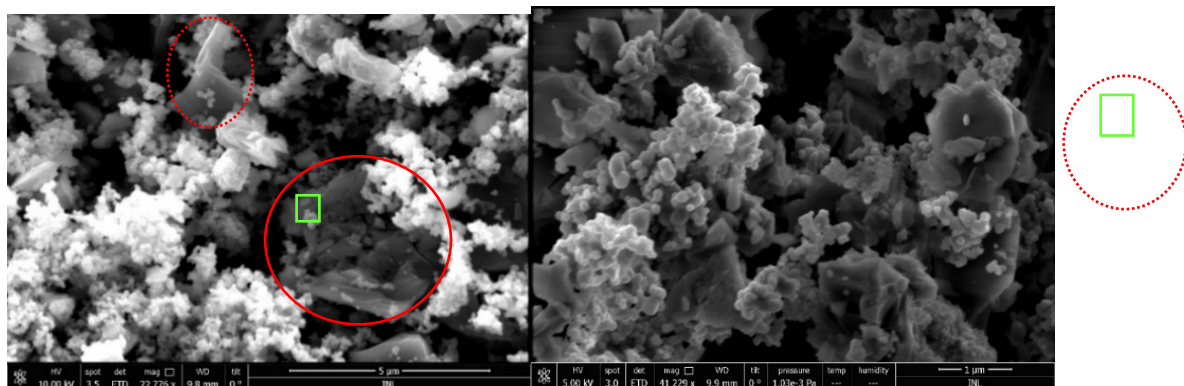
(a)

TiO_2 nano- sized

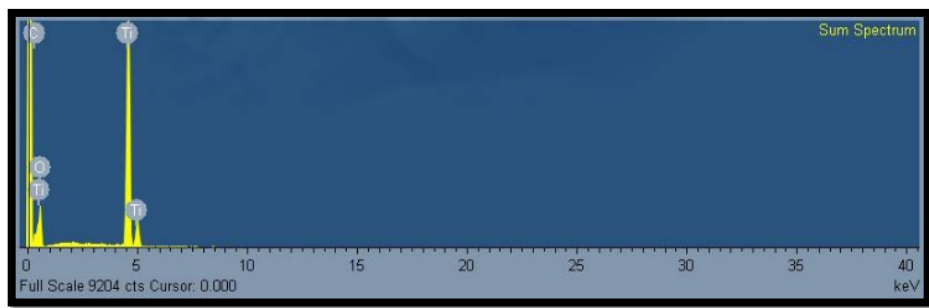
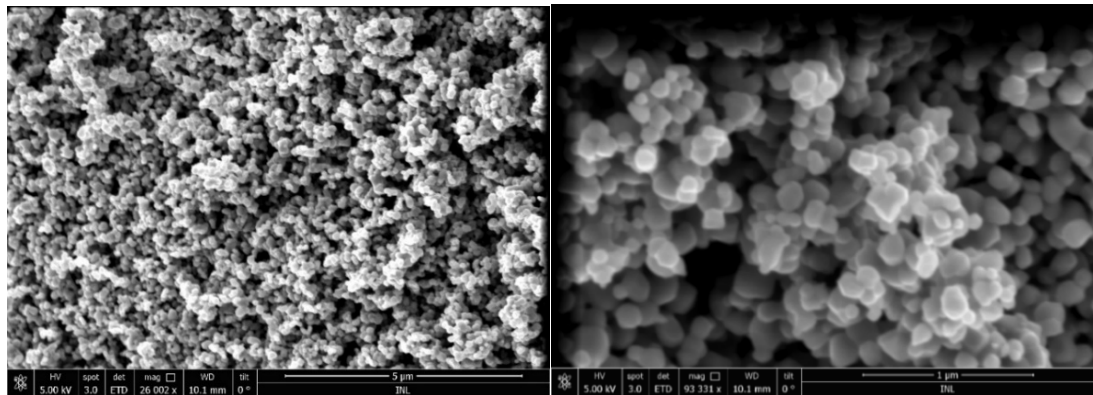


TiO_2 micro- sized





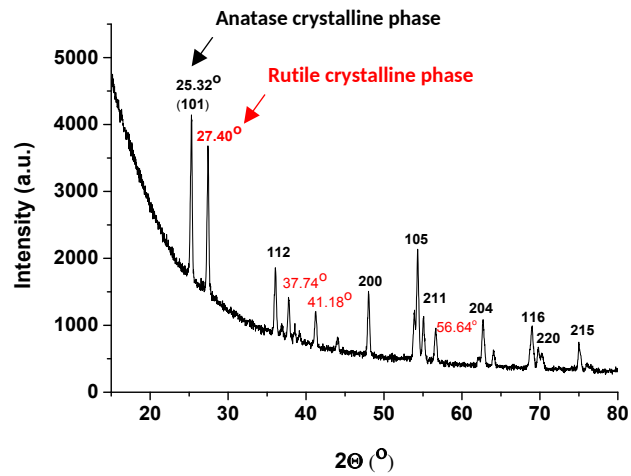
TiO₂ micro- sized (Foodchem®)



(b)

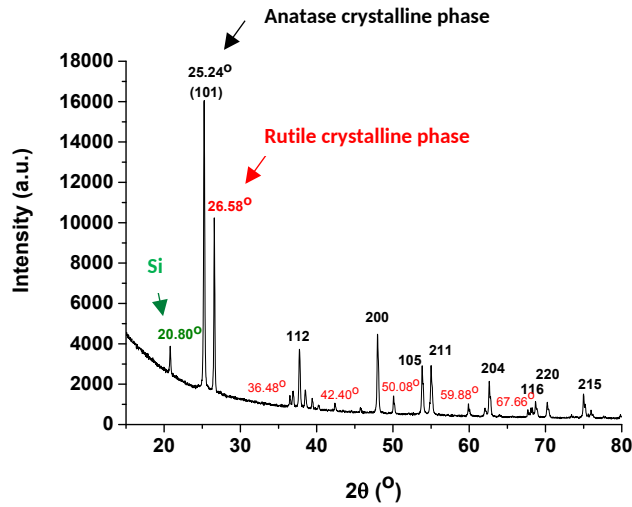
TiO₂ nano- sized

(TiO₂ - Anatase vs Rutile phase- ref. [Wang et al., 2012](#))



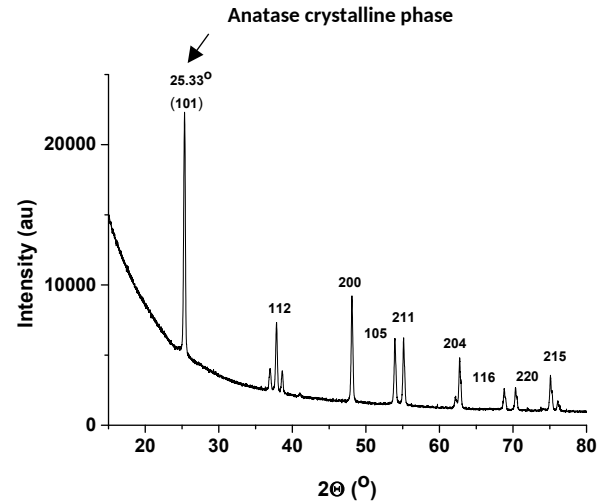
TiO₂ micro- sized

(TiO₂ coated with silica- ref. [Ekka et al., 2016](#))



TiO₂ micro- sized (Foodchem®)

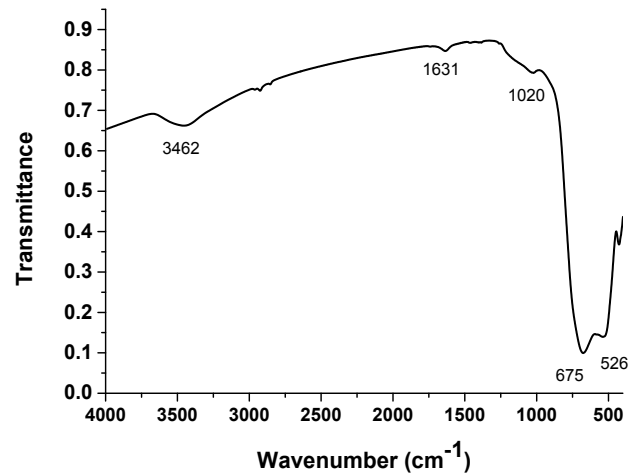
(TiO₂ - Anatase phase- ref. [Srinivasu et al., 2011](#))



(c)

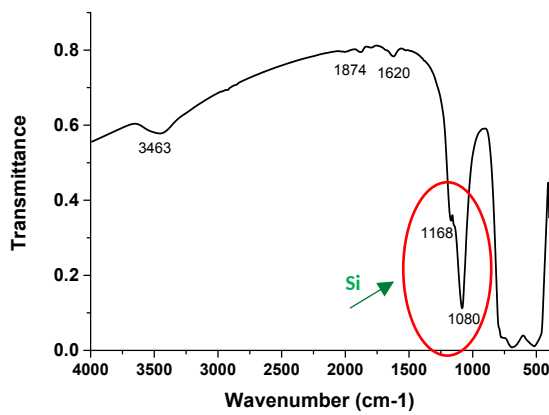
TiO₂ nano- sized

(TiO₂ - Anatase vs Rutile phase- ref. [Bobrova et al., 1968](#))



TiO₂ micro- sized

(TiO₂ coated with silica- ref. [Park, & Kang, 2005](#))



TiO₂ micro- sized (Foodchem®)

(TiO₂ Anatase phase- ref. [Bobrova et al., 1968](#))

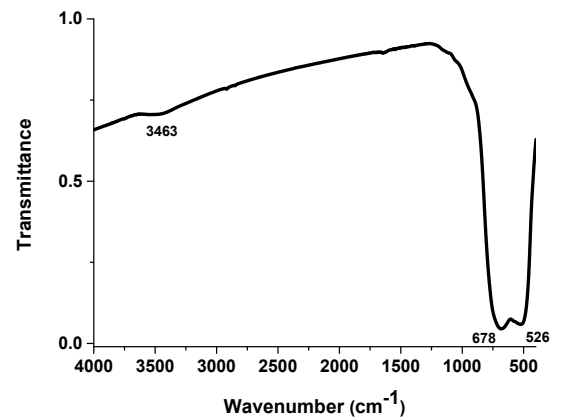


Fig. 1

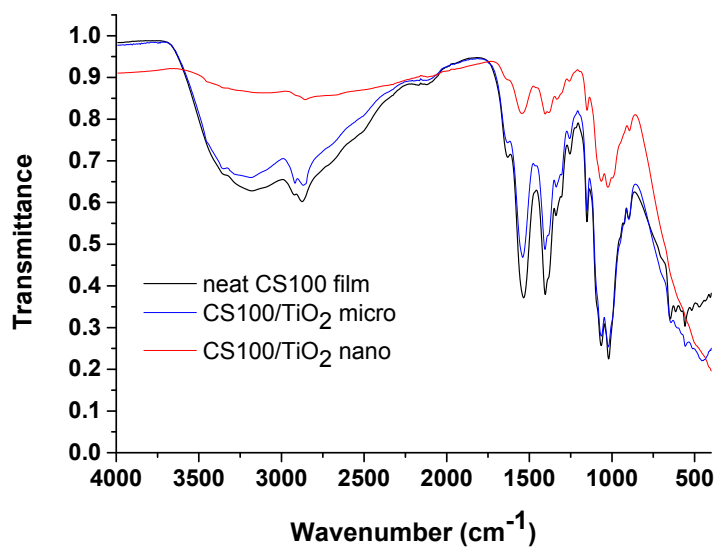
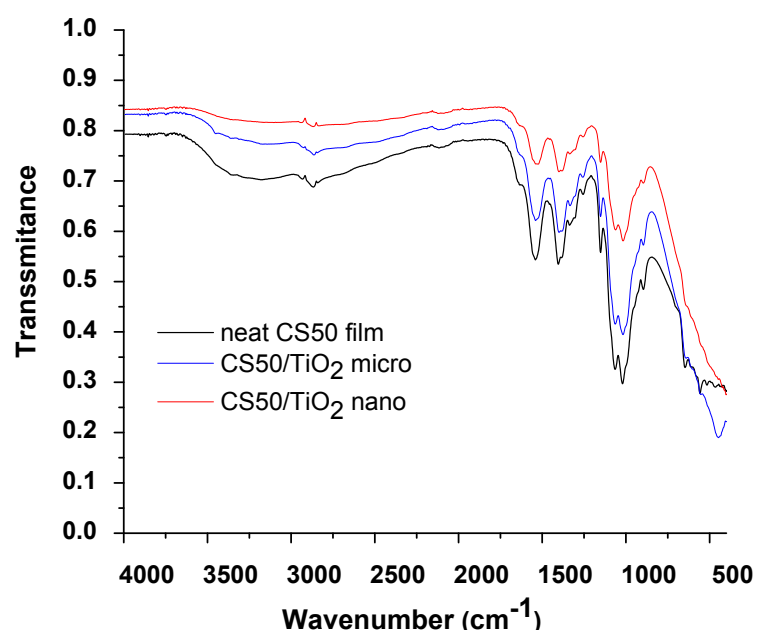
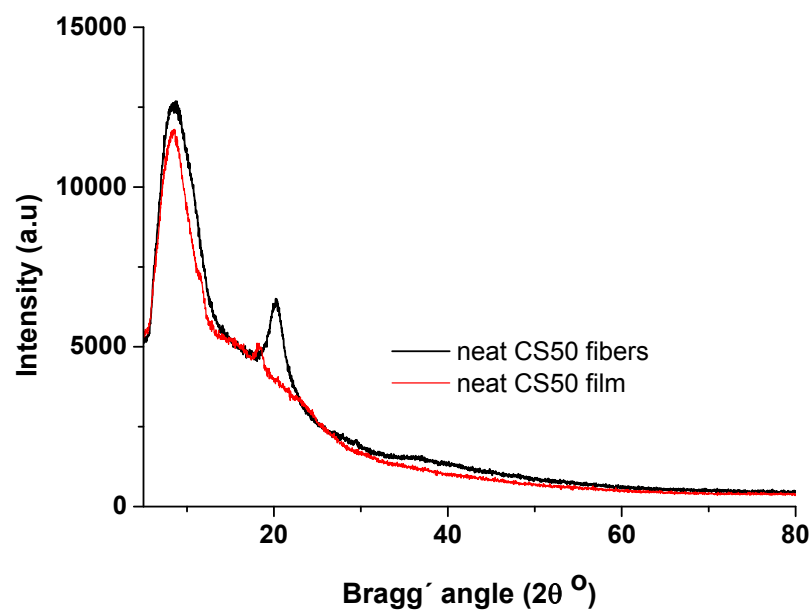
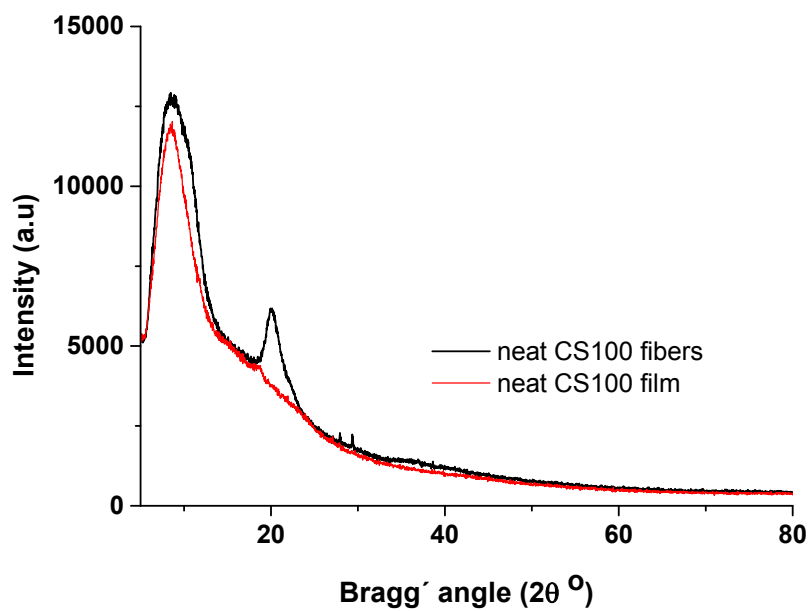


Fig. 2

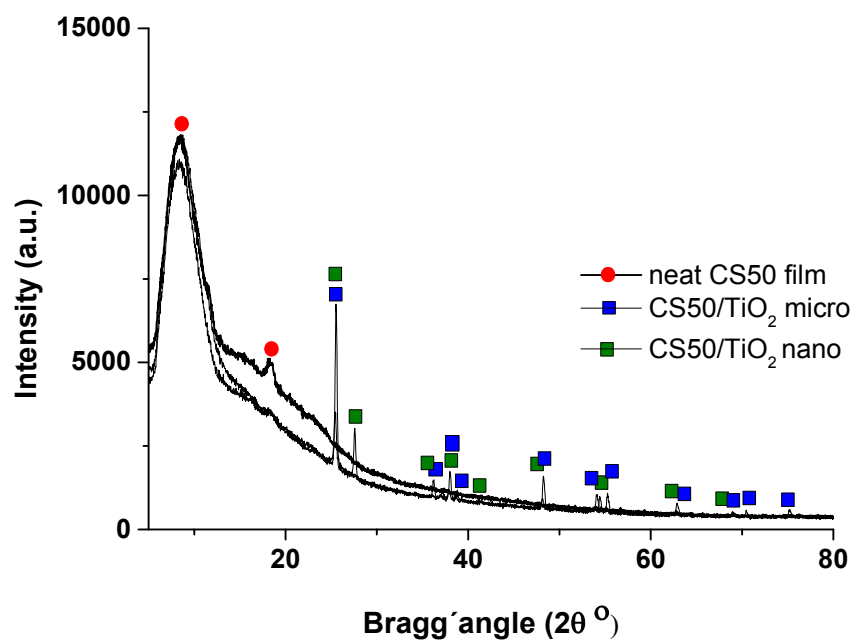
(a)



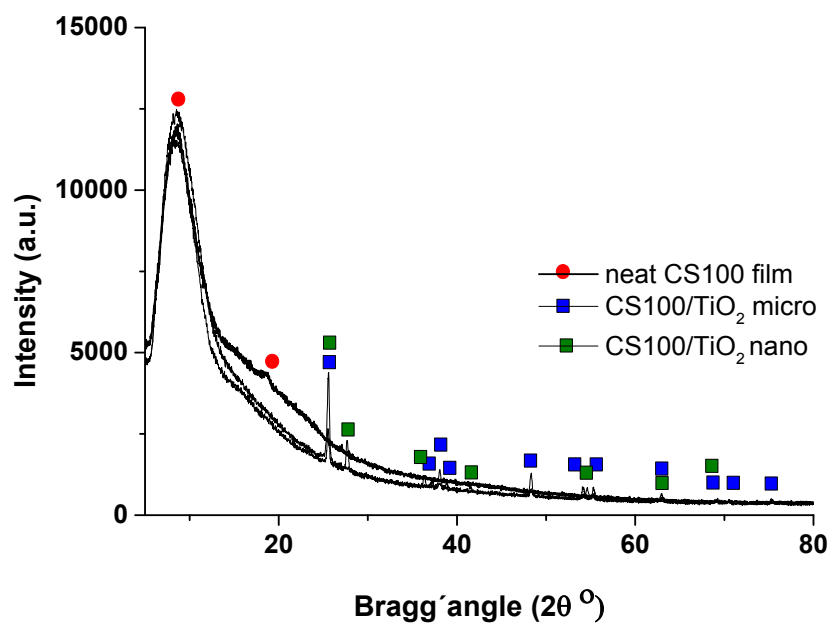
(b)



(c)

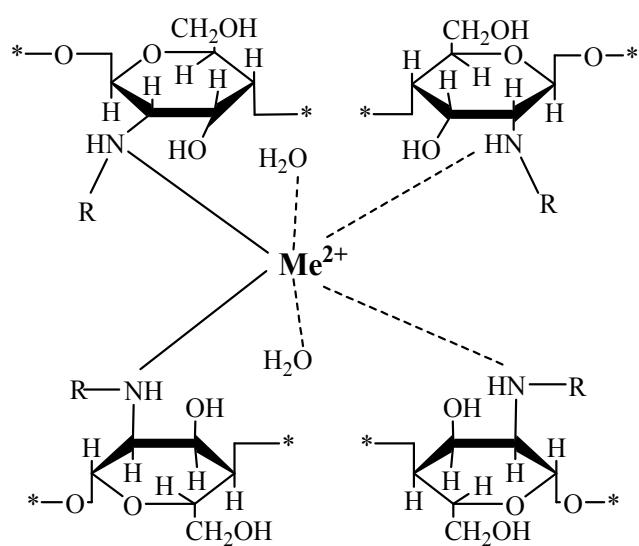


(d)



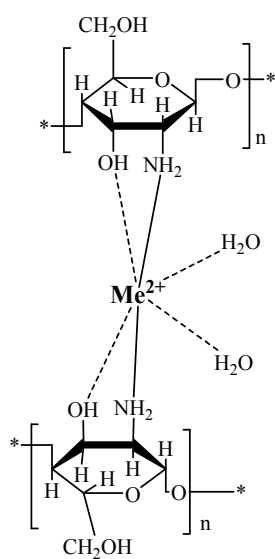
Figs. 3

a)

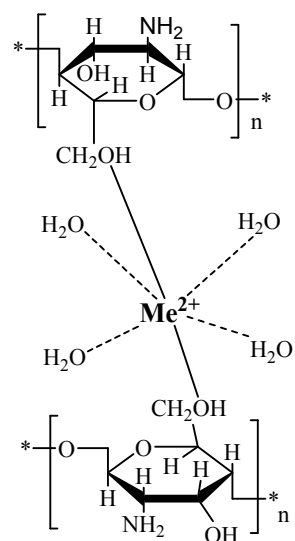


where R is: H or CO-CH₃

b)



c)



Scheme 1

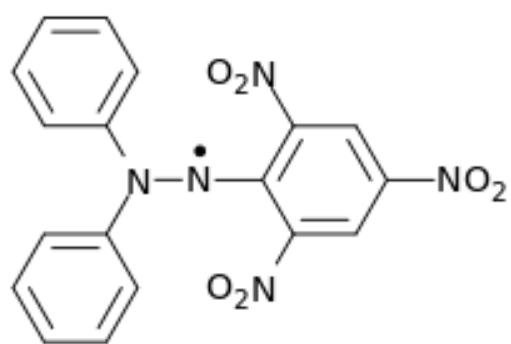


Fig. 4

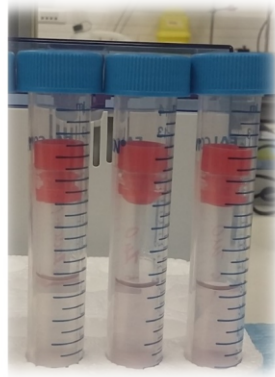
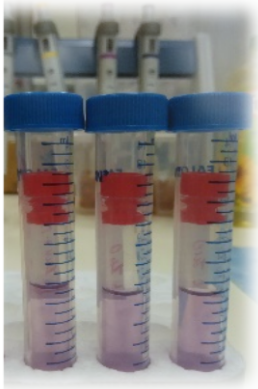
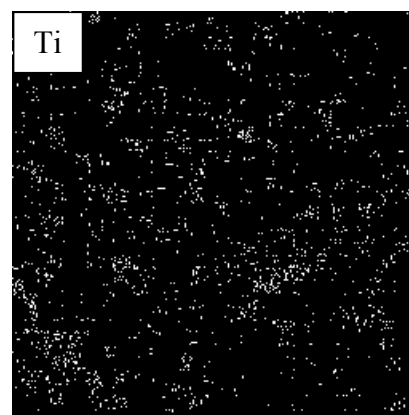
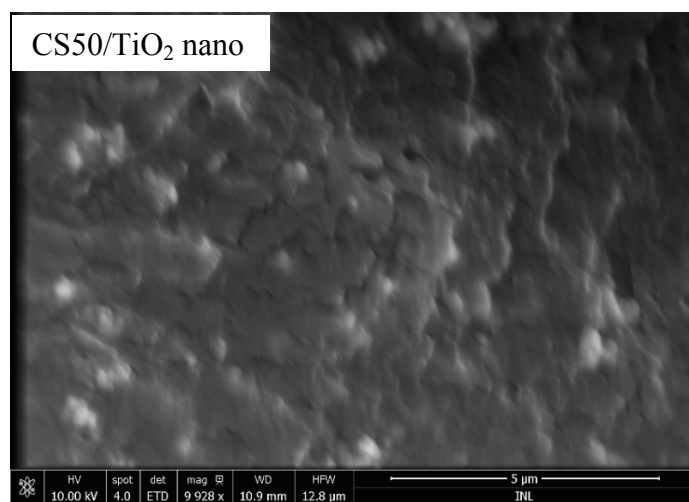
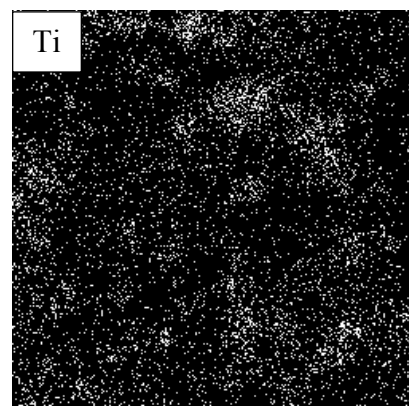
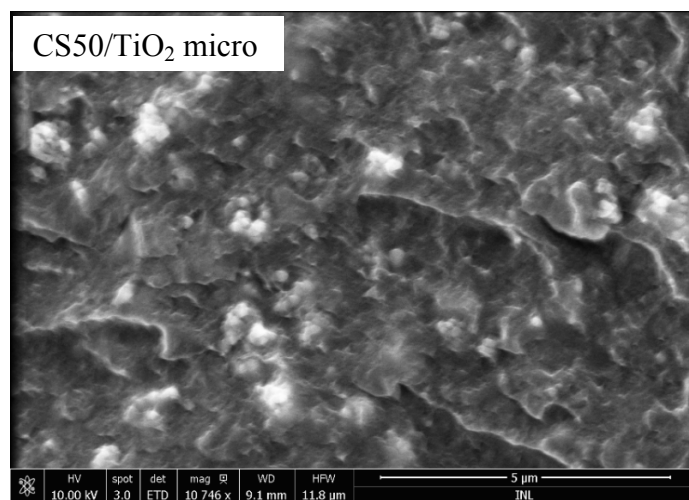
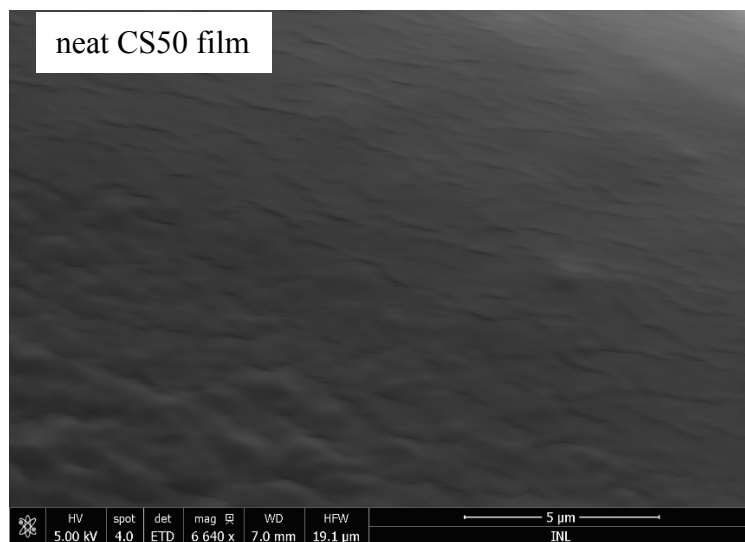
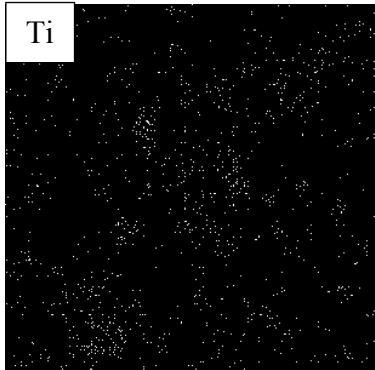
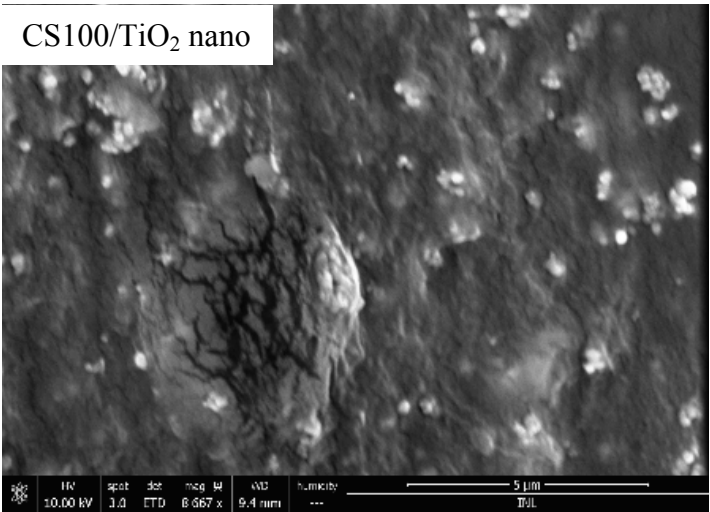
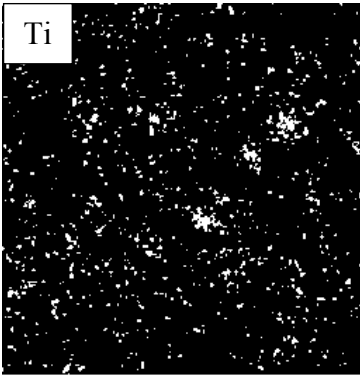
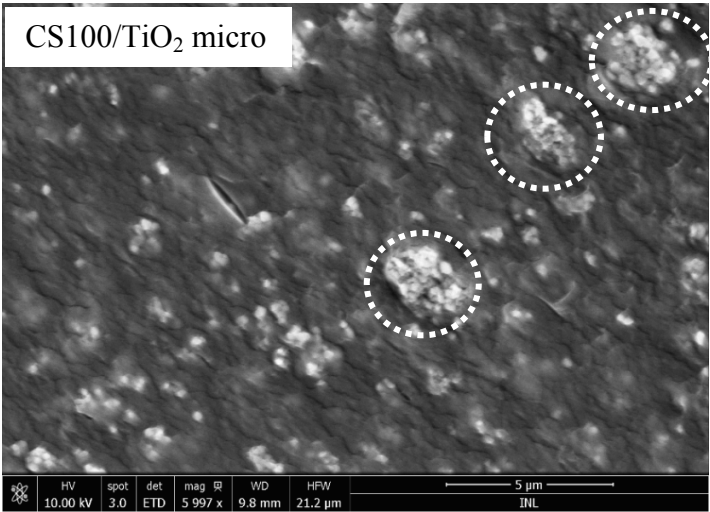
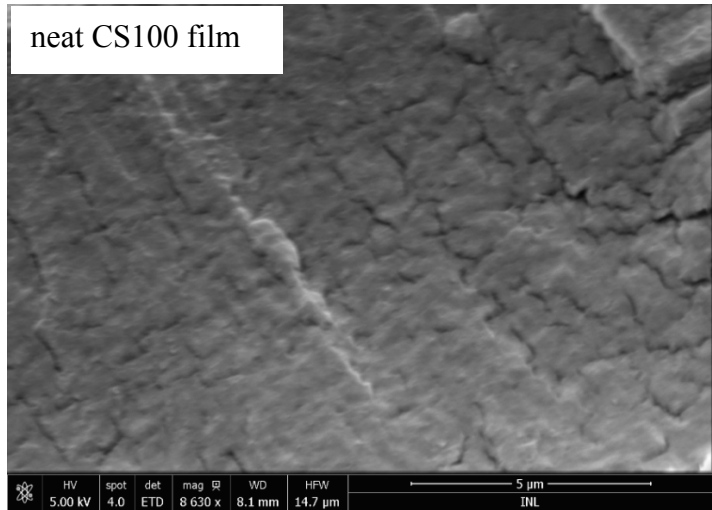


Fig. 5





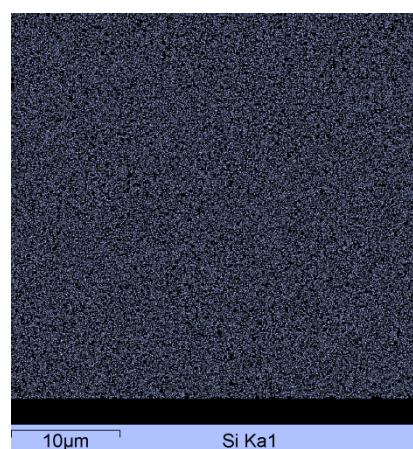
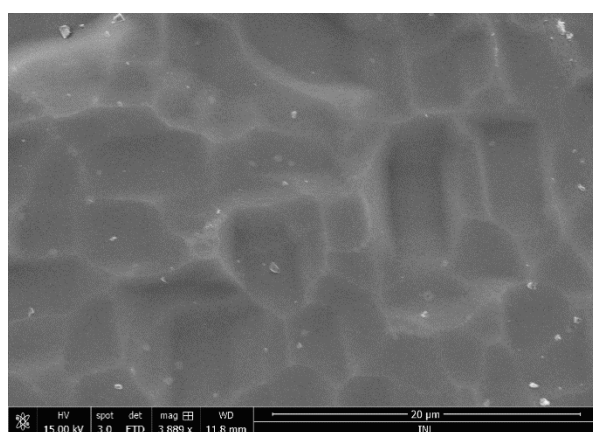
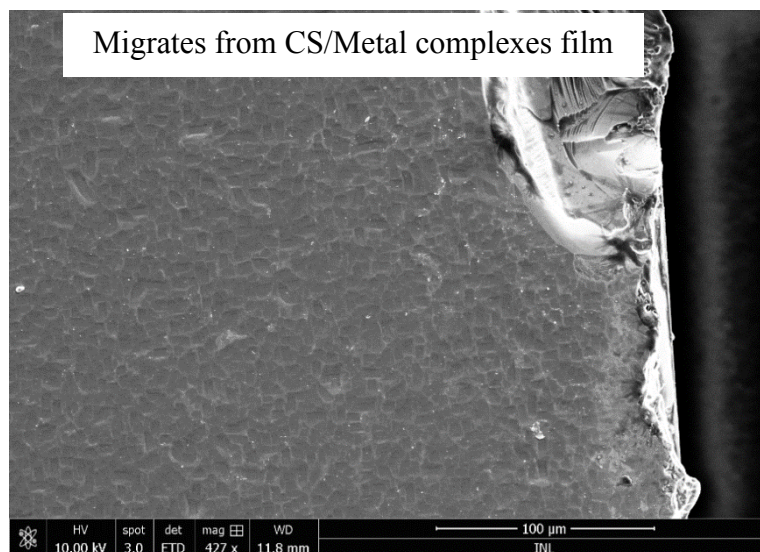
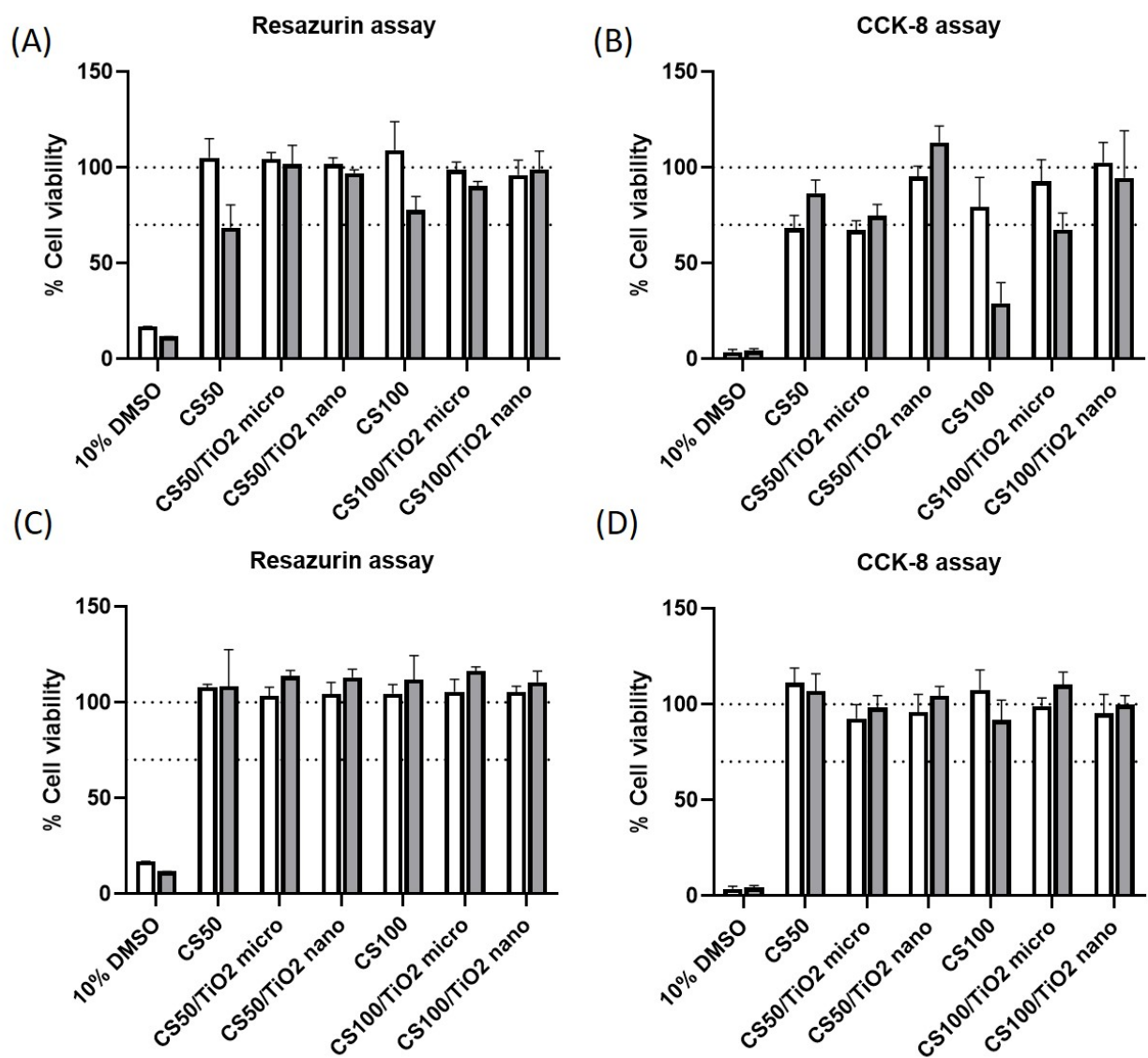


Fig. 7



Figs. 8

Table 1

RSA of CS/Metal complexes films

Sample Code	RSA (%) [*]	TE ^{**}
		($\mu\text{M/g}$)
CS50	25.98 ± 0.7^a	15.0 ± 0.002
CS50/TiO ₂ micro	27.13 ± 0.8^a	14.8 ± 0.002
CS50/TiO ₂ nano	41.51 ± 0.6^a	10.0 ± 0.005
CS100	11.75 ± 3.4^b	18.6 ± 0.008
CS100/TiO ₂ micro	25.48 ± 2.1^a	15.0 ± 0.006
CS100/TiO ₂ nano	34.44 ± 0.6^b	12.7 ± 0.001

*Values given are averages of triplicate samples \pm standard deviations. Average values with different superscript letters differ statistically ($p \leq 0.05$); (a and b: indicate statistically difference among formulations CS50 vs CS100, values followed by the same letter are not statistically different according to Tukey's Multiple Range Test.

**Trolox equivalent.

Table 2

Determination by ICP-MS of the amount of titanium migrated from CS-Metal complexes films into different food simulant after incubation period of 10 days at 40 °C or 10 days at 5 °C, conditions established by European Normative EN 13130-1:2004.

Sample Code	Food simulants			
	95 % (v/v) aqueous ethanol		Olive oil	
	Ti [ng/L]*,**	Ti [ng·dm ⁻²]*	Ti [ng/L]*,**	Ti [ng·dm ⁻²]*
CS50/TiO ₂ nano	220.4±5.9****a	36.7	173.5±1.7 ^a	28.9
CS50/TiO ₂ nano	198.7±4.8 ^a	33.1	163.0±7.8 ^a	27.2
CS50/TiO ₂ nano	200.7±4.5 ^a	33.5	186.1±6.8 ^a	31.0
CS50/TiO₂ nano	206.6±11.9 (Avg.)	34.4±1.9 (Avg.)	174.2±11.5(Avg.)	29.0±1.9 (Avg.)
CS50/TiO ₂ micro	104.0±2.1 ^a	17.3	70.0±4.1 ^a	11.7
CS50/TiO ₂ micro	109.7±1.6 ^a	18.3	78.0±3.5 ^a	13.0
CS50/TiO ₂ micro	99.0±1.0 ^a	16.5	86.3±4.9 ^a	14.4
CS50/TiO₂ micro	104.2±5.3 (Avg.)	17.4±0.9 (Avg.)	78.1±8.1 (Avg.)	13.0±1.3 (Avg.)
CS100/TiO ₂ nano	245.0±3.1 ^b	40.8	312.2±2.0 ^b	52.0
CS100/TiO ₂ nano	239.2±2.3 ^b	39.9	281.6±4.6 ^b	46.9
CS100/TiO ₂ nano	233.0±1.9 ^b	38.8	279.9±4.6 ^b	46.7
CS100/TiO₂ nano	239.1± 6.0 (Avg.)	39.8±1.0 (Avg.)	291.2±18.1 (Avg.)	48.5±3.0 (Avg.)

CS100/TiO ₂ micro	99.8±1.2 ^a	16.6	79.9±1.5 ^a	13.3
CS100/TiO ₂ micro	82.0±1.2 ^a	13.7	80.8±0.5 ^a	13.5
CS100/TiO ₂ micro	98.4±1.6 ^a	16.4	97.3±0.5 ^a	16.2
CS100/TiO₂ micro	93.4±9.8 (Avg.)	15.6±1.6 (Avg.)	86.0±9.7 (Avg.)	14.3±1.6 (Avg.)

*All results were blank subtracted. Values are the means ± standard deviation (n=3).

**Values given are averages of triplicate samples ± standard deviations. Average values with different superscript letters differ statistically ($p \leq 0.05$); (a and b: indicate statistically difference among formulations CS50 vs CS100, values followed by the same letter are not statistically different according to Tukey's Multiple Range Test.

*** Values are the means ± standard deviation of three repetition on the same samples.

Table 3

Determination of titanium content in CS-Metal complexes films by ICP-MS using different digestion methods (i.e., microwave digestion and dry ashing digestion).

Sample Code	Methods	Weight of film	Ti (μg)	
		samples* (g)	Theoretic value	Measured value
CS50/TiO ₂ nano	Microwave digestion	0.0692±0.0001	10175.2±8.4	7998.68±38.8
	Dry ashing	0.0668±0.0002	9817.6±7.9	7637.12±37.07
CS50/TiO ₂ micro	Microwave digestion	0.0666±0.0001	9788.2±14.7	8814.10±51.9
	Dry ashing	0.0664±0.0001	9758.8±14.6	8679.41±51.35
CS100/TiO ₂ nano	Microwave digestion	0.0720±0.0002	10601.4±22.4	8912.68±35.4
	Dry ashing	0.0666±0.0002	9788.2±20.7	8160.65±32.51
CS100/TiO ₂ micro	Microwave digestion	0.0742±0.0001	10919.8±14.6	8870.66±37.9
	Dry ashing	0.0668±0.0001	9817.6±13.1	7876.95±33.80

*Values are the means ± standard deviation (n=3)

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

DEnescu
9 January 2020