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Self-Sterilized EVOH-TiO₂ Nanocomposites: Interface Effects on Biocidal Properties**

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Nanocomposite materials obtained by TiO₂ incorporation into ethylene–vinyl alcohol copolymers, extensively used in food packaging, are prepared via a straightforward melting process. The structural characteristics of the nanocomposites are examined using wide and small angle X-ray scattering (WAXS/SAXS), and vibrational infrared and Raman spectroscopies. A microscopy (SEM/TEM) study shows that the materials obtained are highly homogeneous at the nanometric scale, exhibiting an intimate contact between both the organic and inorganic components. TiO₂ incorporation into this polymer matrix renders self-sterilized nanocomposite materials upon light excitation, which are tested against nine micro-organisms (gram-positive and gram-negative bacteria, cocci, and yeasts) typically involved in food contamination and/or degradation. Overall, the nanocomposites display an impressive performance in the killing of all micro-organisms with a maximum for an oxide content between 2–5 wt %. The measurement of the physico-chemical properties together with the structural characterization of the materials provide conclusive evidence that the nanocomposites biocidal capability born of the nanometric organo-inorganic interface and rationalize the existence of a maximum as a function of the TiO₂ content.

1. Introduction

Hybrid or nanocomposite organic–inorganic materials that combine attractive qualities of dissimilar components have received immense interest for a wide range of mechanical, electronic, magnetic, biological, and optical properties. These

novel materials are not simply physical mixtures (vide supra) but can be broadly defined as complex materials having both organic and inorganic components intimately mixed. The scale of mixing or, in other words, the degree of homogeneity would influence or even command the properties of the nanocomposite solid materials when the components mixture is adequately reached, typically at the nanometer range. The control of the surface/interface interaction between components appears thus of prime importance in the development of advanced materials having novel functionalities, out of those characteristic of the components.^[1]

Modification of food packaging polymeric matrices to prevent growth or reduce adhesion of detrimental microorganisms is a highly desired objective. Hence, there is a significant interest in the development of antimicrobial biomaterials for application in the health and biomedical devices, food, and personal hygiene industries.^[2] Those agents need to combine desirable attributes such as potent bactericidal and fungicidal efficiency, environmental safety, low toxicity, and easy, cost-effective fabrication. Three broad classes of materials are currently used for rendering antimicrobial properties: a) synthetic macromolecules/polymers that mimic naturally occurring bactericidal molecules/peptides; b) microbe-repelling antiadherence polymers; and c) inorganic–organic materials with a slow release of biocides such as heavy metals or oxides (particularly based on silver), small molecule biocides, halogen species, or nitric oxide.^[2,3] Options a) and b) are usually cost ineffective for a general application while option c) requires the release of an agent potentially toxic for humans and the environment, and typically with weaknesses in the attack to certain class

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(gram-negative or positive bacteria, virus and fungus) or to specific microorganisms.

TiO₂ is an inert, non-toxic and cheap material whose use does not require any release from a nanocomposite material but has potential activity against all kind of microbes. We stress here the well known non-toxicity of the oxide (even in nanometric scale) for human related applications which allows using it as an additive in cosmetics, pills or tooth paste.^[4] Additionally, TiO₂ is able to eliminate (within an extended period of time) dead cells rendering CO₂, opening in this way a path for the auto-regeneration of the system.^[2a,5] All these together characteristics lead to a safer, cost-effective technology of universal application as an alternative to current inorganic- and organic-based biocidal agents. Under light excitation with energy above the TiO₂ band gap (3.2 eV), the formation of energy rich electron-hole pairs is produced. Once at the surface of the material, such charge carriers are able to interact with microorganisms rendering biocidal properties to the TiO₂-containing nanocomposite material. A point of relevance is the control of the TiO₂ polymorphism (e.g., to ensure the presence of the Anatase phase) and primary particle size (high surface materials would lead to high chemical activity as well as stabilizing the correct polymorph) of the inorganic nanoparticles, as this would yield a correct handling of the UV-light matter interaction (excitation and de-excitation) and would ultimately lead to an optimized photo-activity in the elimination of micro-organisms.^[6] This way of reasoning implies that the inorganic oxide component used here must be prepared in a previous step, before the synthesis of the nanocomposite. Besides that, optical properties are strongly dependent on the length scale of compositional inhomogeneities within the material as they influence light scattering if their characteristic size is in the range of photon wavelengths.^[7] This last point obviously adds further complexity to the system under study and emphasizes the requirement for adequate synthesis methods.

In this work ethylene-vinyl alcohol copolymer (EVOH)-TiO₂ nanoparticled composites are described for their application in the food packaging industry.^[2a,b] A straightforward, cost-effective method was devised to introduce different contents of the inorganic solid into the EVOH polymeric matrix. After ultrasonic dispersion of TiO₂, the nanocomposites were prepared through a melt compounding process without incorporation of coupling agents. Although conceptually simple, the ultrasonic dispersion of the oxide is good enough to yield nanocomposite systems having a rather good homogeneity up to a 5 wt % loading of TiO₂. Note that, as detailed below, the resulting preparation favors the oxide dispersion,^[8a,b] and may display significant advantages over other solid-state methods as ball milling which usually produces inhomogeneities in the polymeric matrix (e.g., molecular weight segregation),^[8c] or over inherently expensive preparation methods using chemical modification of the organic and/or inorganic components (e.g., coupling agents) and/or liquid-phase (solution processing) or in-situ polymerization.^[8c,d] A multi-technique approach will be used here to characterize the

state of the components as well as the whole nanocomposite materials and to study their main physicochemical characteristics. Finally, an extensive screening will be made on biocidal properties of the systems regarding common microorganisms involved in food spoilage and poisoning and, consequently, feasible causal agents of infections in humans. The battery of tests concerns gram-positive and gram-negative bacteria (*Escherichia coli*, *Erwinia caratovora*, and *Pseudomonas fluorescens*), gram-positive cocci (*Staphylococcus aureus*), and yeasts (*Zygosaccharomyces rouxii* and *Pichia jadini*). In this way, we intend to give a clear idea about the biocidal potential of our nanocomposite system.^[9]

2. Results and Discussion

2.1. Characterization of the Nanocomposite Materials

Figure 1 displays the DRIFT spectra of several nanocomposite and EVOH-reference systems. The presence of TiO₂ in the solid films is confirmed by the skeletal vibration observed around 650–750 cm⁻¹.^[10a] The spectra show additional peaks attributable to the EVOH component as evidenced by comparison with the single organic component and which have been ascribed elsewhere.^[10b–d] For our purposes, the main point is that the inclusion of the oxide component mainly disturbs OH-related frequencies (1430 cm⁻¹) indicating, as expected, that it interacts preferentially with the hydrophilic (OH groups) part of the organic component.

The EVOH copolymer is semicrystalline and WAXS experiments were performed to determine the influence of TiO₂ nanoparticle incorporation on the crystal lattice developed in the different nanocomposites. Similar WAXS profiles are found at room temperature for all Ti_x samples and the EVOH polymeric component as depicted in Figure 2. Therefore, all of these Ti_x quenched specimens show an orthorhombic lattice, without noticeable change with respect to that exhibited by the neat polymeric component. In addition

Figure 1. DRIFT spectra of the EVOH component and selected Ti_x nanocomposites.

Figure 2. WAXS profiles of the different Tix nanocomposite materials. Diffraction peaks are labeled by their *hkl* parameters.

to the rather constant 101 and 200 diffraction peaks characteristic of the EVOH orthorhombic cell,^[11] the 101 reflection ascribed to the TiO₂ anatase polymorph (centered at ca. 25°; JCPDS-84-1286) is seen in the nanocomposites and its intensity increases as the TiO₂ content does. The crystallinity degree of the different nanocomposites can be estimated from these WAXS patterns by their decomposition into the crystalline diffractions and the amorphous component. The amorphous peak of the different samples was found to be centered at $2\theta = 19.9^\circ \pm 0.2^\circ$, the upper limit for the EVOH reference and the lower one for Ti13 sample. The results reported in Table 1 point out that crystallinity does not practically change by the introduction of the TiO₂ nanoparticles. Regarding the long spacing also estimated at ambient temperature from the Lorentz-corrected SAXS profiles, it has to be said that a slight increase is observed as the TiO₂ content

Table 1. Main physicochemical characteristics of the organic component of Tix nanocomposites and for the EVOH copolymer reference: T_g : glass transition temperature; T_m : melting temperature; ΔH : melting enthalpy (thermal data obtained by DSC), f_c^{WAXS} : crystallinity degree determined by WAXS, L : long spacing estimated by SAXS, and l_c : most probable crystal size.

Sample	T_g [°C]	T_m [°C]	ΔH_m [J g ⁻¹]	f_c^{WAXS}	L [nm]	l_c [nm]
EVOH	47	190	88	0.39	13.7	5.4
Ti0.25	47	190	83	0.39	14.6	5.7
Ti0.50	47	189	86	0.41	15.3	6.3
Ti1	47	189	80	0.41	16.2	6.6
Ti2	47	189	84	0.39	17.0	6.6
Ti5	47	188	98	0.40	17.4	7.0
Ti10	47	190	98	0.37	17.3	6.4
Ti13	47	188	97	0.38	17.6	6.7

Standard errors ($\pm$): 1 °C for T_g and T_m ; 4 J g⁻¹ for ΔH_m ; 7% for f_c^{WAXS} ; 0.5 nm for L and l_c .

raises (see Table 1). Consequently, the most probable crystal size corresponding to the polymeric matrix, determined from the values of long spacing and crystallinity assuming a two-phase model, increases with nanoparticle content.

A parallel study of the TiO₂ state on the nanocomposite materials was performed using Raman spectroscopy (see Supporting Information Fig. S1). Only anatase peaks at ca. 145, 395, 515, and 640 cm⁻¹ were discerned over a background dominated by fluorescence effects but here we will focus our attention on the lowest energy, larger intensity E_g mode.^[12] The analysis of this peak intensity, position and width is presented in Figure 3. While a non-linear behavior of the intensity with the inorganic content of the nanocomposite is observed, both the position and width observables appear

Figure 3. Raman detail of the anatase E_g mode signal, intensity, position and width as a function of the TiO₂ content of the Tix nanocomposite materials.

constant within error throughout the set of samples giving a detectable anatase-related Raman signal. Thus, as judged by the invariance of the E_g mode position and width, no change in the oxide component primary morphology (e.g., primary particle size and shape) is detected through the preparation procedure. The non-linear behavior of the E_g peak intensity suggests strong light scattering differences among samples, potentially related to changes on the morphological properties of the nanocomposite solid films. To further confirm this point and analyze homogeneity within the nanocomposites, SEM pictures were obtained. Figure 4 primarily provides evidence of the high degree of the oxide dispersion within the polymeric matrix. Absence of detectable inorganic domains across the film is noticed for samples up to a 2 wt % of TiO₂ (micrographs *a* to *e*). In the case of specimens having TiO₂ contents equal or above 5 wt %, the SEM pictures point out the existence of two types of zones: one with absence of oxide agglomerates and another with presence of those aggregates, having characteristic sizes ranged from 1 to 3 μ m. Zones free of oxide agglomerates were dominant (90–70%) in the case of Ti5 and Ti10 samples but clustering of agglomerates at a higher content is exemplified in micrograph *h* of Figure 4 for the Ti13 sample. Further structural details of the organic-inorganic interaction are provided by the TEM study of the Ti2 sample (Fig. 5). In this case, the oxide is dispersed in the polymer matrix exhibiting nanometer-scale aggregates ranging from 10 to 200 nm, with an average size (Feret diameter)^[13] of 90 nm (± 20 nm). Considering that our preparation makes use of an oxide previously calcined at high temperature to ensure the exclusive presence of anatase and the strict control of its biocidal capabilities, the nanometric dispersion of the oxide reached for loadings below 2 wt % is significant, particularly with respect to micro-sized or bulk oxide specimens.^[1,2a,8] Improved dispersion of the oxide can be obtained by using low temperature routes which does not require a calcination step to synthesize the oxide but this at the cost of a limited phase control (e.g., presence of titania amorphous phases).^[14]

2.2. Physicochemical Properties

The thermal behavior of neat EVOH and nanocomposite materials was evaluated using DSC (see Fig. 6 and Table 1). The values of glass transition and melting temperatures (T_g and T_m , respectively) do not differ significantly from each other, indicating a slight effect of TiO₂ nanoparticles on the transitions related to the amorphous and crystalline organic-component phases. Note however that the overlapping of the glass transition with an enthalpic relaxation process complicates the analysis of the first observable.

Microhardness, MH, measures primarily the resistance of the material to plastic deformation and also involves a complex combination of other bulk mechanical properties as elastic modulus, yield strength, strain hardening and toughness.^[15] Figure 7 shows the MH dependence in the Tix nanocomposites on oxide amount added. A significant MH increase is observed

as the TiO₂ weight fraction is raised in the nanocomposite materials. In fact, the contribution of TiO₂ nanoparticles, which are much harder than the polymer, is noticeable even at incorporations as low as 0.25 and 0.50 wt %. This behavior can be ascribed to the favorable interfacial characteristics reached during the preparation protocol used. On the other hand, taking apart the step-like growth observed for the initial presence of the oxide (0.25 wt %), the MH displayed a linear increase along with the TiO₂ composition in the whole range scanned (Fig. 7), which could be related to both the thicker polymer crystallites (e.g., l_c parameter in Table 1) obtained as the oxide content grows and, particularly, to the nanoparticle reinforcement effect. As shown by IR (Fig. 1), the latter comes out from the fact that TiO₂ nanoparticles OH-groups act as crosslinking sites via hydrogen bonding with the organic component.

Optical properties are rather important considering the need of efficient light absorption by TiO₂ to become a biocidal material. The absorption spectra of the EVOH single component and Tix nanocomposite films are displayed in Figure 8. The spectrum of the TiO₂ component has been reported elsewhere and is characterized by a band gap of 3.1 eV (ca. 380 nm).^[6b,16] The EVOH polymeric component would show a typical insulator behavior with a band gap of ca. 200 nm, but also displayed localized absorption transitions at ca. 220 and 280 nm ascribed to chromophores existing in the antioxidant additives incorporated because of its industrial nature. Incorporation of the inorganic component to the polymeric matrix produces important changes in the absorption profile. While the characteristic shape of the EVOH absorption spectrum is retained for Tix solid films containing up to a 2 wt % of oxide, a significant shape distortion is detected for samples with higher oxide loadings. Below Ti2, there is however an important intensity enhancement for wavelengths above ca. 350 nm. On the other hand and similarly to previous results,^[14] the anatase band gap is clearly visible on top of a very high background in Ti5, Ti10, and Ti13 nanocomposite films. Presence of the inorganic component has thus two effects; enhances the intensity of the signal in the UV-A and visible ranges and shows the characteristic oxide band gap feature for samples having a Ti content above 5 wt %. Light interaction with the nanocomposite materials would thus display a complex behavior, with an enhancement of the absorption spectrum which is not easily ascribed to any of the components (which do not absorb in the visible range). This will be further studied with the analysis of the de-excitation phenomena.

Photoluminescence (PL) spectra obtained using two excitation energies (marked as dotted vertical lines in Fig. 8) were thus used to further progress in the interpretation of the optical properties of the nanocomposite systems. Figure 9 shows the PL spectra of samples excited with UV-light (333 + 365 nm). Figure 10 depicts the intensity behavior of the PL intensity through the series using UV (333 + 365 nm) and visible (457 nm) laser excitations. Note that in the first case both the EVOH and TiO₂ components can be excited while in the second none of them should be excited. The spectra reported in

Figure 4. SEM micrographs showing cross-section views of (vacuum cleaved) Tix nanocomposites: a) neat EVOH; b) Ti0.25; c) Ti0.50; d) Ti1; e) Ti2; f) Ti5; g) Ti10; and h) Ti13. Micrographs marked as f1, g1, and h1 display small agglomerates at isolated zones found in the corresponding films.

Figure 5. TEM image of the Ti2 nanocomposite (top) and detail showing a nanometer-scale oxide agglomerate (bottom).

Figure 9 display a single, broad peak centered at 420–430 nm echoing that of the EVOH parent component. As TiO₂ is an indirect band gap semiconductor, band-edge luminescence is very difficult to observe due to the low emission probabilities of indirect transitions.^[6b,17b] This means that the dominance of the EVOH component in the photoluminescence spectrum is by no means a surprise. The analysis of the intensity of the

Figure 6. Normalized heating run DSC traces of the different Tix nanocomposites.

Figure 7. Microhardness values versus TiO₂ content of the Tix nanocomposite materials.

organic-like transition is nevertheless rather informative. As can be seen in Figure 10 (top), a growth of the PL intensity is observed for samples having oxide content up to 0.50 wt %, while the intensity underwent a strong decay afterwards. Tix films having an inorganic content above 2 wt % display lower PL intensity than the EVOH single component. The whole intensity behavior shows the existence of energy/charge transfer(s) through the polymer/oxide interface, evidencing again the intimate contact between these two components. For samples with TiO₂ content around/above 2 wt %, the strong decay of the signal would indicate, according to the band gap and position of the components, a potential exciton (from the organic component) and/or hole (from the inorganic component) transfer(s), followed by an efficient charge (electron and hole) separation, leaving in this way holes into organic-like electronic states while electron could be preferentially located into oxide-like states.^[17b,c] This would appear as a key information to interpret the biocidal behavior of the nanocomposite films. The PL signal observed under visible light excitation gives some clues as to allow a definitive interpretation of the visible absorption features appearing in the corresponding spectra of the nanocomposite films (Fig. 8). While a growing light scattering phenomenon (with the inorganic component content) may be envisaged to contribute to the strong UV-A and visible absorption increase observed for the Tix samples in Figure 8 with respect to the parent EVOH/TiO₂ systems, the PL intensity showed in Figure 10 (bottom) provides conclusive evidence for the presence of true, new electronic states unequivocally ascribable to the nanocomposite system. Note that the intensity of the organic/inorganic components is close to zero and reaches very high values for the Tix nanocomposite films. It thus appears that these new states, not present in the parent systems, suffer a strong charge recombination upon de-excitation for Ti0.25 to Ti1 films, moderating this behavior for Ti2 and Ti5 samples. To conclude, the physical contact of the organic and inorganic components produces potential beneficial effects in terms of charge separation (for Tix films with $x \geq 2\%$) and the presence of novel electronic states allowing visible light absorption.

Figure 8. UV-visible absorption spectra of the EVOH component and Tix nanocomposite materials.

2.3. Biocidal Properties

To analyze the biocidal properties of the nanocomposite systems the performance of the EVOH reference as a control sample and the Ti0.5, Ti2, and T5 films were tested. Experiments measuring the UV-alone effect on the microorganism survival ratios were also run. Nine different microorganisms including gram-positive and negative bacteria, cocci, and yeasts were employed. Representative examples of gram-positive bacteria (*B. stearothermophilus*) and cocci (*S. aureus*), gram-negative bacteria (*E. coli*), together with one yeast (*Z. rouxii*) are presented in Figure 11. Figure S2 (Supporting Information) gives the corresponding plots for the other five microorganisms. Table 2 summarizes the most interesting information derived from the biocidal tests and includes the cell inactivation initial rates and the (cell inactivation) log-reduction values obtained by the end of the experiment, after 30 minutes of continuous irradiation. As mentioned in the introduction, the survey of nine microorganisms intends to establish the potential of our nanocomposite films against pathogens involved in the contamination and/or degradation of packaged foods.^[2,9] We note that our systems were also tested

Figure 10. Intensity of the Luminescence spectra for the EVOH component and Tix nanocomposite materials obtained upon UV-A (333 + 365 nm; top) and visible (457 nm; bottom) excitations.

against pathogens related to hospital infections (*P. aeruginosa* and *Enterococcus faecalis*).^[18] On such work we also tested the auto-degradability potential of the nanocomposites and confirmed the absence of significant degradation within the commercial lifetime of a food packaging polymer.

A first general point to stress out is that both control experiments (e.g., UV-alone and UV + EVOH) produce only moderate effects on both the cell inactivation initial rate and the total log-reduction, as can be expected from the sub-lethal energy fluences employed through the experiment.^[19] Taking into account the data reported in Table 2, more than 1 h should be required for all microorganisms to allow a 1 log-reduction in the combined presence of UV-light and the EVOH component. The presence of TiO₂ extremely affects the biokilling action of the nanocomposite materials. While a moderate enhancement between 2 and 4 times is observed in the initial rate, by the end of the experiment we typically detected ca. 5 times log-reduction enhancement with respect to the control experiment mentioned. As a result we ended with a 4–5 log-reduction (e.g., 99.99 to 99.999 % killing). This killing level is commonly understood as being bactericidal and, as detailed elsewhere, is sufficient to maintain a good (human health) safety control, helping in eliminating the need for sterilization or other aggressive treatment of foods.^[20] Concerning the

Figure 9. Luminescence spectra of the EVOH component and Tix nanocomposites. Excitation line 333 + 365 nm (see text for details).

Figure 11. Process come-up time logarithmic reduction of microorganism population suspended in appropriate liquid media according of the microorganisms tested. Survival curves for four microorganisms as a function of the irradiation time for Ti_x and EVOH control samples.

optimum TiO₂ loading of the nanocomposites, Table 2 gives evidence of the optimum Ti2–Ti5 performance. Thus, whereas two of the nine microbes tested was slightly more affected by Ti2 (*S. aureus*, *P. fluorescens*), Ti5 maximizes the biokilling activity for four of them, and three were rather similar (*Z. rouxii*, *E. carotovora*, and *Bacillus sp.*). As differences among them are within experimental error, this would mean that the optimum oxide loading is in between 2 and 5 wt %. The maximization of the biokilling action is somehow parallel to the morphological behavior as a function of the TiO₂ content evidenced by the SEM study reported in Figure 4. The physical bases underlying the morphological-biocidal properties link rely on the correct handling of the matter–light interaction. The efficient charge carrier separation achieved upon the intimate mixing of the polymer and oxide components for samples having a TiO₂ content above 2 wt % is of particular

importance. As discussed, holes are preferentially located in the organic component after charge carrier separation, being thus able to interact with micro-organisms without the need of a direct oxide–biological contact. This would boost photo-activity for samples above this (2 wt %) cut-off point. The maximum of biocidal potential which, according to our study, will be obtained for a point between 2 and 5 wt %, could be thus additionally related to significant differences among samples in terms of the available oxide surface area by effect of changes in the oxide secondary particle size (e.g. agglomeration) within the polymeric matrix. The trade off between the available oxide surface area and the enhanced photo-activity induced by an optimized charge carrier separation appears as the key feature in optimizing the activity of our nanocomposite materials. Note, on the other hand, that both (“opposite”) physical properties are driven by the organo–inorganic inter-

Table 2. Cell inactivation initial rate (10^{-1} min^{-1}) and maximum log-reduction obtained in the experiments displayed in Figure 10.

Micro-organism	Control: EVOH	TiO ₂ 0.50	Ti2	Ti5
<i>B. stearothermophilus</i>	0.59(0.04)/0.8	0.96(0.09)/1.8	1.08(0.06)/3.2	1.18(0.08)/>5
<i>S. aureus</i>	0.59(0.09)/0.7	0.83(0.09)/2.2	1.40(0.03)/3.9	1.2(0.1)/3.4
<i>E. coli</i>	0.66(0.02)/0.7	0.90(0.04)/1.4	1.07(0.05)/3.5	1.07(0.08)/4.0
<i>Z. rouxii</i>	0.70(0.06)/0.8	0.9(0.1)/3.9	0.99(0.06)/3.9	0.97(0.08)/4.0
<i>Bacillus sp.</i>	0.65(0.09)/0.7	1.40(0.04)/3.1	2.0(0.1)/>5	2.0(0.1)/>5
<i>L. platatum</i>	0.55(0.04)/0.5	1.10(0.03)/1.4	1.00(0.07)/3.2	1.00(0.04)/>5
<i>E. caratovora</i>	0.58(0.09)/0.6	0.83(0.09)/3.1	1.15(0.08)/2.8	1.20(0.06)/2.9
<i>P. jadinii</i>	0.51(0.04)/0.9	1.00(0.03)/2.7	1.10(0.06)/4.0	1.10(0.06)/>5
<i>P. fluorescens</i>	0.60(0.06)/0.4	0.82(0.07)/1.8	1.00(0.08)/4.0	0.80(0.09)/3.8

Standard error in the initial rate is given in parenthesis. Standard error in the log-reduction: ± 0.2 units.

face and born of the nanometric scale contact between the two components.

Comparison with previous results concerning the efficiency of inorganic/organic biocidal agents is easy in the case of the *E. coli*^[21] and *S. aureus*^[22] microorganisms. There is also an important number of works devoted to both pathogens,^[23] and *P. fluorescens*,^[24] but very few detailed the case of *B. stearothermophilus*,^[22b,25] *E. caratovora*,^[26] and *Z. rouxii*.^[27] To the best of our knowledge, there are not available reports concerning the other three microorganisms that are here studied by the first time in the context of their control and killing. To made appropriate comparison of biocidal properties, we must consider at least three points: the first one relates to the rather different excitation energies and/or fluences (power $\times$ time) used in experiments with titania as both influence significantly the results. The second concerns the state and nature of the biocidal agent; while certain biocidal agents as Ag are typically release to the media, others like TiO₂ works by surface/near-surface contact and display significant variability in efficiency while used as powders or are immobilized in a support. As well known, as a powder it may present typically a 1-fold order of magnitude higher activity (for example, in the case of *E. coli*)^[28a] due to the fact that nanoparticles in suspension/powders can be ingested by microorganisms by phagocytosis, causing rapid cellular damage in addition to that caused by photo-activity.^[21a] The third point is specific for the measurements of cell inactivation reaction rates as they show a linear relationship with the initial bacteria concentration, a fact that must be taken into account when comparing results.^[28b]

E. coli control and elimination have received a lot of attention as a general model system, with reports using TiO₂ powders,^[21a–e,23a] immobilized/supported TiO₂ on polymers (acetate; polystyrene, Plexiglas),^[21f,23b,c,28a] or Ag^[21g,23e,f] biocidal agents. Cell inactivation rates have been measured only with TiO₂ powders and were in the 0.11–0.29 min^{−1} interval.^[21a,b] Considering that our films contains a “immobilized” TiO₂ oxide, the maximum 0.11 min^{−1} rate here observed favorably compares with those obtained by using the biocidal in suspension. Our maximum log-reduction (4.0) obtained after 0.5 h of treatment further confirms the goodness of the

nanocomposite films. Measurements using immobilized/supported TiO₂ systems give values of ca. 5.4/2/1 log-reduction for 1.5/0.5/1 hour(s) of treatment.^[21f,23b,c] To end, the comparison can be extended to Ag-based biocidal agents; while a general higher activity is observed for our system we can made a direct, useful comparison with the immobilized AgION[®] on stainless steel, which showed a 5.6 log-reduction after 4 hours of contact.^[23f] Overall, the situation is repeated when we consider the killing of *S. aureus*. Literature reports mentioned the use of TiO₂ powders,^[23a] immobilized/supported TiO₂ on polymers (polystyrene, Plexiglas),^[23b,c] and Ag^[22a,23e,f] systems but also the use of negative air ions generated with the help of electrical fields.^[22b] None of them display comparable results in terms of efficiency or time response, being this particularly true for the immobilized TiO₂ systems. An additional and illustrative comparison comes from the data reported by the use of different antibiotics/biocidals coating the EVOH polymer.^[23g] Only the most powerful antibiotic, gatifloxacin, gives comparable results in terms of log-reduction but for a certainly extended period of times (24 hours), derived in the case of the antibiotic for its slow release from the polymer matrix.

To finish with the available data we must refer to the earlier studies on the elimination of *P. fluorescens*,^[24] *B. stearothermophilus*,^[22b,25] *E. caratovira*,^[26] and *Z. rouxii*.^[27] To be brief, the use of TiO₂-based biocidals^[24a,26a] display comparable results to those reported here (ca. 4 log-reduction/30 min), although the titania P25 system supported in a cellulose acetate membrane showed a higher rate (0.22 vs. 0.10 min^{−1}), measured, however, in favorable conditions (e.g., higher bacteria concentration 10⁷–10⁸ vs. 10⁵ cfu ml^{−1}).^[24a] Other interesting papers concern the use of chemicals (acids, ozone, chlorine, H₂O₂, ethanol) and/or UV-alone light but do not show comparable efficiency expect in the case of Minicare[™] (hydrogen peroxide + peracetic acid) for *P. fluorescens*,^[24c] which allow obtaining a 4 log-reduction after 20 min of treatment while here we use 30 min.

We can thus conclude that the whole set of data above analyzed is illustrative of the biocidal potential of our nanocomposite films. Overall, our systems can be satisfactorily compared with the most powerful biocidal agents reported up to date, irrespective of their nature and way of killing (contact,

release, etc.). Stress can be put in the fact that our system is highly active against both gram-positive and negative bacteria and cocci but also higher eukaryotic microorganisms such as yeasts.

3. Conclusions

In summary, self-sterilized polymeric nanocomposites have been developed by a straightforward preparation protocol that allows incorporation of nanoparticle TiO₂ within a polymeric EVOH matrix. A complete structural and electronic characterization of the EVOH–TiO₂ nanomaterials gave evidence of an intimate, nanometric contact between the polymer and oxide components. This aspect holds responsible of the exceptional antimicrobial properties exhibited by these novel nanocomposite materials against gram-positive and gram-negative bacteria, cocci and yeasts, which favorably compare and typically outperform all known biocide agents. Optimization of the antimicrobial properties of the nanocomposites films is achieved with a TiO₂ loading between 2–5 wt %. This optimum appears as a consequence of the trade off between the loss of interface contact derived from the increasing aggregation state of the oxide component as the inorganic content grows up to 5 wt % and the enhanced photo-activity presented by samples having a loading above 2 wt % and induced by the optimized charge carrier separation produced at the organic-inorganic interface upon light absorption.

The exceptional antimicrobial characteristics together with the invariable thermal behavior and the improved mechanical response, even exhibited at small contents of nanoparticles, makes the EVOH–TiO₂ nanocomposites a feasible and promising material for biomedical devices and food packaging.

4. Experimental

Sample Preparation: The TiO₂ component was prepared using a microemulsion synthetic route by addition of titanium(IV) isopropoxide (Aldrich) to an inverse emulsion containing an aqueous phase dispersed in *n*-heptane (Scharlau), using Triton X-100 (Aldrich) as surfactant and 1-hexanol (Aldrich) as cosurfactant. The resulting mixture was stirred for 24 h, centrifuged, decanted, rinsed under stirring five consecutive times with methanol (2), water (2) and acetone (1), in order to eliminate any portion from the organic and surfactant media, dried at 110 °C for 24 h and calcined at 500 °C for 2 h [16]. A commercially available ethylene-vinyl alcohol copolymer (EVOH; Solvay), containing a nominal 71 mol % vinyl alcohol content, was used as polymeric matrix in the preparation of EVOH–TiO₂ nanocomposites with different TiO₂ nano-particle contents: 0.25, 0.5, 1, 2, 5, 10, and 13 wt %. These novel materials were prepared through melt processing at 195 °C and at 60 rpm for 5 min in an internal mixer with a volumetric capacity of 3 cm³, prototype built in our machine shop scaling from a commercial Haake internal mixer. Previous to this, the nanoparticles and EVOH copolymer were subdued to ultrasonic (Sonics VC505) and stirring cycles to homogenize the batch. After blending and homogenization of these two components, specimens were obtained as films by compression molding in a Collin press between hot plates (210 °C) at a pressure of 1.5 MPa for 5 min. A quench was applied to the different films from the melt to room

temperature. Materials are labeled as EVOH/TiO₂ for the single component solids or Tix for the nanocomposite materials, where x described the above mentioned weight percent of the inorganic component in the nanocomposite material. Note that the EVOH reference hereafter used is surrendered to the same preparation steps suffered by the nanocomposite materials.

Strains and Bacterial Cultures: The microorganisms used in this study include *B. stearothermophilus* CECT47, *Bacillus* sp. CECT40, *L. plantarum* CECT 220, *E. coli* CECT 101, *E. caratovora* CECT 225T, *P. fluorescens* CECT 378, *S. aureus* CECT 240, *Z. rouxii* CECT 1232, and *P. jadini* CECT 1062. Microorganisms were maintained according the recommendations of the manual of the Spanish Type Culture Collection (CECT; <http://www.uv.es/cect/>). Cells were streaked from a glycerol stock onto an appropriate agar Plate [29], grown overnight at 28 °C (*Bacillus* sp., *P. fluorescens*, *E. caratovora*, *P. jadini* and *Z. rouxii*), 37 °C (*S. aureus*, *E. coli* and *L. plantarum*) and 45 °C (*B. stearothermophilus*), and subsequently used for photochemical cell viability assays.

Photochemical Viability Assays: To study the anti-microbial activity of the TiO₂ component, two suspensions were prepared. The first suspension was an aqueous solution of the material at 1 mg ml^{−1} using sterilized water. The second contain 10 μl of microbial cells (ca. 10⁵ cfu ml^{−1}) suspended in 1 ml broth solution. Grow cultures were prepared as described elsewhere [29]. Aliquots of 1 ml from both suspensions were added to a 4 ml quartz cubic cell containing 1 ml of sterilized water. The oxide-cell slurry was placed in the UV spectrometer chamber (UVIKON 930) and irradiated with a UV light of 280 nm for different periods of time. A sub-lethal, maximum radiation energy fluence of ca. 1 kJ m^{−2} was used throughout the study. After irradiation and for different time intervals, aliquots of 100 μl were transferred to a 10 ml LB broth test tube. The order of cell dilution at this stage was 10^{−2}. Loss of viability after each exposure time was determined by the viable count procedure on LB agar plates after serial dilution (10^{−2} to 10^{−5}). All plates were incubated at 28 °C (*Bacillus* sp., *P. fluorescens*, *E. caratovora*, *P. jadini*; and *Z. rouxii*), 37 °C (*S. aureus*, *E. coli*, and *L. plantarum*) and 45 °C (*B. stearothermophilus*) for 24 h prior to enumeration. A similar scheme was used when the nanocomposites or the control materials were measured. A minimum of three experimental runs were carried out to determine antimicrobial activity.

Characterization techniques: Diffuse reflectance Infrared Fourier Transform Spectra (DRIFTS) were taken in a Bruker Equinox 55 FTIR spectrometer fitted with an MCT detector. Absorption spectra were recorded in transmission with a Cary 4000 from Varian. Raman and photoluminescence measurements were performed at room temperature with different laser lines of an Ar⁺–Kr⁺ laser: 333 nm + 363 nm, 457 nm; and 514 nm. A home-made micro-Raman system was utilized; it consists on a Jobin-Yvon HR 460 monochromator, a N₂ cooled CCD and a Kaiser Super-Notch-Plus filters (the latter used to suppress the elastic scattered light at 514 nm or 457 nm). The excitation light was focused on the sample with an Olympus microscope (except for 333 and 365 nm UV laser lines) which is also used to collect the scattered light. Spectra are corrected by the instrumental function recorded with a calibrated white source and a CaF₂ pellet.

Scanning electron microscopy experiments for cross-section material analysis were carried out at room temperature in a XL30 ESEM PHILIPS equipment working at 25 KV. Samples were *in-situ* cryofractured prior observations of the film cross section. The samples were coated with gold-palladium (80:20) with a Sputter Coater (Polaron SC7640) working at 800 V and 5 mA. Transmission electron microscopy experiments were carried out at room temperature in a 200 Kv JEM-2000 FX JEOL microscope. Samples were embedded in Spurr resine (low viscosity epoxi, cured at 60 °C for 48 h) to obtain parallel cuts of the films surface in thin (80 μm) sections by ultramicrotomy (Reichert-Jung Ultracut E), which were picked up on cooper grids and coated with a thin layer of carbon graphite (MED 010 Balzers evaporator) to improve heat conductivity. The crystal

lattice characteristics of the nanocomposites were examined by wide angle X-ray scattering, WAXS, in the reflection mode at room temperature using a Rigaku Rotaflex RTP300 diffractometer connected to a computer having a Geiger counter and using a Ni-filtered Cu K α radiation. The diffraction scans were collected at a rate of 1° min⁻¹ between 2 θ values from 4 to 43°, using a sampling rate of 1 Hz. The goniometer was calibrated with a standard of silicon. The long spacing determination was performed at room temperature using small angle X ray scattering, SAXS, employing synchrotron radiation (λ = 0.150 nm) at the beamline A2 at HASYLAB (Hamburg, Germany). A MARCCD detector was used for data acquisition, sited at a distance of 265 cm from the sample and calibrated with the different orders of the long spacing of rat-tail cornea (L = 65 nm). Differential scanning calorimetry measurements were performed in a Perkin Elmer DSC/TA7DX, PC series with an Intra-cooler for low temperatures. The temperature scale was calibrated from the melting point of high purity chemicals (lauric and stearic acids and indium). Samples (~10 mg) weighed were scanned at 10 °C min⁻¹ under dry nitrogen (20 cm³ min⁻¹). The first heating scan was performed after decreasing (10 °C min⁻¹) the temperature from room temperature to -20 °C, then the sample was heated up to 210 °C. A Vickers indenter attached to a Leitz microhardness tester was used to perform microindentation measurements undertaken at 23 °C. A contact load of 19.235 N and a contact time of 25 s were employed. Microhardness (MH) values (in MPa) were calculated according to Equation 1 [15]:

$$MH = 2 \sin(68^\circ) \frac{P}{d^2} \quad (1)$$

where P (in N) is the contact load and d (in mm) length of the projected indentation area.

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