COLLAGEN/TiO_2-Ag COMPOSITE NANOMATERIALS FOR ANTIMICROBIAL APPLICATIONS

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Pure TiO_2 nanoparticles or composite materials based on TiO_2 and silver nanoparticles, for instance, are known as exhibiting antimicrobial properties. The main purpose of this work was to synthesize nanoparticles and nanocomposite materials and to incorporate them into an adequate support to obtain cosmetic formulations with sunscreen properties. Collagen was used and it showed that it does not change the antimicrobial activity but increases the biocompatibility as well the skin regeneration. FTIR, XRD, SEM and TEM characterized the nanobiocomposites obtained. The antimicrobial activity was tested against S. aureus. The antibacterial activity is induced by the presence of silver; it is also worth to note that synthesis route and the reducing agent are important.

Keywords: TiO_2 nanoparticles, silver nanoparticles, antimicrobial activity, collagen, cosmetic formulations

1. Introduction

Nowadays, the tendency in technology is to use particles in nano-size range because of their increasing ability to synthesize and manipulate into small materials [1]. So, a nanomaterial has to have at least one dimension that measures

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less than 100 nm and because of that they often exhibit new properties than their bulk material counterparts [2].

Everyday, people are faced to a lot of type of microorganisms such molds, bacteria and viruses. To overcome this issue there has been running tests and researches to develop antimicrobial materials which contain antibiotics as well as nanometric inorganic particles such as TiO_2, Ag and ZnO [3, 4].

One of TiO_2 uses is as a pigment because of its brightness, high refractive index, and resistance to discoloration. Nearly 70% of TiO_2 is used as a pigment in paints, but also used in glasses, plastics, paper, fibres, food, pharmaceuticals, cosmetics and toothpastes. Because of his interesting photocatalytic properties, TiO_2 has been used in decontamination, purification, deodorization of air and wastewater but it has also the ability to kill cancer cells, bacteria and viruses under mild UV illumination [3, 5, 6]. TiO_2 proves to be the most suitable safe and broad-spectrum antimicrobial agent [3] Titanium alloys have many applications because of theirs good mechanical properties, high corrosion resistance, and excellent biocompatibility. It has been used as a biomaterial for bone implants and dental applications [7, 8]. TiO_2 is a well-known semiconductor used in photo catalysis, with high reactivity, good photo stability, low price and no toxicity [9-12]. And because of all these special properties, TiO_2 mesoporous materials became an excellent support material for silver nanoparticles which will be a promising composite material with antimicrobial applications [3, 13].

Another interesting material due to its excellent antibacterial properties is silver, which has applications such drug delivery, molecular imaging of cancer cells, antimicrobial agent that exhibits low toxicity in human [14-17]. Silver nanoparticles have recently come into the spotlight due to their unique optical, electrical, and thermal properties but also strong antibacterial activity and low toxicity on human beings [18, 19]. Silver plays an important role in drug delivery, diagnostics, artificial implants and tissue engineering. New studies have found that silver nanoparticles can now be synthesized from bacteria, fungus and plants [20-23].

Even if TiO_2-Ag system is considered to be the most long lasting antimicrobial agent, it also has disadvantages [24]. TiO_2 exhibits photocatalytic activity under UV irradiation, and because is harmful to humans, the researchers developed antibacterial agents by doping it with noble or transition metal ion like silver [9].

Development of synthetic biomaterials had the explosive need to use them in the form of implants and medical devices, so it has been developed biomaterials with anti-infective properties [25-27]. For instance, natural polymers, such as collagen are known to be a wound healing matrix protein. Clinical acceptance has shown that it is a safe material and can be used to modify

the biomaterial surface, due to the fact that it easily attaches to cells [28, 29,30-33]

Collagen is the most abundant protein and it can be extracted from bovine skin, tendons and bone. Collagen is used in many applications because of its good biocompatibility, low immunogenicity and cell-adhesive properties [34, 35].

In this work, nanocomposite materials were encapsulated in collagen to produce new formulations aimed to protect the skin against UV radiation and regenerate the skin. The synthesis process and structure of TiO_2/ Ag encapsulated in collagen were investigated by FT-IR, XRD, SEM and antibacterial tests.

2. Materials and methods

The TiO_2 photocatalyst was obtained as a result of a sol-gel process, from titanium tetra-isopropoxide as a precursor (Sigma Aldrich, reagent grade). On the samples prepared from titanium tetra-isopropoxide was added silver nitrate and glucose; to obtain a proper pH=8-9 was added sodium hydroxide in the solution. Silver was synthesized by precipitation from silver nitrate. The final solution was filtrated and dried, and then the samples were characterized through FT-IR, X-ray diffraction, scanning electron microscopy and antimicrobial activity. All other chemicals were reagent grade and were used without further purification.

The TiO_2/Ag composite materials were obtained according to Fig. 1. The TiO_2/ Ag powders were prepared from TiO_2, silver nitrate solid and glucose (or ascorbic acid). On this mixture was poured a water-ethanol solution. After stirring for 2h the mixture became a white thick gel. On this gel was added NaOH until the solution reached up to pH=8-9, then it changed into a grey gel, because of the precipitation of the silver nanoparticles which was due to the chemical reduction of Ag^+ to Ag^0 in the presence of glucose or ascorbic acid. The synthesis was conducted in order to obtain 300 ppm Ag NPs. After filtration, the gels were dried for 12h. The obtained solids were grounded and send to analysis.

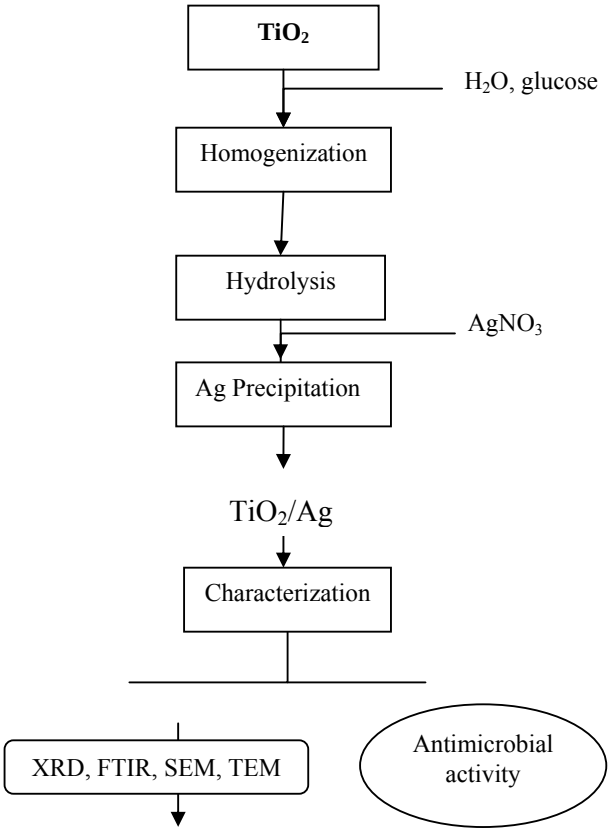

Fig.. 1. Synthesis of TiO_2/ Ag

0.25 g from the TiO_2/Ag composite materials obtained by precipitation with 10 g of type I bovine collagen gel (2.42%) and 13 mL water were mixed through fast stirring. 15 mL of this stable suspension was further crosslinked, freeze dried and used for characterization purposes. 200 µL glutaraldehyde (GA) (0.66%) was added to the 15 mL of obtained sample and mixed until it becomes homogeneous; samples were placed into Petri dishes and kept at 4^0C for 24 h and then subjected to lyophilization process using Martin Christ Alpha 2-4 LSC lyophilizer [17]. The composites were obtained according with the flowchart presented in Fig. 2.

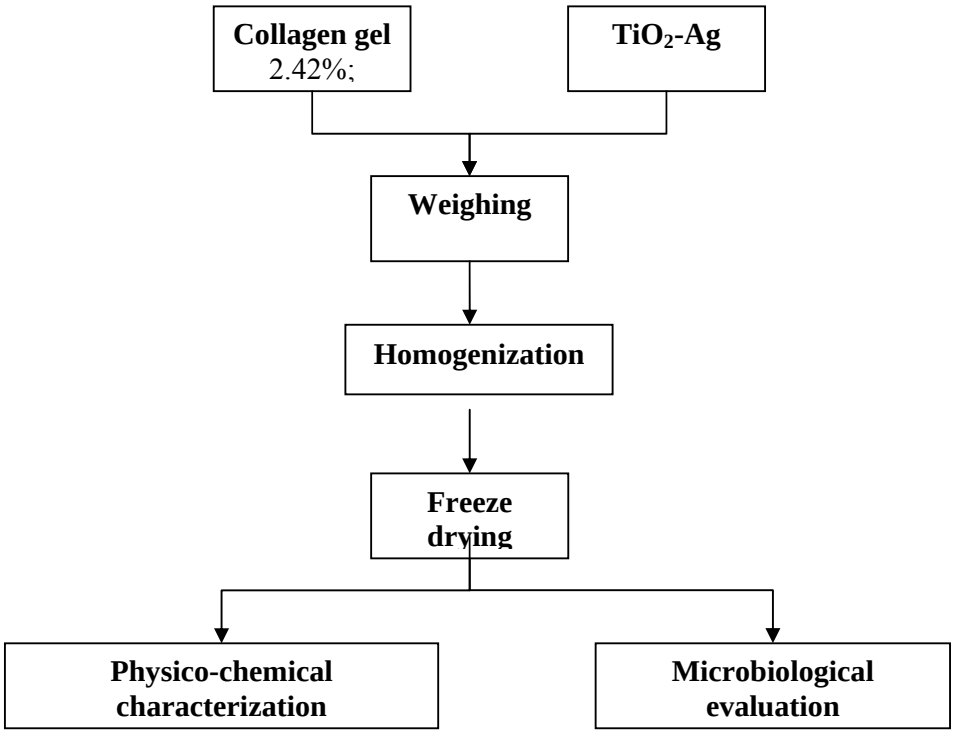

Fig. 2. Schematic representation of Coll/ TiO_2-Ag composite material obtaining

The synthesized Coll/TiO_2-Ag composite materials were investigated by Fourier transform infrared spectroscopy-FTIR, X-ray diffraction-XRD (only the TiO_2-Ag sample), scanning electron microscopy-SEM, transmission electron microscopy-TEM (only the TiO_2-Ag sample), as well as by determining the *in vitro* antimicrobial activity against *S. aureus* [36].

Infrared spectroscopy (IR) measurements were performed on an iN10 MX mid infrared FT-IR microscope operated in transmission, reflection or Ge-ATR mode. The spectra of the Coll/TiO_2-Ag samples were recorded in ATR mode without no sample preparation, over the wavenumber range of 675–4000$cm^{-1}$by co-adding 32 scans with a resolution of 4cm^{-1}, using the cooled MCT detector [36].

X-ray diffraction analysis was performed using a Shimadzu XRD 6000 diffractometer at room temperature. In all the cases, Cu K_α radiation from a Cu X-ray tube (run at 15mA and 30 kV) was used. The powdered samples were scanned in the Bragg angle, 2θ range of 10 – 70^o, with a sampling interval of 0.02^o [36].

SEM images were recorded on a HITACHI S2600N instrument equipped with an EDS probe. Before imaging, all samples were covered with a thin gold layer [36].

For antibacterial assays *S. aureus* ATCC 25923 (Gram (+) bacterium) was grown in Luria Bertani Agar (LBA) plates at 37 °C with following composition: peptone (Merck), 10 g/L; yeast extract (Biolife) 5 g/L, NaCl (Sigma-Aldrich) 5 g/L and agar (Fluka) 20 g/L. We choose *S. aureus* for antibacterial test because it is capable of causing various infections of the skin. *S. aureus* infections are specific to people with frequent skin injury, particularly if the skin is dry [37, 38].

The Kirby-Bauer disk-diffusion method was performed to determine antibacterial activity of the tested samples, the strain used being *S. aureus*. After a predetermined time the inhibition zone was measured (in millimeters) on the agar surface around the samples. In this study, triplicate plates were prepared for each sample. The mean zone of inhibition was calculated with standard deviation procedure; standard deviation was calculated as the square root of variance using STDEV function in Excel 2010 [39, 40].

3. Results and discussion

The main purpose of this paper was to obtain multifunctional materials such as Coll/TiO_2-Ag nanocomposites for using them in sun creams as these creams are designed for protecting skin against UV radiation.

First TiO_2-Ag powder was characterized. The three components of the cream are added to induce certain functionality; collagen assures faster healing, silver assures antimicrobial and antiseptic activity, while TiO_2 was especially added for its UV-protecting role. Desired antimicrobial and UV protective properties can be achieved by varying ratio between Ag and TiO_2, The increasing amount of silver nanoparticles induces stronger antimicrobial activity while the increasing amount of TiO_2 induces increased UV protection.

- X-ray diffraction

X-ray diffraction analysis was used to identification of crystalline phases existing in the multifunctional materials such as Coll/TiO_2-Ag nanocomposites of the TiO_2 nanoparticles (Fig. 3).

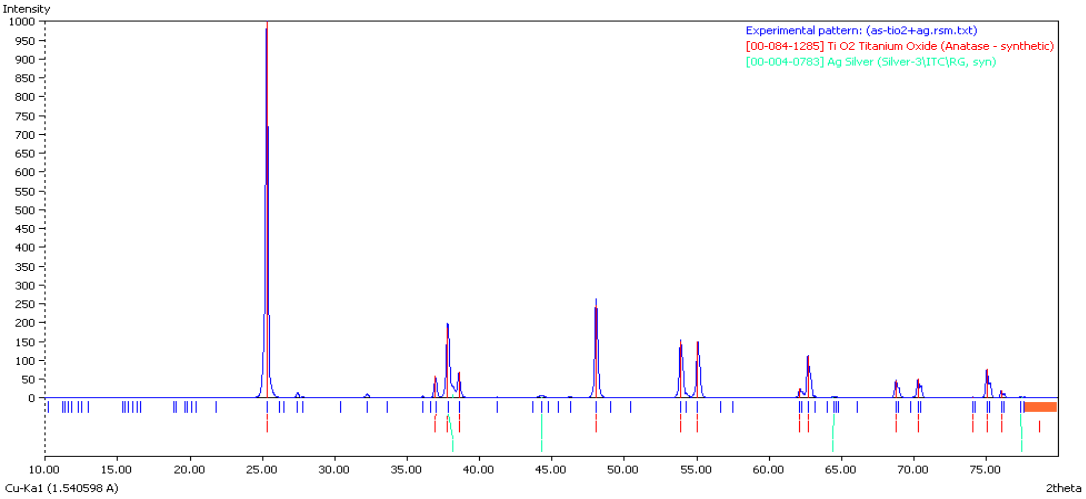

Fig. 3. XRD of TiO_2-Ag

From the X-ray diffraction pattern (Fig. 3) it can be easily identify the main characteristic peaks of TiO_2 in anatase form as the main component of the powder (based on the ASTM file 084-8285). Peak assignments were made based on the values of 2θ and their relative intensities. Even if the content of Ag is 300 ppm, these peaks can be identified based on ASTM file 04-0783. Based on the shape of the diffraction pattern, it can assume that the obtained nanopowder is highly crystalline.

- SEM

The SEM images of TiO_2-Ag and Coll/TiO_2-Ag are presented in Figs. 4-6 at different magnifications. Scanning electron microscopy was used especially to analyze the morphology of the agglomerates of the synthesized TiO_2-Ag powder, as well as the morphology of the Coll/TiO_2-Ag multifunctional materials, the direct characterization of the Coll/TiO_2-Ag gel being not possible. However, due to the freeze-drying method, it can assume that the morphology of the gel is similar with that of the freeze-dried composite Coll/TiO_2-Ag.

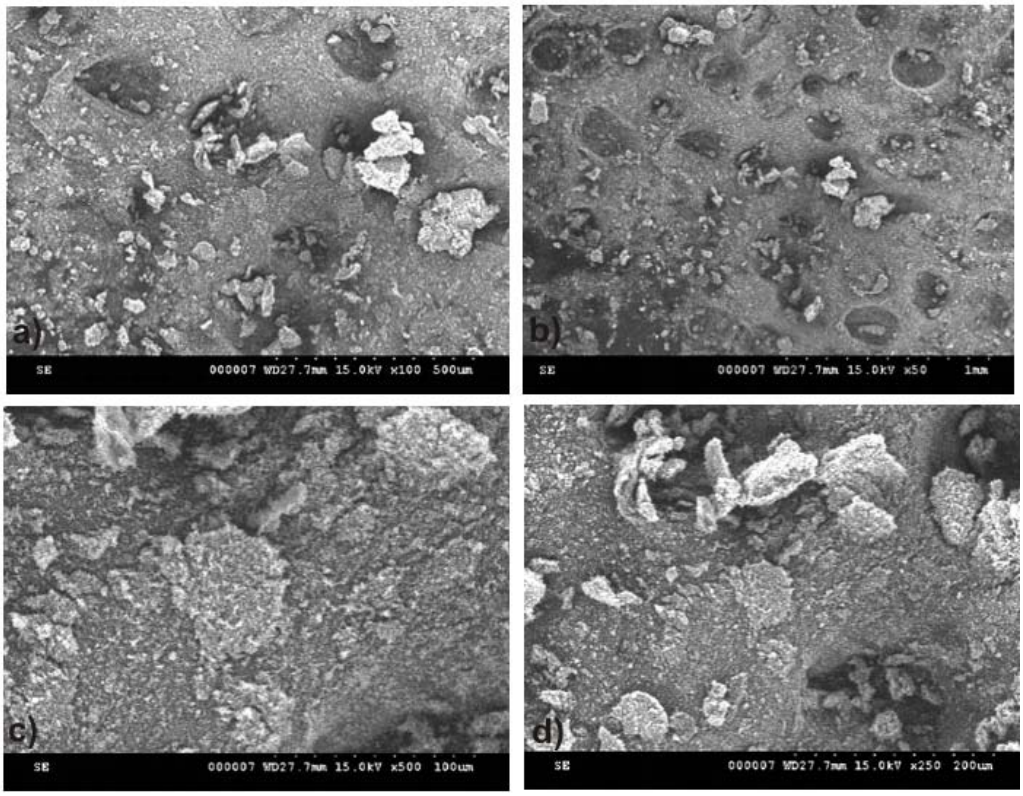

Fig. 4. SEM images of TiO_2-Ag

Looking at images from Fig. 4 it can be observed some small agglomerates. Based on the 10kx magnification was confirmed that the obtained nanometric particles have not a specific determination.

Fig. 5 and 6 showed the SEM images of Coll/ TiO_2-Ag obtained starting form TiO_2-Ag via glucose and ascorbic acid route. The Coll/ TiO_2-Ag sample obtained via glucose route (Fig 5) exhibits a slight tendency of stratification, which is visible at low magnification (100x – Fig 5b). As usually, collagen is structured in fibrils, fibres and even plackets and for this reason, it incorporates well the TiO_2-Ag mineral phase. At 1500 x magnification (Fig 5c), the well-embedded mineral phase is highlighted. Even if the samples are homogeneous, there are some larger agglomerates, which can reach up to 20µm.

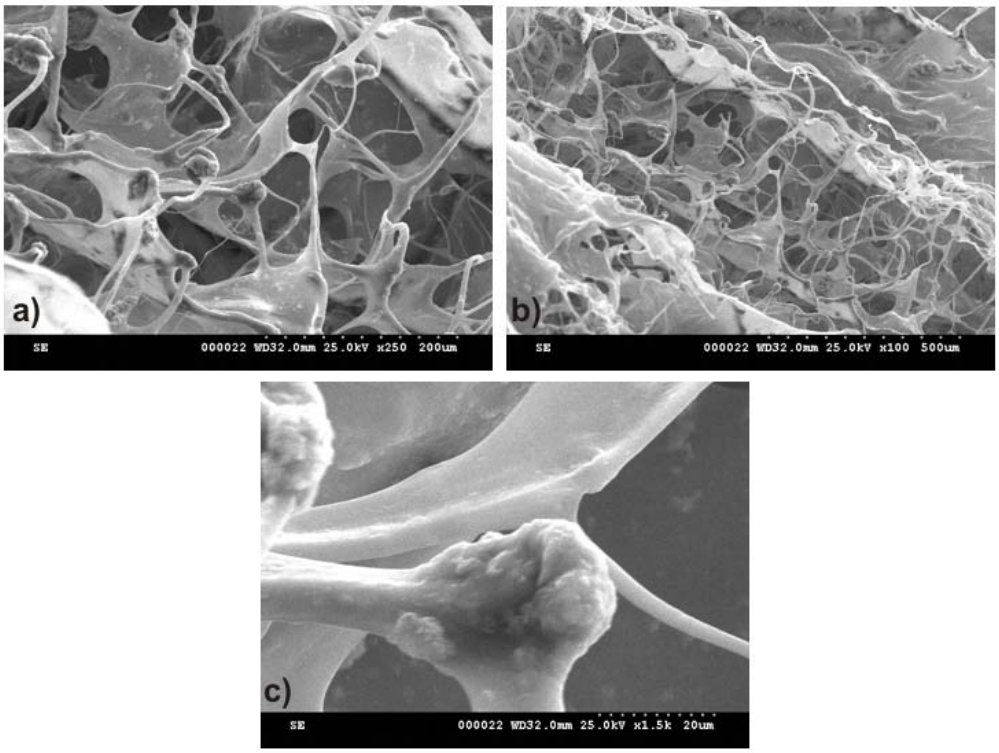

Fig. 5. SEM images of Coll/ TiO_2-Ag obtained via glucose route

Fig. 5 shows the structure of nanocomposites, which are well connected and form a homogeneous structure. Coll/ TiO_2-Ag sample obtained via ascorbic acid route exhibit no stratification tendency. The pore sizes present a large dispersity, with–diameters ranging from a few microns to a–100μm, which are arbitrary disposed.

The fibrils and fibres are arbitrary disposed and measure between 100 μm and 10 μm. The tendency of forming plackets seems to be less than in the previous case. Agglomerates can be also identified embedded into the collagen matrix and reach less than 10μm, in diameter.

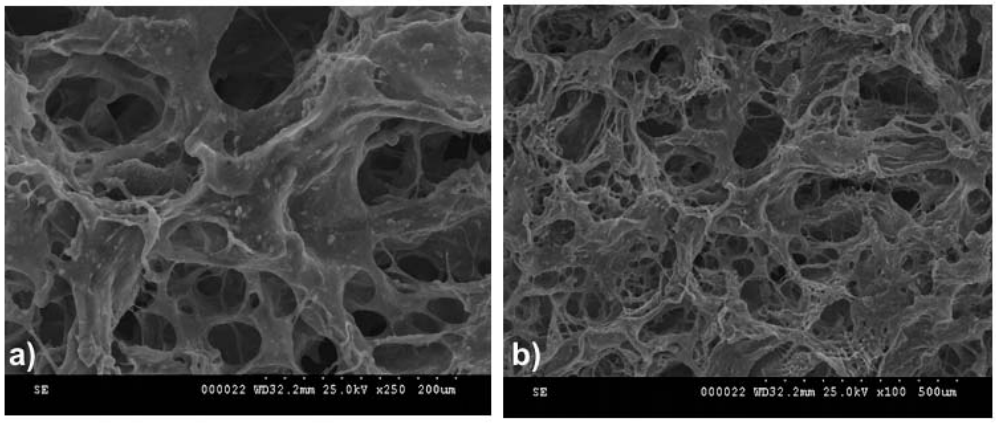

Fig. 6. SEM images of Collagen/TiO_2-Ag obtained via ascorbic acid route

- TEM

The TEM images of TiO_2-Ag *obtained via* glucose and ascorbic acid routes are presented in Fig. 7. The morphology of the TiO_2-Ag *obtained via* glucose route (Fig 7a) is heterogeneous; there are small (dark) particles, which are assigned to the silver nanoparticles, and large particles, which are assigned to TiO_2. The average size of silver is 3-5 nm while the size of TiO_2 particles reaches up to 200 nm. The shape of silver nanoparticles is spherical while TiO_2 have rounded edge polyhedral form, at both magnifications.

The morphology of the TiO_2-Ag *obtained via* ascorbic acid route (Fig 7b) is quite different heterogeneous comparing with the particles obtained via glucose route. In this case, the silver nanoparticles cannot be visualized, most probably because these nanoparticles are smaller. The reducing agent is also influencing the formation of the TiO_2, the particles. These are smaller and rather spherical than polyhedral.

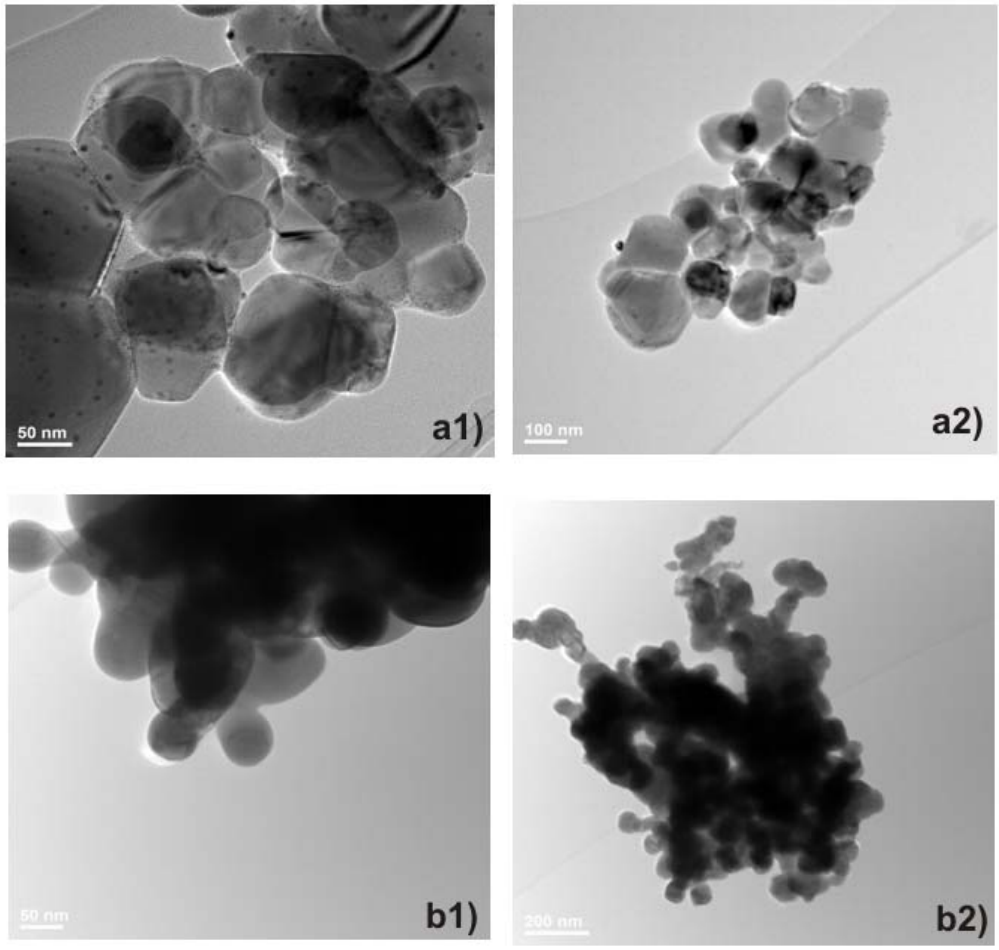

Fig. 7. TEM imagines of TiO_2-Ag obtained via glucose route (a1, a2); and TiO_2-Ag obtained via ascorbic acid route (b1, b2)

Fig. 8 shows the FT-IR spectra of *Coll/TiO_2-Ag* represents the composites obtained via glucose (a) and acid ascorbic route (b). The two spectra are similar which means that collagen is not denatured during the synthesis route; so the overall processing route of preparation of the Coll/TiO_2-Ag gels do not alter the nature of the collagen.

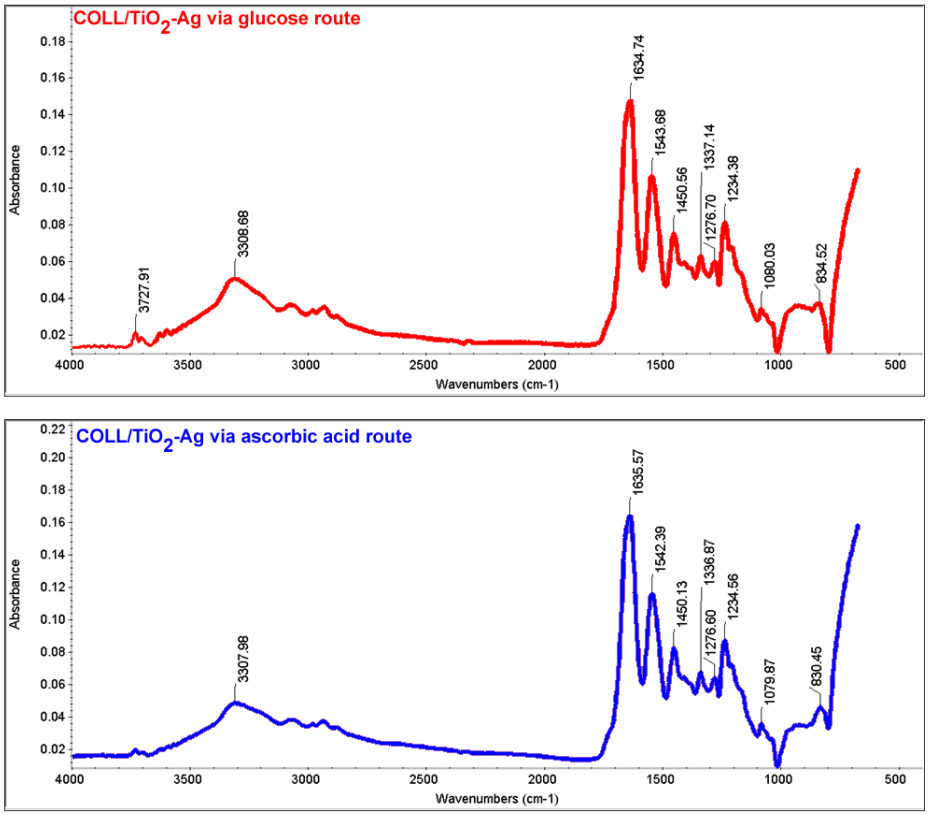

Fig. 8. FTIR spectra of Coll/TiO_2-Ag obtained via glucose route (a); and Coll/TiO_2-Ag obtained via ascorbic acid route (b)

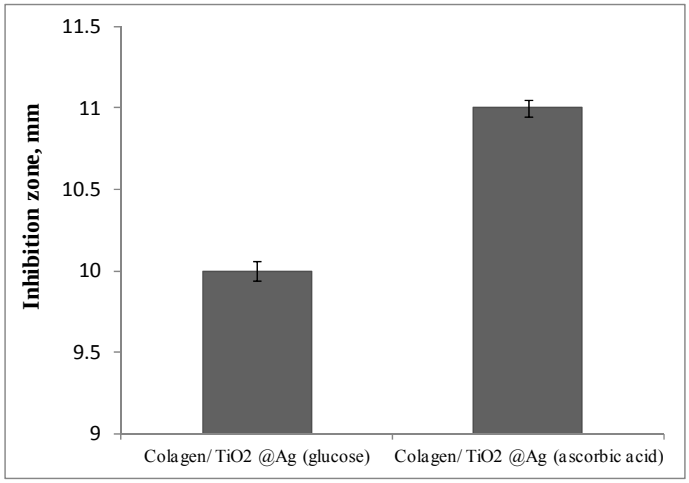

Fig. 9. Antibacterial activity against S. aureus bacterium

Fig. 9 shows a higher antibacterial activity in case of nanocomposites obtained by using TiO_2/Ag via ascorbic acid route. This result can be explained especially based on the transmission electron data. It is well known that antimicrobial activity is strongly dependent on size and shape of the nanoparticles [44]. Both samples exhibit good antimicrobial activity, because ascorbic acid induces a specific morphology and in consequence, it induces a stronger antimicrobial activity against *S. aureus*. Further works will be done in order to correlate the antimicrobial activity with BET (Bruner, Emmett & Teller – used for determination of surface area and pore size distribution) and TEM (transmission electron microscopy) analysis [41-43].

Ascorbic acid has an antibacterial effect against many microorganisms [41-42] but mechanism of action and the chemical group responsible for the bacterial inhibition are not known [43].

4. Conclusion

The synthesized TiO_2-Ag precursor materials as well as the composite materials consisting in TiO_2/Ag embedded in collagen matrix were investigated by FT-IR, XRD, SEM and TEM and were tested against *S. aureus* by the Kirby-Bauer disk-diffusion method. Based on the obtained results it can be concluded that silver nanoparticles were homogeneously distributed and assured improved antibacterial activity of the nanobiocomposites. The antibacterial assessment was made with *S. aureus* (Gram-positive bacterium) because it has been found that it is capable to cause different infections of the skin. As many tests confirm, ascorbic acid has an excellent reducing capacity of silver ions to colloidal silver and, due to the fast reduction, the particles are smaller than those obtained by reduction with glucose. Further tests will be done for other microorganisms and for testing the UV-protecting capacity of these systems, the final goal of this work being to obtain creams for multifunctional purposes, as regenerative, UV-protective and antimicrobial cream.

In vitro microbiology tests showed a good resistance of the nanocomposites with silver nanoparticles against *Staphylococcus aureus* (*S. aureus*), also able to print an antimicrobial activity to the cosmetic products.

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