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1 **Thermal, mechanical and microstructural characterization and antioxidant potential of**
2 ***Rhinobatos cemiculus* gelatin films supplemented by titanium dioxide doped silver**
3 **nanoparticles**

4
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26 **Abstract**

27 Hybrid composites films were prepared by the incorporation of titanium dioxide doped
28 silver nanoparticles (TiO₂-Ag NPs) at different concentrations (1, 2, 3 and 4 wt%) into
29 *Rhinobatos cemiculus* gelatin (RCG) matrix. The microstructure analysis, realized by
30 scanning electron microscopy and atomic force microscopy revealed a homogenous structure
31 and a smooth surface of the composite films. The mechanical properties, light transmission
32 and moisture content of composite films are greatly dependent on the amount of added TiO₂-
33 Ag NPs. A gradual decrease in elongation at break was observed with the increase in TiO₂-Ag
34 nanoparticles content (reaching 16.05% at an addition level of 4%). Moreover, the addition of
35 TiO₂-Ag NPs considerably enhanced the antioxidant activity of nanocomposite films
36 evaluated by DPPH radical scavenging activity and ferrous chelating activity. It is, thus,
37 concluded that the incorporation of TiO₂-Ag NPs into the gelatin matrix promotes the
38 improvement of gelatin films' performance.

39

40 **Keywords:** *Rhinobatos cemiculus*, gelatin, nanoparticles, nanocomposite films, thermal
41 analysis, microstructural and mechanical properties.

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51 **Introduction**

52 Biopolymers are considered as a promising alternative to petrochemical based polymers
53 for the sake of environment protection and sustainable development (Félix, Lucio-Villegas,
54 Romero, & Guerrero, 2016). Several market studies showed that with feedstock's low cost
55 and technologies, the biopolymer demand exceeds an annual growth rate of 4.5 times of the
56 current production between 2014 and 2019 (Galiano, et al., 2018). Moreover, consumers are
57 also more and more interested in products based on biopolymers regarding their low toxicity,
58 biodegradability and biocompatibility (Galiano, et al., 2018).

59 Currently, the fish processing industries as well as the fish markets are providing large
60 amounts of by-products and wastes serving as raw materials for the extraction of various
61 biopolymers. Among these wastes, skin (Arumugam, Sharma, Balakrishnan, & Ettiyappan,
62 2018), bones (Maccari, Galeotti, & Volpi, 2015), exoskeletons (Hamdi, et al., 2017) and
63 viscera (Abdelhedi, Nasri, Souissi, Nasri, & Jridi, 2016) are the most exploited. The skin
64 gelatin from multiple fish species such as *Nile tilapia* (Zheng, et al., 2018), *Hemiramphus far*
65 (Abdelhedi, et al., 2019), *Cyprinus carpio* (Tkaczewska, Morawska, Kulawik, & Zajac,
66 2018), *Loligo formosana* (Hamzeh, Benjakul, Sae-leaw, & Sinthusamran, 2018) and
67 *Probarbus jullieni* (Ali, Kishimura, & Benjakul, 2018) has been extracted and characterized.
68 Gelatin is a protein produced by thermal denaturation of collagen. It is widely used in food
69 industries as a thickening agent in desserts, a stabilizer in ice cream preparations, a texturizer
70 in confections' production, and as food foam and gelling agent (Handbook, 2012; Haug,
71 Draget, & Smidsrød, 2004; Karayannakidis & Zotos, 2016; Karim & Bhat, 2008; Schrieber &
72 Gareis, 2007). In addition, the intrinsic properties of gelatin, especially its excellent film
73 forming ability (Arfat, Benjakul, Prodpran, & Osako, 2014) nowadays offer substantial
74 advantages for uses in membrane technology. In fact, gelatin-based membranes showed a
75 growing interest in water treatment (Kamal, Pochat-Bohatier, & Sanchez-Marcano, 2017), gas

76 separation, micro and ultra-separation as well as for tissue engineering (Tayebi,
77 Rasoulianboroujeni, Cui, & Ye, 2018) where they are used not only as barriers to volatile
78 compounds, oxygen and carbon dioxide, but also as carriers of a wide variety of substances,
79 such as antioxidants, antibacterial agents, colorants, and nutrients (Ahmad, Benjakul,
80 Prodpran, & Agustini, 2012).

81 Nevertheless, gelatin membranes exhibit poor mechanical and thermal properties, which
82 restrict their use as a packaging material (Gómez-Guillén, et al., 2009). Using nanoparticles in
83 biopolymer-based films is a promising alternative to improve both mechanical and thermal
84 properties (Shankar & Rhim, 2018). Among these nanoparticles, titanium dioxide and silver
85 nanoparticles (TiO₂-NP and Ag-NP) are the most commonly manufactured (Chen, et al.,
86 2016). In fact, TiO₂-NPs are gaining special attention thanks to their low cost, high stability,
87 photo-catalytic and anticorrosive properties (Feng, et al., 2007). A recent study has shown that
88 TiO₂-NPs could avoid light induced oxidation and decomposition when used in food
89 packaging systems (Vejdan, Ojagh, Adeli, & Abdollahi, 2016). In addition, these NPs have
90 been used as an UV protective skin compound as they have wide UV spectrum-attenuation
91 characteristics (Schulz, et al., 2002). On the other hand, silver nanoparticles displayed an
92 inhibitory and bactericidal effect thanks to their high surface area (Mangalaraj & Devi, 2017;
93 Yan, et al., 2007). They are considered as a non-specific bactericide agent able to act against a
94 broad spectrum of bacterial and fungal species (Raghunath & Perumal, 2017). A
95 supplementary layer, of titanium dioxide as an example, is often added to prevent silver
96 oxidation and reduce its concentration (Mangalaraj, et al., 2017).

97 In this respect, this work aimed to develop novel films based on *R. cemiculus* gelatin
98 supplemented with TiO₂-Ag nanoparticles. The physicochemical, thermal, mechanical and
99 antioxidant properties of the prepared films were then investigated.

101 **2. Materials and methods**

102 **2.1. Gelatin extraction**

103 Fresh blackchin guitarfish (*R. cemiculus*) skin was supplied from the local fish market
104 located in Sfax, Tunisia. Samples were placed in ice and then transported in polyethylene
105 bags to the laboratory. The skin was rigorously washed and stored at -20 °C before gelatin
106 extraction.

107 Gelatin was extracted according to the protocol described by Jridi, et al. (2013) with
108 slight modifications. Once thawed, the blackchin guitarfish skin was cut into small pieces
109 with an electric carving knife, and then soaked in 0.05 M NaOH (1:5, w/v) under continuous
110 stirring for 2 h at 25 °C. The medium was changed every 30 min to ensure better elimination
111 of non-collagenous species. Thereafter, the alkaline treated skin pieces were washed with
112 distilled water until a neutral pH value of the washing solution was reached. They were then
113 soaked in 0.2 M acetic acid solution (ratio 1:5, w/v) for 18 h at 25 °C. Thereafter, the pH of
114 the medium was adjusted to 7.0 using NaOH solution. The gelatin extraction was realized at
115 50 °C for 18 h under continuous stirring. The centrifugation was carried out at 8 000 g for 30
116 min at 4 °C to remove insoluble residues. Finally, the supernatant was collected and freeze-
117 dried (Moduloyd freeze dryer thermo fisher, USA) to obtain *R. cemiculus* gelatin (RCG)
118 powder.

119 **2.2. Electrophoretic analysis**

120 The protein patterns of the extracted gelatin were determined using the sodium dodecyl
121 sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) as described by Laemmli (1970)
122 with slight modifications. The gelatin (10 mg) was dissolved in 1 ml of distilled water at 50
123 °C. 50 µg of each sample were mixed with loading buffer (5% mercaptoethanol, 2% SDS and
124 0.002% bromophenol blue) at a ratio of 1:5. The prepared mixtures were heated at 90 °C for
125 10 min and then loaded onto 5% w/v stacking gel and 7.5% w/v separating gel.

126 **2.3. Turbidity determination**

127 The gelatin turbidity was evaluated using the method described by Fernández-Díaz,
128 Montero, & Gómez-Guillén (2001). Briefly, the absorbance at 360 nm was measured after
129 dissolving the dry gelatin powder in distilled water at a concentration of 6.67% (w/v). Then an
130 adjustment of the pH ranging from 2 to 10, using either 10 mM HCl or 10 mM NaOH, was
131 carried out. Experiments for each sample were fulfilled in triplicate (Fernández-Díaz,
132 Montero, & Gómez-Guillén, 2001).

133 **2.4. Films' preparation**

134 The gelatin/titanium dioxide-silver films were developed through a simple casting
135 approach. Nanoparticles with size ranging within 15 – 20 nm, were obtained as described by
136 Elleuch, et al. (2020). First, the RCG powder (4 g) was solubilized in 100 ml of distilled water
137 under magnetic stirring at 50 °C. The titanium dioxide doped silver nanoparticles (TiO₂-Ag)
138 were added at different ratios to the solution (1, 2, 3 and 4 wt %). Glycerol was then added to
139 the mixture as a plasticizer at a concentration of 15 % (w/w, plasticizer/polymer dry matter),
140 and the mixture was stirred for 30 min. The sonication was subsequently applied using an
141 Elmasonic S10 H ultrasound cleaner in order to guarantee a homogeneous dispersion of
142 nanoparticles in the mixture. 25 ml of each solution was then poured into a Petri dish of 13.5
143 cm diameter, and allowed to dry under an air flow of a laboratory hood at 25 °C for 48 h.
144 Finally, the films were peeled off and preserved at 25 °C before use.

145 **2.5. Moisture content**

146 The moisture content (MC) of each sample was determined by drying 100 mg of film
147 specimens in an oven (Memmert UF1060) at 105 °C for 24 h. The MC values, evaluated in
148 triplicate, were obtained by weighing samples before and after oven drying. The results were
149 expressed as g moisture/100 g film.

150

151

152 **2.6. Water solubility**

153 The films' water solubility (WS) was carried out via the method described by
154 Gennadios, Handa, Froning, Weller, and Hanna (1998). Samples (100 mg) were weighed and
155 then placed in a centrifuge tube containing 30 ml of distilled water. The tubes were then
156 shaken at 25 °C for 24 h, and then centrifuged at 10000 g for 10 min at 25 °C (Labogene,
157 ScanSpeed 1248R centrifuge). The supernatant was discarded, and the pellet corresponding to
158 the non-solubilized film was dried at 105 °C for 24 h. The water solubility was carried out in
159 triplicate and expressed as follows:

$$WS (\%) = \frac{(m_i \times (100 - M_C) - m_f) \times 100}{m_i \times (100 - M_C)}$$

160 where m_i is the initial film mass (g), m_f the final dry film mass (g), M_C the moisture content
161 (%).

162 **2.7. Color parameters**

163 The color of RCG-TiO₂-Ag NPs films was evaluated using a bench-top colorimeter
164 (CR-5; Konica Minolta). The values were expressed by referring to a white standard color
165 plate used as a background. The color parameters, L^* , a^* and b^* expressing
166 lightness/brightness, redness/greenness and yellowness/blueness, respectively, were
167 determined. The difference in color ΔE was determined as described by Sinthusamran,
168 Benjakul, Hemar and Kishimura, (2018).

$$\Delta E = \sqrt{(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2}$$

169 **2.8. Ultraviolet-visible absorption spectrum**

170 The difference in gelatin films' absorption as a function of the nanoparticles' content
171 was determined in a wavelength ranged from 200 to 800 nm using a spectrophotometer

172 (JENWAY, 7315 spectrophotometer), as described by Nagarajan, Benjakul, Prodpran, and
173 Songtipya (2012).

174 **2.9. Fourier transform infrared spectroscopy**

175 The Fourier transform infrared spectroscopy (FTIR) spectra were obtained using
176 Thermo Fisher Scientific spectrometer (Model: Nexus). The sample compartment was
177 equipped with an attenuated total reflectance (ATR) accessory. The reflection incidence angle
178 of the diamond crystal was about 45° with respect to the IR beam. A resolution of 4 cm⁻¹ and
179 a number of 32 scans were used to obtain the spectra in the 4000-600 cm⁻¹ range at room
180 temperature.

181 **2.10. X-ray diffraction analysis**

182 The X-ray diffraction (XRD) patterns were collected on Bruker D5000 ray
183 diffractometer using a Cu K α radiation source. The analyses were operated at 40 kW and 20
184 mA and the patterns were recorded in the range of 2 Θ = 7-40° at a scanning rate of 1°/min.

185 **2.11. Thermal analysis**

186 The differential scanning calorimetry (DSC) was carried out using DSC Q20, TA
187 Instrument in order to determine the thermal properties of different prepared films. Samples
188 (5 mg each) were subjected to two cycles of heating-cooling from -50 °C to 200 °C with a
189 heating rate of 10 °C/min. The nitrogen purge was applied at a flow rate of 50 ml/min. The
190 obtained DSC thermograms were treated using TA Universal Analysis 2000 software (version
191 4.5 A, TA instruments). The glass transition temperature (T_g) was determined as the mid-
192 point of the second heating cycle. In order to determine thermal stability, thermogravimetric
193 analysis (TGA) was performed using TGA Q500 High Resolution, TA Instruments. Samples
194 (5 mg each) were heated from 25 to 800 °C at a heating rate of 20 °C/min under air flow. The
195 thermograms were obtained using TA Universal Analysis 2000 software (version 4.5 A).

196

197 **2.12. Mechanical properties**

198 The mechanical properties of RCG-films were measured in terms of tensile strength
199 (TS) and elongation at break (EAB) using a rheometer apparatus (Physica MCR 301, Anton
200 Paar, GmbH, France) equipped with a measuring geometry (CTD 450). Rectangular samples
201 of 1.0 cm x 4.5 cm were prepared for measurements. Prior to testing, all samples were
202 conditioned at 25 °C and 50 % relative humidity for two weeks, and the thickness was
203 measured. The film samples were fixed on the extension grips, and then deformed uniaxially
204 under a deformation rate of 5 mm/min until breaking, based on the ISO standard conditions.
205 The stress-strain curves were attained using the Rheoplus software. The maximum TS and the
206 final EAB were determined. Values between 4 to 6 measurements were saved and used for
207 calculations.

208 **2.13. Microstructure and surface topography analysis**

209 The microstructure of RCG-TiO₂-Ag NPs films was examined using Hitachi S4800
210 scanning electron microscope (SEM). Prior to analysis, the samples were sputtered with a thin
211 gold layer of 5 nm. An accelerating voltage of 2.0 kV was applied to observe the surface and
212 the cross-section morphology of the film samples. The surface topography of the film samples
213 was further examined using a dimension 3100 atomic force microscopy (AFM) apparatus
214 (Digital Instruments, Veeco, CA). The experiments were conducted at ambient temperature in
215 an intermittent tapping mode. Data were processed using Gwyddion software (version 2.14).

216 **2.14. Antioxidant activities**

217 **2.14.1. DPPH radical-scavenging activity**

218 The DPPH radical scavenging activity of the gelatin films was determined according to
219 the method described by Bersuder, Hole, and Smith (1998). The absorbance was determined
220 at 517 nm. The DPPH radical scavenging activity was calculated as expressed in the
221 following formula:

$$\text{DPPH radical – scavenging activity (\%)} = \frac{OD_{control} + OD_{blank} - OD_{sample}}{OD_{control}} \times 100$$

222 where OD_{blank} , $OD_{control}$ and OD_{sample} are the absorbance of the blank, DPPH control
 223 and sample reaction, respectively. The experiments were carried out in triplicate.

224 **2.14.2. Ferrous chelating activity**

225 The chelating effect of the gelatin films was determined according to the method of E.
 226 A. Decker and Welch (1990) with slight modifications. The method is based on inhibiting the
 227 formation of Fe^{2+} -Ferrozine complex in presence of the studied sample. Initially, 100 mg of
 228 each film was mixed with 100 μ l of distilled water to which 50 μ l of 2 mM $FeCl_2$ and 450 μ l
 229 of distilled water were added. After 5 min of incubation, 200 μ l of ferrozine solution (5 mM)
 230 were added, and the mixture was incubated again for 10 min at room temperature. Ethylene-
 231 diamine-tetra-acetic acid (EDTA) was employed as a positive control. The absorbance was
 232 determined at 562 nm for each sample. The ferrous chelating activity was calculated as
 233 expressed in the following formula:

$$\text{Metal chelating activity (\%)} = \frac{OD_{control} + OD_{blank} - OD_{sample}}{OD_{control}} \times 100$$

234 where $OD_{control}$, OD_{blank} , and OD_{sample} are the absorbances of the control, blank and sample
 235 reactions, respectively.

236 **2.15. Statistical analysis**

237 The statistical analyses were performed with SPSS version 17.0, professional edition
 238 using ANOVA analysis. A standard deviation at a confidence level of 95% was used to
 239 compare all parameters analyzed for all elaborated films.

240 **3. Results and discussion**

241 **3.1. Electrophoretic analysis for RCG**

242 The molecular weight distribution of RCG was analyzed by SDS-PAGE (Fig. 1). The
 243 resulting pattern showed the presence of all gelatin characteristic chains, specifically β and

244 α_1/α_2 with MW of 200 and 120-130 kDa, respectively, as previously reported by Gomez
245 Guillen et al. (2009) and Tümerkan, Cansu, Boran, Mac Regenstein, and Özoğul (2019). In fact,
246 α_1 and α_2 chains intensities for *R. cemiculus* skin gelatin were almost similar. Additionally,
247 high molecular-weight components resulting from residual heat-stable cross-links were found
248 in RCG, as proved by the high-intensity band at the top of the polyacrylamide gel. On the
249 other hand, owing to the higher molecular weight of β -chain, dimer of α -chain, it might not
250 be extracted effectively as demonstrated by the lower band intensity in the obtained gelatin.
251 Nagarajan et al. (2012) demonstrated that a higher content in α -chain reflected better
252 functional properties for splendid squid skin gelatin. Liu, Li, and Guo (2008) confirmed that
253 β - and γ - chains would permit a more organized structure with a higher gel strength. Large
254 amounts of β - and γ -chains have been shown to negatively affect some of the functional
255 properties of fish gelatins, such as lower viscosity and melting point (Muyonga, Cole, &
256 Duodu, 2004).

257 **3.2. Turbidity analysis**

258 The gelatin solution turbidity was evaluated by measuring the absorbance measurement
259 at 360 nm and results were illustrated in Fig. 2. The results showed that the highest turbidity
260 of RCG was obtained at pH 5. For higher and lower pH values, turbidity values decreased.
261 Similarly, Lassoued, et al. (2014) reported that the turbidity was influenced by pH variation.
262 Poppe (1997) reported that the gelatin solutions showed the maximum of turbidity at their
263 isoelectric point. This could be explained by the fact that at pH close to pI, the protein
264 molecules lean to form aggregate and less water interaction might take place with the protein
265 molecules.

266 **3.3. Film solubility**

267 Solubility is an essential property of edible films since they are used as protective
268 coatings on food. Potential applications may necessitate water insolubility to enhance product

269 integrity and water resistance (De Moura, Lorevice, Mattoso, & Zucolotto, 2011). For this
270 purpose, the RCG films' solubility was determined and the values are represented in Table 1.
271 Native gelatin films exhibit almost 33 % of solubility in pure water due to its hydrophilic
272 nature caused by the presence of polar peptides in gelatin. With the addition of TiO₂-Ag NPs
273 to the film matrix, a decrease in the solubility was detected, which could be attributed to the
274 formation of hydrogen bonds between the gelatin matrix and the nanoparticles resulting in a
275 reduction of interactions between the biopolymer and water molecules, causing solubility
276 decrease (Voon, Bhat, Easa, Liong, & Karim, 2012). Similar findings were obtained in
277 previous studies where gelatin films were added with SiO₂ nanoparticles and showed a
278 decrease in water solubility. Additionally, for other biopolymer-based films, a decrease in
279 water solubility has been perceived once the nanoparticles, such as chitosan nanoparticles in
280 hydroxypropyl methylcellulose films (M. De Moura, Avena - Bustillos, McHugh, Krochta, &
281 Mattoso, 2008) and sodium montmorillonite in methyl cellulose films (Tunç & Duman,
282 2010), were added.

283 **3.4. Color**

284 Color is an important parameter that should be controlled as it affects consumers'
285 acceptance (Pereda, Dufresne, Aranguren, & Marcovich, 2014). Color parameters in terms of
286 rectangular coordinates (a^* , b^* and L^*) and total color difference (ΔE) of the various films are
287 presented in Table 2. With the addition of TiO₂-Ag NPs, the values of a^* and b^* increased
288 while the L^* value decreased, indicating the leaning toward red and yellow colors. In fact, the
289 RCG films supplemented with 4% of TiO₂-Ag NPs presented the highest a^* , b^* and ΔE values
290 of 0.76 ± 0.11 , 4.16 ± 0.37 and 14.85 ± 1.0 , respectively and the lowest value of L^* of about
291 94.09 ± 0.57 . The reduced L^* value demonstrated that the films became darker with the
292 addition of TiO₂-Ag NPs, which is in accordance with the visual observations. These changes
293 in color were most likely attributed to TiO₂-Ag NPs color which is initially brownish-gray as

294 described by Naoi, Ohko, and Tatsuma (2005). Therefore, if the composite is intended to be
295 employed as edible coating material, the percentage of TiO₂-Ag NPs has to be carefully
296 controlled to keep the initial color of the coated product, as mentioned by Jamróz, Kulawik,
297 and Kopel (2019).

298 **3.5. Light transmittance**

299 The transmission of UV and visible light of the films in the wavelength ranged from
300 200 to 800 nm is illustrated in Fig. 3. The UV light transmission for all films was low at 200-
301 280 nm. These results may be due to the presence of chromophore groups in aromatic amino
302 acids constituting the gelatin matrix such as tyrosine and phenylalanine, which have strong
303 absorption of UV light (Gómez-Guillén, et al., 2009; Kchaou, et al., 2017; Nagarajan, et al.,
304 2012). Figure 3 also shows that the light transmission of the films rapidly increased in the
305 range from 280 to 400 nm, and then leveled off in the visible range. It is also noted that the
306 light transmission decreased with the increase of nanoparticles content, suggesting that TiO₂-
307 Ag NPs were well integrated into the gelatin matrix. Therefore, their UV light transmission
308 was reduced.

309 **3.6. FTIR analysis of RCG based nanocomposite films**

310 FTIR was used to investigate the molecular interaction in RCG films incorporated with
311 different amounts of TiO₂-Ag NPs. The FTIR spectra of all prepared gelatin films in the
312 spectral ranged between 600 and 4000 cm⁻¹ are shown in Fig. 4. Generally, the prepared films
313 showed similar spectra in the range of 600-1800 cm⁻¹, presenting amide I, amide II and amide
314 III bands as found by Arfat, et al. (2016). The most pronounced absorptions appeared at
315 wavenumbers ranging between 1560-1680 cm⁻¹, 1540-1610 cm⁻¹ and 1230-1340 cm⁻¹, which
316 are attributed to typical absorptions normally detected for gelatin-based films as amide I
317 (C=O stretching at ~ 1630 cm⁻¹), amide II (N-H groups coupled with C-N stretching
318 at ~ 1540 cm⁻¹) and amide III (C-N and N-H stretch and vibrations of CH₂ groups of glycine),

319 respectively (Nur Hanani, Beatty, Roos, Morris, & Kerry, 2013; Tongnuanchan, Benjakul, &
320 Prodpran, 2012). Peaks related to amide A were detected at 3300-3500 cm^{-1} as described by
321 Fundo, Galvis-Sanchez, Delgadillo, Silva, and Quintas (2015). The peak detected at 1080 cm^{-1}
322 referred to the C-O of the glycerol (Bergo, Moraes, & Sobral, 2013), used as a plasticizer.
323 These results showed that no notable changes in the shifting or density of peaks related to
324 gelatin occurred regardless of TiO_2 -Ag NPs addition, except for the O-H stretch indicating the
325 arrangement of intermolecular hydrogen bonds (Qiao, et al., 2017).

326 **3.7. X-ray diffraction (XRD) analysis**

327 The XRD patterns of gelatin films with various TiO_2 -Ag NPs contents are shown in
328 Fig. 5. An amorphous halo is observed for all the films. In contrast, the composites with 2, 3
329 and 4% TiO_2 -Ag NPs displayed a crystalline peak at $2\theta = 25.8$ characteristic of the diffraction
330 of TiO_2 crystal (Ba-Abbad, Kadhun, Mohamad, Takriff, & Sopian, 2012). No peak was
331 detected for the Ag crystal, indicating that the silver core was amorphous and that the
332 nanoparticles used were effectively composed of a non-oxidized silver core protected by the
333 TiO_2 layer. These results are in accordance with those reported by Li, Xu, Chen, and Chen
334 (2011) who suggested that at low contents of nanoparticles, the protein adsorption on the
335 surface of particles reduced their crystallization which became undetectable in the XRD.
336 Meanwhile, self-assembled nanoparticle agglomerates taking place at high concentration of
337 nanoparticles (2 g/100 g) could regain their crystallization capacity.

338 **3.8. Gelatin thermal properties**

339 **3.8.1. Determination of gelatin glass transition temperature**

340 The thermal properties of gelatin sample were characterized using DSC to determine
341 the corresponding glass transition temperature (T_g). It's demonstrated through the DSC
342 curves (data not shown) that a glass transition temperature of almost 150 $^{\circ}\text{C}$ was obtained for
343 the extracted gelatin. The obtained value was higher than the T_g values of commercial bovine

344 (74.8 °C) and fish (68.1 °C) gelatins (Al-Hassan, 2020). The glass transition temperature is
345 defined as the temperature at which the polymer relaxes and changes from the glassy state to
346 the elastic state, for a given heating rate due to the onset of long-range coordinated molecular
347 motion of the amorphous structure (Sperling, 2005). The difference between RCG and the
348 cited commercial bovine and fish gelatins T_g values could be related to its structural
349 characteristics that might change as a function of molecular mobility (Rahman, Al-Saidi, &
350 Guizani, 2008). The rigidity and mobility of the polymer particularly play a crucial role in the
351 improvement of the glass-forming ability (Minecka, et al., 2020). In addition, Bell & Touma
352 (1996) explicated that the variation of T_g may be due to different types of gelatin transformed
353 through different extraction methods. Besides, it could also be related to characteristics of
354 animals' skins based on species and age.

355 **3.8.2. Evaluation of gelatin thermal stability**

356 The TGA curves of the extracted gelatin are presented in Fig. 6a. The studied gelatin
357 powder shows a minor loss in weight below 100 °C, corresponding to moisture release. A
358 major weight loss was detected from 250 to 450 °C, corresponding to the thermal
359 decomposition of gelatin. Chuaynukul, Prodpran, and Benjakul (2014) suggested that the
360 water adsorbed influenced the protein-protein interaction by reducing the thermal stability of
361 the gelatin system. A high content in water leads to a possible protein degradation that is more
362 likely to take place in a greater degree especially at high temperature. Thus, a greater loss of
363 thermal stability of protein is attained.

364 **3.9. Nanocomposite films thermal properties**

365 The differential scanning calorimetric (DSC) analysis was carried out to determine the
366 thermal properties of nanocomposites. The results of Table 1 summarized the values of the
367 glass transition (T_g) of RCG-Ag-TiO₂ films. The RCG control film exhibited a T_g value of
368 about 59.52 °C which is higher than those found by Hazirah, Isa, and Sarbon (2016) and

369 Theerawitayaart, Prodpran, Benjakul, and Sookchoo (2019) estimated at 48.4 and 56.4 °C,
370 respectively. The T_g of composite films decreased with the increase of TiO₂-Ag NPs content,
371 reaching 52.61°C for 4% of NPs. This decrease may be attributed to a disorder state among
372 gelatin molecules or the net-like structure of the gelatin molecules according to the theory
373 related to molecular interactions and chain stiffness previously described by Luecha, Sozer,
374 and Kokini (2010) with corn zein/montmorillonite gelatin. Moreover, the T_g decrease may
375 also be caused by the hydrophobic feature due to TiO₂-Ag nanoparticles added in the gelatin
376 matrix (Bahadur & Uludağ, 2016). This phenomenon facilitated water evaporation and
377 consequently decreased hydrogen bonding as described by Wu, Liu, Wang, Han, and Liu
378 (2017). Furthermore, lower T_g values were probably related to the decrease in hydrogen
379 bonds initially existing in the gelatin based film (Chak, Kumar, & Visht, 2013).

380 The thermal stability of composite films was evaluated using TGA and DTG. As shown
381 in Fig. 6c, the RCG control film and that added with 1% of TiO₂-Ag NPs exhibited a five-step
382 weight loss pattern, whereas other composite films showed 3 decomposition phases. As
383 shown in Fig. 6c, the first phase started around 60 °C for control RCG film and between 80
384 and 100 °C for the composites owing to the evaporation of free and bound water remaining in
385 the films (Rhim, Hong, Park, & Ng, 2006). As the temperature increased from 120 to 800 °C,
386 a peak at 250 °C appeared in all thermograms due to the blending of glycerol as a plasticizer in all
387 prepared gelatin films. The third weight loss, which exhibits a strong exothermic peak at 320–
388 350 °C for all films, was due to the decomposition of lower molecular weight gelatin fractions
389 (Arfat, et al., 2016). The peak at 500 °C, which almost disappeared for films prepared with 2, 3
390 and 4% of TiO₂-Ag NPs, was related to the degradation of highly interacted proteins in the
391 gelatin matrix (Arfat, et al., 2016). The absence of this decomposition step for the composites
392 with TiO₂-Ag NPs contents above 2% proved the enhancement of the thermal stability of
393 these films at 500 °C, mostly due to the heat-stable TiO₂-Ag NPs, in agreement with data

394 reported by Rhim, et al. (2006) for gelatin-silver nanoparticle added-composite films. The last
395 degradation stage occurs between 550 and 700 °C for films added with 0%, 1% and 4% and
396 corresponds to the thermal decomposition of the gelatin networks (Mishra, Majeed, &
397 Bantia, 2011). These results proved that RCG films added with 2 and 3% are thermally
398 stable until approximately 800 °C.

399 **3.10. Mechanical properties of RCG/TiO₂-Ag NPs composite films**

400 Gelatin films are widely used in various industrial packaging processes. Thus, their
401 resistance to stretching is of major importance for their applications. The effect of TiO₂-Ag
402 nanoparticles' addition on the mechanical properties of RCG-films was investigated in terms
403 of TS and EAB as summarized in Table 1. The control film exhibited the highest EAB
404 (28.1±2.5%) compared to nanoparticles-added films. In fact, the EAB decreased from
405 21.6±0.3 to 16.1±1.8% with increasing NPs content from 1 to 4%. Some reported differences
406 might be due to the source from which the gelatin was extracted, the composition, the type
407 and ratio of the added plasticizer as well as the preparation and storage methods. Moreover,
408 the moisture content of the native gelatin film (49 g moisture/100 g wet basis film) decreased
409 after the addition of nanoparticles to reach 26.5, 28.4, 27.8 and 31.3 for films added with 1, 2,
410 3 and 4%, respectively. Serrano-León, et al. (2018) proved that a decrease in moisture content
411 values leads to a decrease in the molecular motion. Thus, mechanical properties of the
412 prepared films could be affected by water content as well as nanoparticles addition. Data in
413 Table 1 also show that the TS of the films decreased with the increase of TiO₂-Ag NPs.
414 Compared to the value of 7.1 MPa for the control film, the TS decreased to 5.9, 5.4, 5.3 and
415 5.2 MPa for composites with 1, 2, 3 and 4% of TiO₂-Ag NPs, respectively. This decrease
416 could be related to the configuration of the film's macromolecular network. In fact, the
417 decrease of the mechanical characteristics of the nanocomposite films could be due to the
418 heterogeneous distribution of TiO₂-Ag NPs resulting in the slight aggregation occurring once

419 the concentration of nanoparticles is increased in protein matrix. The aggregate could inhibit
420 the arrangement of protein domains and distant gelatin macromolecules will be found to
421 construct the nanocomposite matrix resulting then in reduced mechanical properties of the
422 prepared films. Hence, the concentration of TiO₂-Ag nanoparticles was a main factor
423 affecting both the spatial organization and the structure of protein aggregates in the film
424 matrix. Similar findings were presented by Zhou, Wang, and Gunasekaran (2009) for whey
425 protein/titanium oxide films and by Arfat, et al. (2016) for fish skin gelatin / fish protein
426 isolate-zinc oxide nanocomposite films. Hence, the incorporation of TiO₂-Ag NPs at a
427 particular level could control the strength of the elaborated gelatin/ NPs films.

428 **3.11. Microstructure of RCG/TiO₂-Ag NPs composite films**

429 The surface and cross section microstructure of the composite films was examined with
430 SEM as illustrated in Fig. 7. The surface of the control film (Fig. 7B) revealed a homogenous
431 and smooth structure without cracks. Concerning the nanocomposite films, the NPs were
432 dispersed uniformly, and the degree of dispersion increased as the content in nanoparticles
433 increased (Fig. 7 B1-B4). Similar findings have been reported by Hosseini, Rezaei, Zandi, and
434 Farahmandghavi (2015) where the chitosan nanoparticles were added to reinforce fish gelatin-
435 based films. The cross section graphs were represented in Fig. 7 A1-A4 and revealed that the
436 addition of Ti-Ag NPs caused changes in the microstructure of films, whereas the non-added
437 film displayed a less undulated surface as supposed for homogeneous material. SEM
438 micrographs of films containing 1%, 2% and 3% of Ti-Ag NPs showed an equi-distribution of
439 particles in the gelatin matrix, without any observed aggregation. To further study the changes
440 in TiO₂-Ag NPs aggregation and film roughness, three-dimensional (3D) images of the films'
441 surface were obtained *via* AFM as illustrated in Fig. 8. The control RCG film was almost
442 smooth. The addition of TiO₂-Ag NPs triggered a rougher surface for nanocomposite films
443 due to the slight aggregation of nanoparticles. Moreover, the surface roughness was about 7.2

444 nm for the control RCG film, and increased from 10.5 to 18.2 nm when varying NPs content
445 from 1 to 4%. This increase was assigned to the level of nanoparticles overlapping and the
446 undulating surface of RCG film as described by Chang, Chen, Lin, and Chen (2012).
447 Consequently, the excessive added TiO₂-Ag NPs amount of 4% is believed to incite the rift of
448 the stable 3D polymeric matrix. Based on SEM and AFM results, it may be concluded that the
449 nanocomposite films are relatively smooth thanks to the uniform distribution of the added
450 nanoparticles and the tight combination with the gelatin matrix.

451 **3.12. Antioxidant activity**

452 The antioxidative potential of some substances could be acquired *via* various
453 mechanisms. In fact, the antioxidant defense systems operate through obstructing the
454 production of free radicals, obstructing the secondary production of inflammatory mediators,
455 scavenging the oxidants, obstructing the chain propagation of these oxidants and improving
456 the endogenous antioxidant defense system of the substance in question. These defense tools
457 act together to defend the body from oxidative stress (H. Decker & Rimke, 1998). For this
458 purpose, the DPPH radical scavenging activity and the metal chelating activity of the films
459 were determined as shown in Fig. 9. The first one is based on free radicals capture, and the
460 latter is based on the chelation of the transition metal involved in the lipid peroxidation
461 reactions. The results clearly indicated that RCG-TiO₂-Ag films displayed an antioxidant
462 capacity (Fig. 9a). The higher the TiO₂-Ag NPs content is, the higher the DPPH radical
463 scavenging activity is. The highest activity (48%) was recorded with TiO₂-Ag NPs content of
464 4%. Similarly, Hajji, et al. (2019) reported that Ag nanoparticles incorporated into the
465 chitosan matrix exhibit a dose-dependent DPPH radical-scavenging efficiency. On the other
466 hand, all tested films present a dose dependence on iron chelating activity, as displayed in Fig
467 9b. The highest activity up to 63 % was recorded for 4% NPs-added films, while the control
468 film exhibited the lowest activity (about 23%). In this context, Nakkala, Mata, Raja, Chandra,

469 and Sadras (2018) demonstrated that silver nanoparticles can accept or lose electrons forming
470 stable compounds, resulting in the free radicals' quench. Hence, the elaborated RCG films
471 supplemented by TiO₂-Ag NPs may act as an antioxidant agent, thanks to their amino and
472 hydroxyl groups, which may perform as proton-donors.

473 **Conclusion**

474 In the present study, TiO₂-Ag NPs were successfully incorporated into a RCG matrix as
475 nanosized fillers. The content of nanoparticles affected the microstructure as well as the
476 mechanical properties and the thermal stability of the elaborated films. Interestingly, the
477 thermal stability of the 3D polymeric matrix of RCG films was tightly related to the
478 concentration of nanoparticles. Furthermore, the RCG films supplemented with the adequate
479 TiO₂-Ag NPs content showed an improvement in their *in vitro* antioxidant activities, thermal
480 stability as well as a gradual decrease in their EAB values leading to the amelioration of the
481 nanocomposite films rigidity. Therefore, RCG/TiO₂-Ag NPs composite films could be
482 promising for potential uses in water-soluble food packaging.

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739 **Table 1.** Moisture content (MC), solubility, the glass transition temperature and mechanical
 740 properties (tensile strength and elongation at break) of gelatin-based films supplemented with
 741 different ratios of TiO₂-Ag NPs

TiO₂-Ag %	MC (g moisture/100 g wet basis film)	Solubility (%)	Tg (°C)	Tensile strength (MPa)	Elongation at break (%)
0	49.3±1.0 ^a	33.12±6.59 ^a	59.52	7.1±0.50 ^a	28.1±2.5 ^a
1	26.5±0.8 ^e	29.02±3.52 ^b	51.77	5.9±0.1 ^b	21.6±0.2 ^b
2	28.4±0.9 ^c	30.53±2.37 ^b	52.19	5.4±0.4 ^b	18.7±0.8 ^c
3	27.8±0.4 ^d	32.28±0.37 ^b	51.80	5.3±0.1 ^b	18.7±1.8 ^c
4	31.3±0.1 ^b	26.57±0.91 ^c	52.61	5.2±0.7 ^b	16.0±1.8 ^d

742 ^{a,b,c,d,e} Letters in the same column within different nanoparticles content indicate significant
 743 differences (p < 0.05)

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753 **Table 2.** Color parameters of *R. cemiculus* gelatin films incorporated with TiO₂-Ag
 754 nanoparticles

TiO ₂ -Ag %	L*	a*	b*	ΔE
0	96.34±0.09 ^a	-0.04±0.01 ^e	1.13±0.02 ^e	-
1	95.84±0.49 ^c	0.27±0.04 ^d	2.14±0.03 ^d	1.38±0.11 ^d
2	96.11±0.06 ^b	0.42±0.04 ^c	2.69±0.09 ^c	2.70±0.09 ^c
3	95.09±0.95 ^d	0.77±0.01 ^a	3.81±0.11 ^b	9.39±1.0 ^b
4	94.09±0.57 ^e	0.76±0.11 ^b	4.16±0.37 ^a	14.85±1.0 ^a

755 ^{a,b,c,d,e} Letters in the same column within different nanoparticles content indicate
 756 significant differences (p < 0.05)

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775 **Figure captions:**

776 **Figure 1.** SDS-PAGE profiles of *R. cemiculus* skin gelatins. SD: standard, RCG: *R. cemiculus*
777 gelatin.

778 **Figure 2.** Absorbance spectrum of *R. cemiculus* gelatin as a function of pH variation.

779 **Figure 3.** Light transmittance of films from *R. cemiculus* skin gelatin containing 1%, 2%, 3%
780 and 4% of TiO₂ doped Ag nanoparticles.

781 **Figure 4.** Fourier transform infrared spectra of *R. cemiculus* skin gelatin films supplemented
782 with different amount of TiO₂-Ag NPs.

783 **Figure 5.** X-ray diffraction patterns of *R. cemiculus* gelatin films with different amounts of
784 TiO₂-Ag nanoparticles.

785 **Figure 6.** TGA thermograms and its temperature derivatives for *R. cemiculus* skin gelatin (a)
786 and *R. cemiculus* skin gelatin supplemented with TiO₂-Ag films (b, c).

787 **Figure 7.** SEM micrographs of cross-section (A) and surfaces (B) of gelatin/TiO₂-Ag.
788 nanoparticles films: 0% (A, B), 1% (A1, B1), 2% (A2, B2), 3% (A3, B3) and 4% (A4, B4).

789 **Figure 8.** AFM 3D images and corresponding roughness curves of RCG gelatin films with
790 different amounts of TiO₂-Ag nanoparticles: 0 wt % (A), 1 wt % (B), 4 wt % (C) on the
791 gelatin basis.

792 **Figure 9.** Antioxidant activities of *R. cemiculus* skin gelatin containing 1%, 2%, 3% and 4%
793 of TiO₂ doped Ag nanoparticles.

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796 **Fig. 1**

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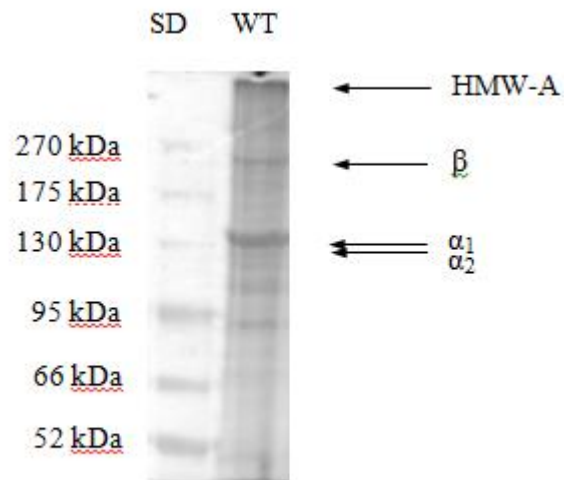
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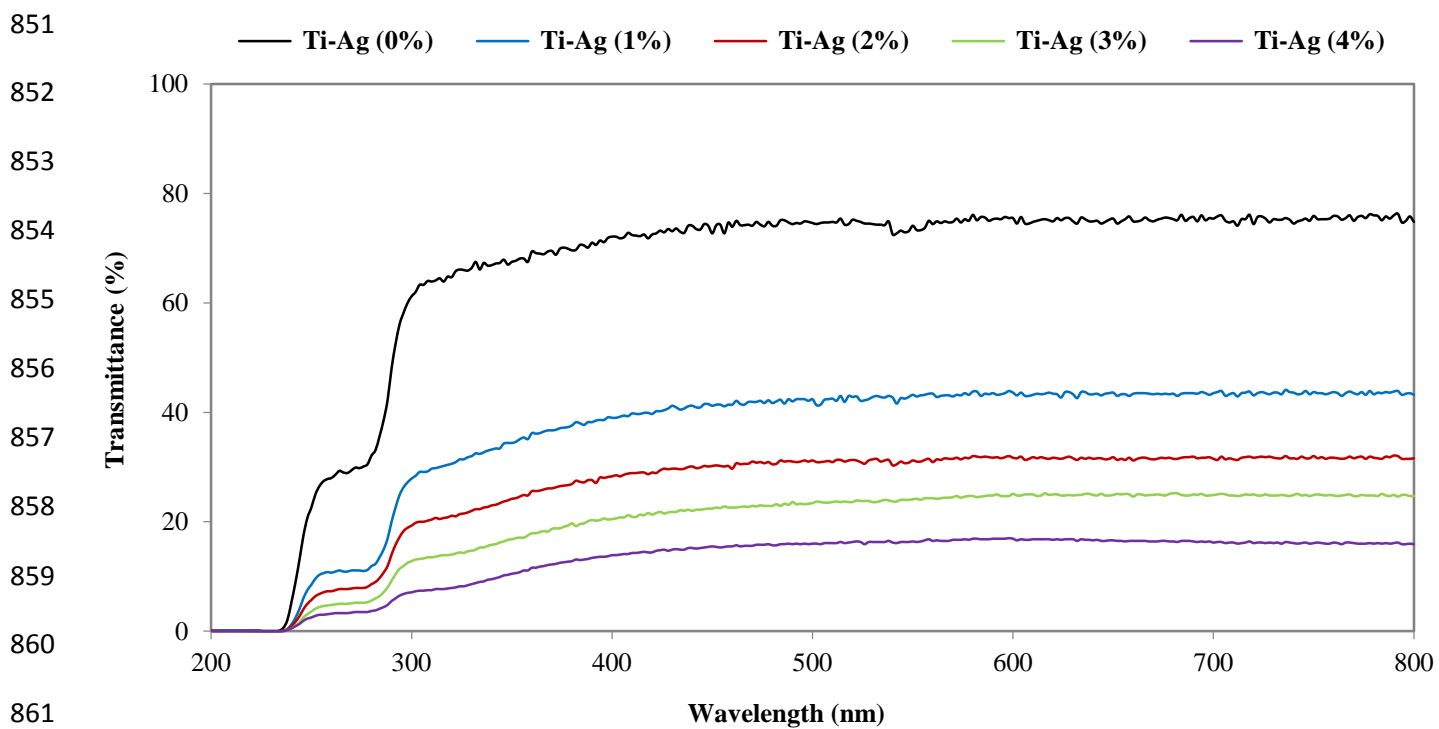
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850 **Fig. 3**



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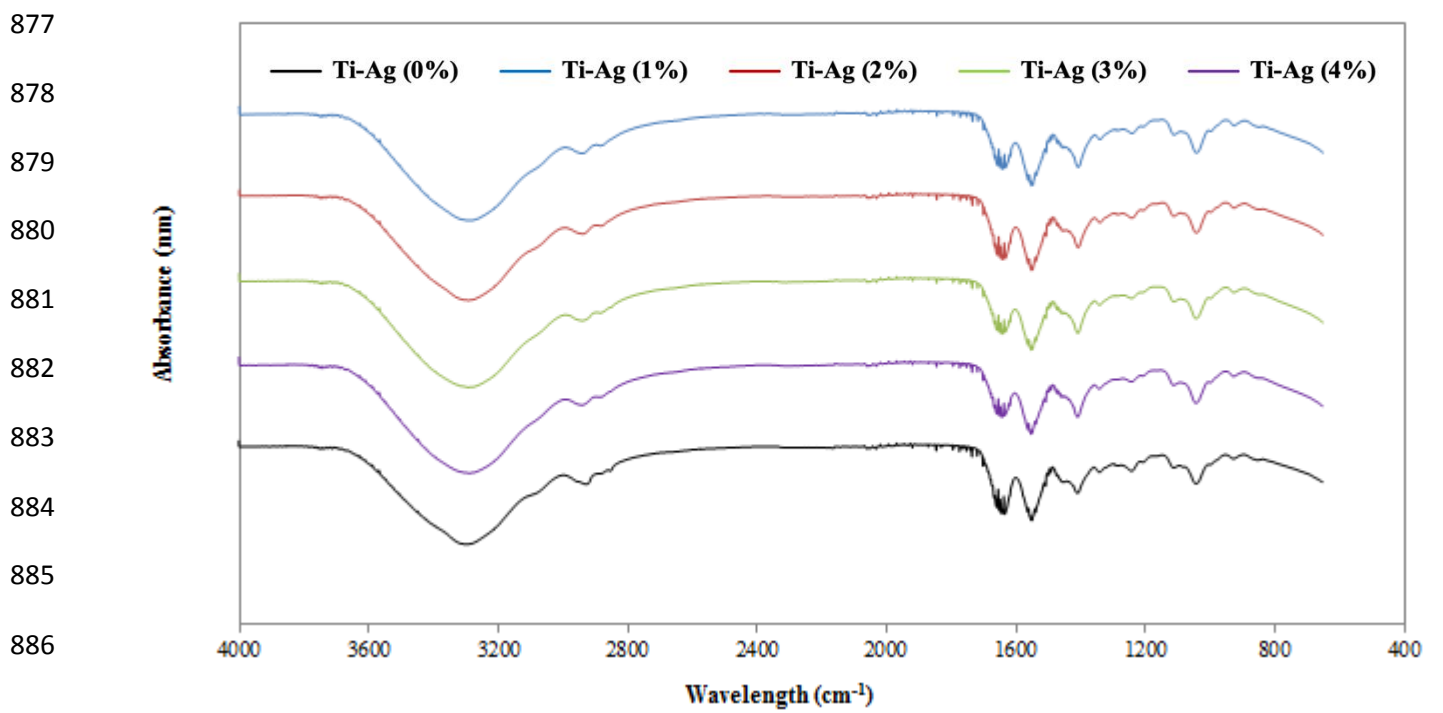
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875 **Fig. 4**

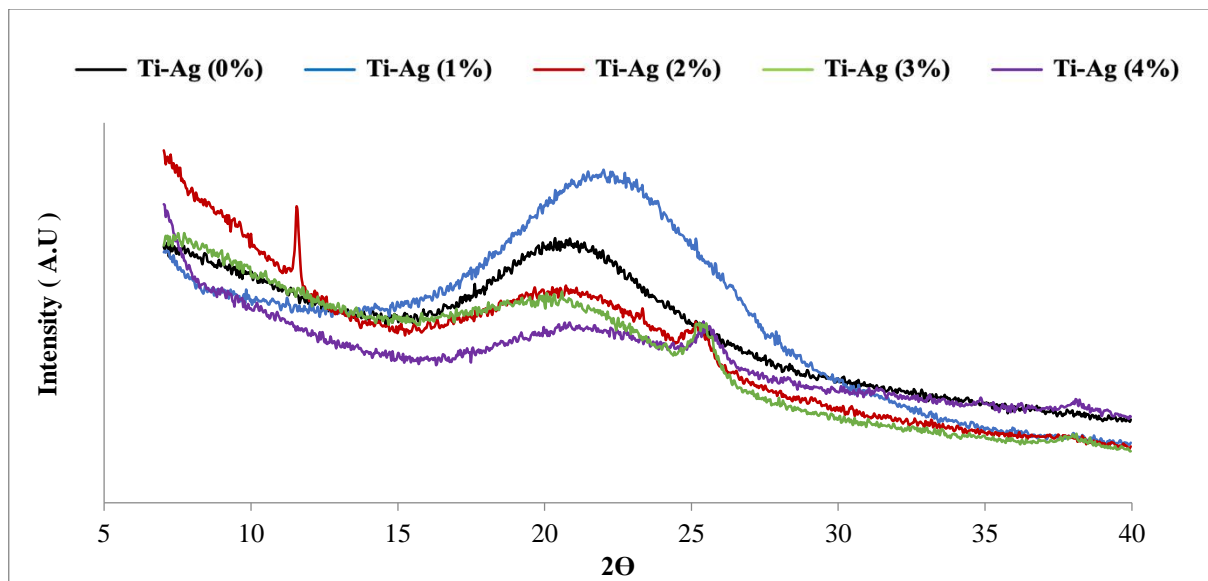
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Fig. 5

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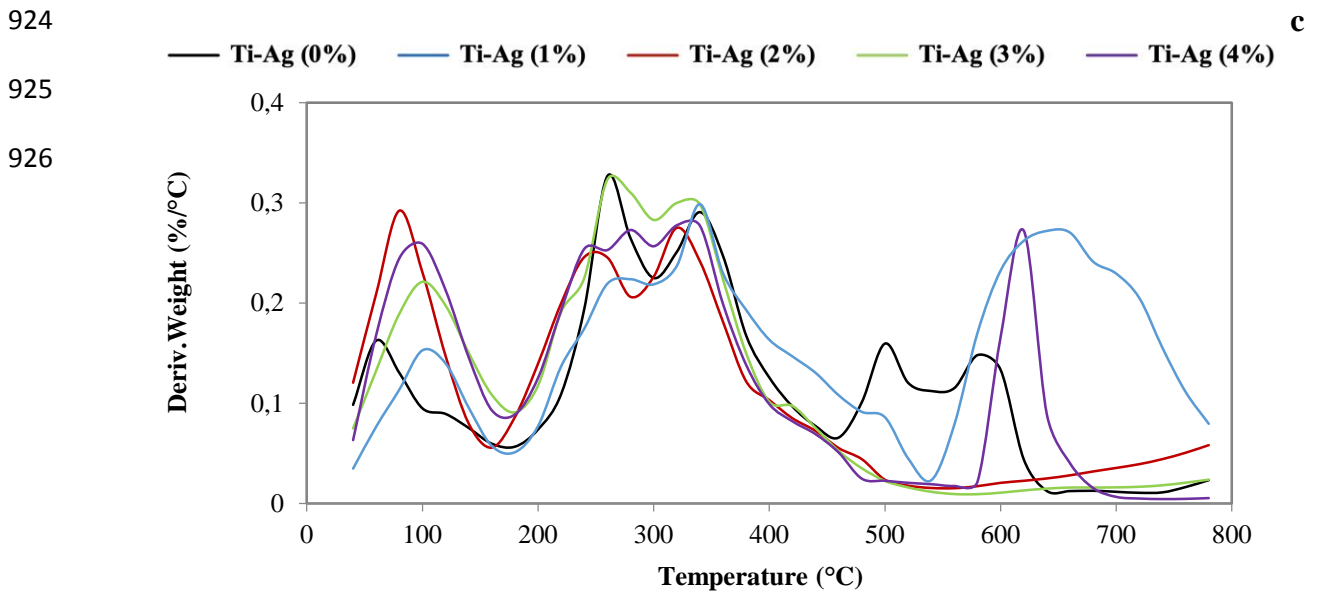
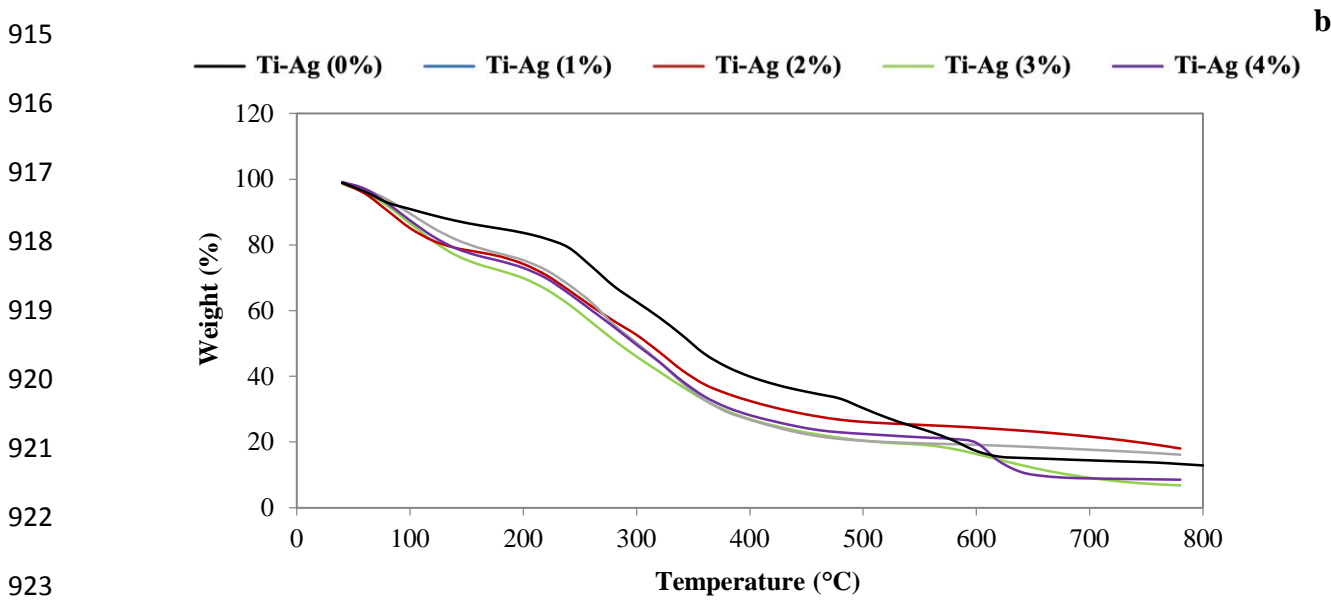
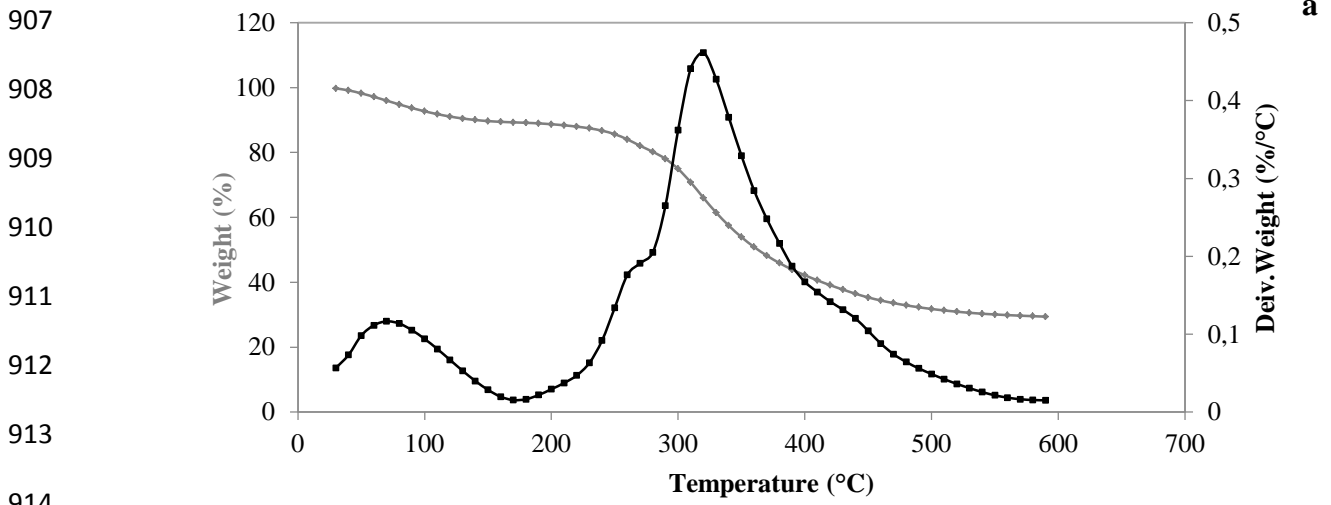
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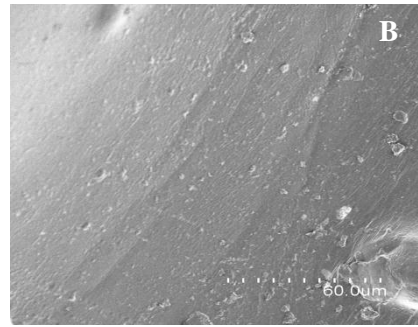
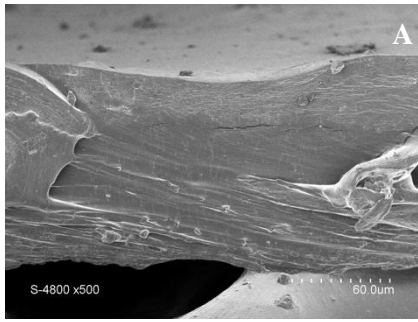
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906 **Fig. 6**



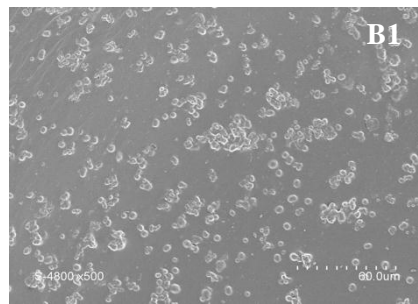
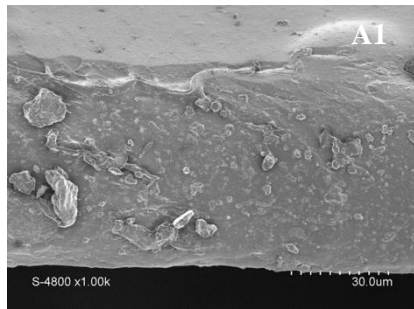
927 **Fig. 7**

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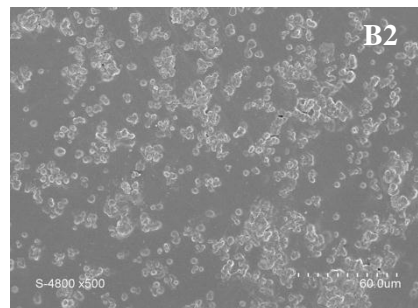
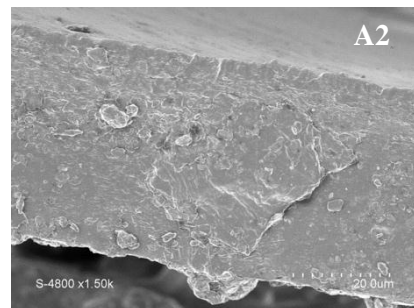
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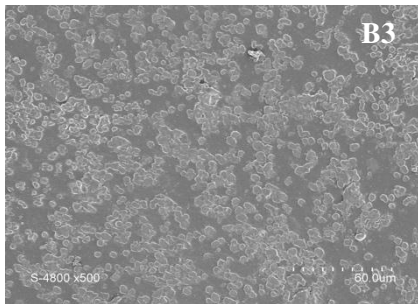
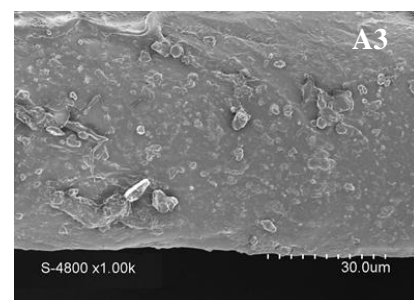
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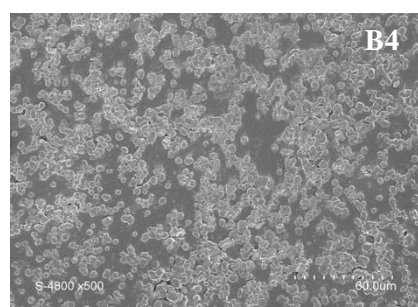
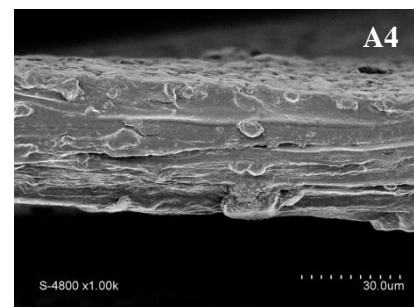
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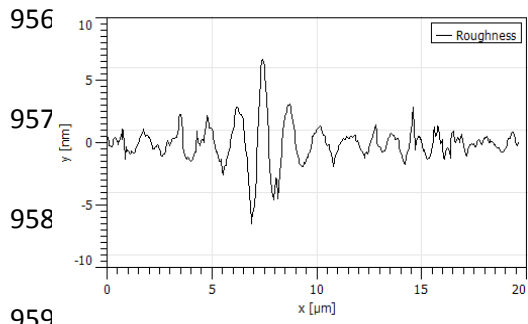
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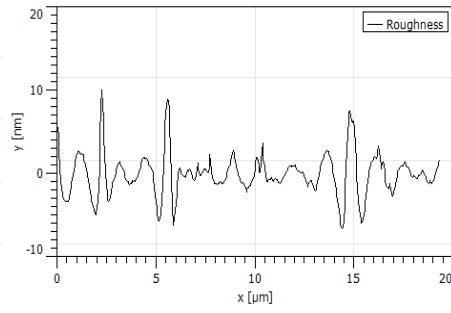
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955 **Fig. 8**

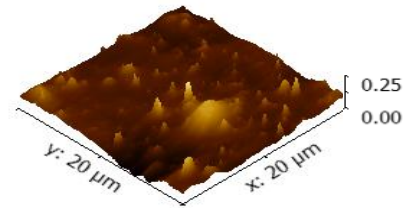
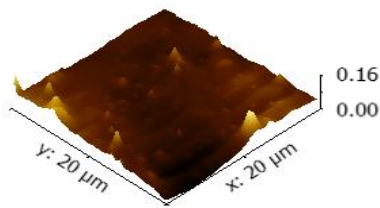
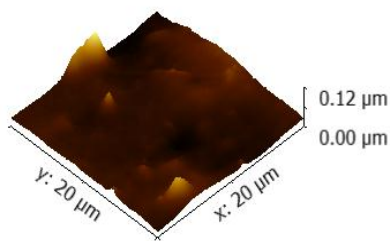
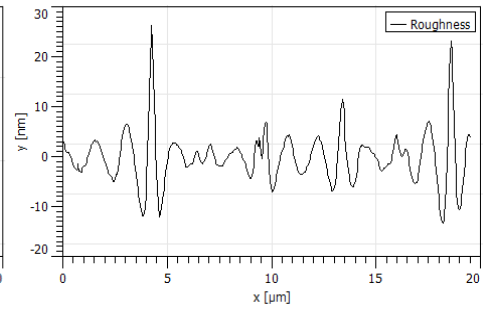
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974 **Fig. 9**

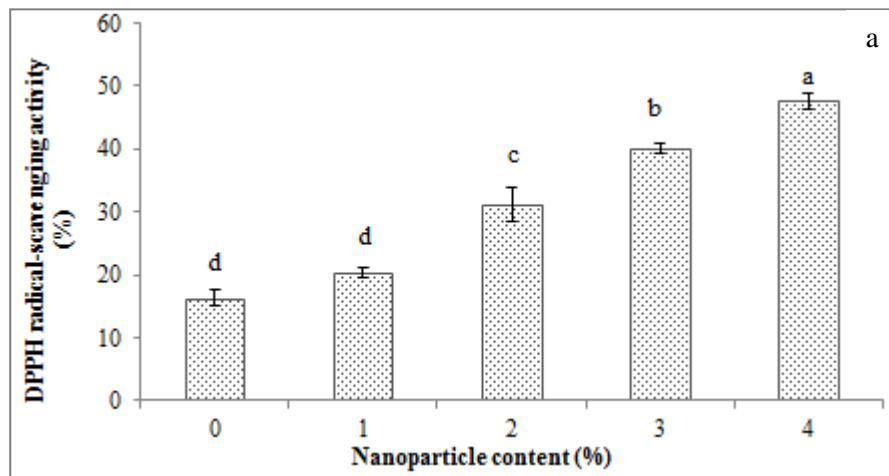
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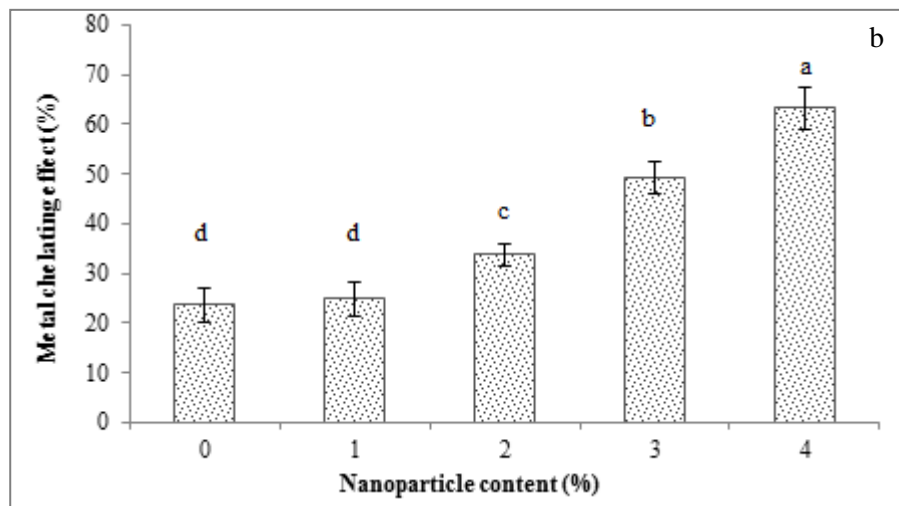
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