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# CELLULOSE DERIVATIVES-SNAIL SLIME FILMS: NEW DISPOSABLE ECO-FRIENDLY MATERIALS FOR FOOD PACKAGING

Maria Francesca Di Filippo<sup>1 §</sup>, Luisa Stella Dolci<sup>2 §</sup>, Letizia Liccardo<sup>1</sup>, Adriana Bigi<sup>1</sup>, Francesca

Bonvicini<sup>3</sup>, Giovanna Angela Gentilomi<sup>3</sup>, Nadia Passerini<sup>2</sup>, Silvia Panzavolta<sup>1\*</sup>, Beatrice Albertini<sup>2</sup>

<sup>1</sup>Department of Chemistry "G. Ciamician", University of Bologna, Via Selmi 2, 40126, Italy;

<sup>2</sup>Department of Pharmacy and BioTechnology, University of Bologna, Via S. Donato 19/2, 40127, Italy;

<sup>3</sup>Department of Pharmacy and Biotechnology, University of Bologna, Via Massarenti 9, 40138, Italy

§ these Authors equally contributed to this work

\*Corresponding author: Silvia Panzavolta

Dipartimento di Chimica "G. Ciamician"  
via Selmi 2 40126 Bologna (Italy)  
tel +39 051 2099566  
fax +39 051 2099456  
silvia.panzavolta@unibo.it

## ABSTRACT

In recent decades, synthetic plastic polymers have been the most practical and economical solution for packaging applications due to their low cost, availability, excellent optical, mechanical and barrier properties and resistance against water. However, most of the plastics used for packaging are hardly biodegradable. With a view to a circular economy, the aim of this work focused on the development of a new material made of commercial cellulose derivatives (hydroxypropyl methyl cellulose or carboxymethyl cellulose) mixed with snail mucus extracted from *Helix Aspersa* Muller. Increasing in Snail Mucus content enhances films elongation and adhesion strength while decreasing water vapor permeability. The cellulose-snail mucus based films are highly transparent but, more interestingly, the mucus confers UV-screening effect. In addition, the composite films exhibit

30 antimicrobial activity against both Gram-positive and Gram-negative bacteria. Furthermore, snail  
31 mucus addition to carboxymethyl cellulose strongly decreases films solubility in water. The  
32 biodegradation tests indicate that all the films degrade in soil between two and four weeks. The  
33 excellent results indicate that these biocomposite films are very good candidate for food packaging.

34

35 **Keywords: snail slime; food packaging; cellulose films; barrier properties; biodegradable films**

36

## 37 INTRODUCTION

38 Nowadays, imagining a world without synthetic plastics seems impossible, though their large-scale  
39 production and their extensive use have only spread since the end of the World War II. In food  
40 packaging it is a mandatory requirement to ensure protection from possible contaminants, as well  
41 as from moisture, gases, dust, temperature, UV and light radiation, odors and mechanical stresses.  
42 Plastic polymers have been the most practical and economical solution for packaging applications  
43 due to their low cost, prompt availability, excellent optical, mechanical and barrier properties and  
44 resistance against water and grease (Marsh & Bugusu, 2007). Traditional plastic packagings are  
45 petroleum-based products, which continuous and massive use will inevitably lead to reduced  
46 availability. However, most of the materials used for packaging, mainly designed for immediate  
47 disposal, are not biodegradable and their use provokes significant environmental pollution.  
48 Biodegradable materials from renewable resources are considered the best answer to the problems  
49 caused by the huge use of plastics. The most abundant renewable polymer is cellulose (Ferrer, Pal  
50 & Hubbe, 2017), which is biodegradable and non-toxic (Al-Tayyar, Youssef & Al-Hindi, 2020). Indeed,  
51 cellulose-based materials are widely employed in the packaging field (Lee, Yam & Piergiovanni,  
52 2008).

For example, hydroxypropyl-methylcellulose (HPMC) is a renewable, largely available and non-ionic vegetable derivative, very attractive because of its interesting properties: it is edible, transparent, odorless, tasteless and able to form oil-resistant and water-soluble films. Moreover, the use of HPMC is approved as food additive by the FDA (21 CFR 172.874) and by the EU (European Commission, 2011) and it is also proposed for the preparation of packaging materials (Wrona, Cranb, Nerín & Bigger, 2017), although it also exhibits a high moisture absorption (Bahrami, Mokarram, Khiabani, Ghanbarzadeh & Salehi, 2018). The highly crystalline derivative sodium carboxymethyl cellulose, CMCNa, which is a GRAS (Generally Recognized As Safe) polymer, is also widely used for film formulations (Arslan & Tog̃rul, 2005) and as a food stabilizer thanks to its peculiar properties like non-toxicity, biocompatibility, biodegradability and hydrophilicity. As well as HPMC, CMCNa is highly soluble in water and this feature limits their use as film-forming polymers.

In fact, to improve the CMCNa water resistance, crosslinking is mandatory: several chemical reactions have been proposed to overcome this problem (Su, Huang, Yuan, Wang & Li, 2010) as well as conjugation of CMC with montmorillonite by means of DOPA formation, as reported by Guo et al (Guo et al., 2019). Incorporation of additives, such as nanoclays, metallic nanoparticles and crosslinkers (Hasheminya, Mokarram, Ghanbarzadeh, Hamishekar & Kafil, 2018; He, Fei & Li, 2019; Kanatt & Makwana, 2020; Liu, Song, Shang, Song & Wang, 2012) have been proposed in order to increase the water barrier permeability, a further drawback of CMC.

However, neither CMC nor HPMC exhibit antibacterial properties, which are highly required in applications aimed to food preservation (Moghimi, Aliahmadi & Rafati, 2017). The research on antimicrobial food packaging films has indeed attracted great attention in recent years (Quintavalla & Vicini, 2002) since they can act as effective physical barriers against bacteria invasion and prolong the food shelf life (Appendini & Hotchkiss, 2002). Compared with the nano-antibacterial agents prepared by different inorganic materials, the introduction of natural extract is considered to be

77 safe and friendly to human and environment (Zhao, Wei, Xu & Han, 2020). Up to now, the studies  
78 aimed to imbue these materials with antibacterial activity are indeed based on the use of essential  
79 oils as additives (Gómez-Estaca, López de Lacey, López-Caballero, Gómez-Guillén & Montero, 2010;  
80 Moghimi, Aliahmadi & Rafati, 2017; Muppalla, Kanatt, Chawla & Sharma, 2014).

81 Herein, we propose the introduction of snail mucus as additive in the preparation of HPMC and CMC  
82 films with the aim to develop new materials with antimicrobial properties and highly improved  
83 water barrier permeability. Moreover, we have recently demonstrated that snail mucus addition to  
84 chitosan films not only provides them with antimicrobial activity, but also remarkably improves their  
85 water barrier and bioadhesion properties (Di Filippo et al., 2020). In this study we further explore  
86 the influence of snail mucus addition on the structural, mechanical, adhesive, barrier and  
87 antimicrobial properties, solubility and biodegradability of HPMC and CMC films. To this purpose,  
88 we utilized HPMC at two different viscosities and CMCNa to prepare and characterize films at  
89 different snail mucus content.

90

91

## 92 **EXPERIMENTAL PART**

### 93 **2. Materials and Methods**

94 Hydroxypropyl methyl Cellulose I (HPMC, Methocel E5 and E50, viscosity at 2% in water = 4-6 mPa·s  
95 and 40-60 mPa·s, respectively) were kindly supplied by Colorcon (UK). Commercial Carboxymethyl  
96 Cellulose sodium salt, (CMCNa Mw = 250 KDa, viscosity at 2% in water= 850 mPa·s) was kindly  
97 donated by ACEF (Piacenza, Italy). Formulae are reported in SI, Figure S1. These Celluloses satisfy  
98 the standards of the United States Pharmacopeia and European Pharmacopeia. Snail mucus or snail  
99 slime (S) from *Helix Aspersa Muller* (kindly offered by “I Poderi” farm, Montemerano, GR, Italy) was  
100 extracted by MullerOne method (<http://www.mullerone.com/it/en/extraction-process>) and stored

101 at 4°C in a sealed polyethylene bottle until use. Analysis of snail mucus was obtained from the  
102 supplier and reported in supplementary materials (Table S1).

### 103 **2.1 Preparation of cellulose films**

104 Cellulose films were obtained by solvent casting. Films forming solutions were prepared by  
105 dissolving 1 g of E5 or E50 in 20 mL of distilled water (5% w/v) or 0.4 g of CMC in 20 mL of distilled  
106 water (2% w/v) under gently stirring overnight. Then, 10.2 g of this solutions were poured in  
107 polyethylene Petri dishes ( $\varnothing$ = 8.5 cm) and allowed to dry under a laminar hood at room temperature  
108 overnight. The obtained films were labeled E5, E50 and CMC respectively and stored at room  
109 temperature between two sheets of plastic-coated aluminum closed inside PVC bags.

### 110 **2.2 Preparation of cellulose films with snail mucus extract**

111 In order to obtain films based on E5, E50 and CMC containing different amounts of snail mucus (S)  
112 (30, 70 and 100 % v/v), the relative volume of S was added to the solution containing 1 g of HPMC  
113 or 0.4 g of CMC, previously dissolved in the remaining volume of water. When S is at 100%, the  
114 polymer is directly solubilized into the snail mucus. After complete dissolution and disappearance  
115 of bubbles, 10.2 g of this solution were poured in each Petri dish ( $\varnothing$ = 8.5 cm) and put under laminar  
116 flow hood overnight. The obtained films were labeled as reported in Table 1, according to the  
117 volume of S used.

118 Table 1. Film compositions and labels.

Label	Polymer type	Polymer % (w/v)	S % (v/v)	Water % (v/v)
E5	HPMC E5	5	0	100
E5_S30	HPMC E5	5	30	70
E5_S70	HPMC E5	5	70	30
E5_S100	HPMC E5	5	100	0
E50	HPMC E50	5	0	100
E50_S30	HPMC E50	5	30	70
E50_S70	HPMC E50	5	70	30
E50_S100	HPMC E50	5	100	0
CMC	CMC Na	2	0	100
CMC_S30*	CMC Na	2	30	70

CMC_S70	CMC Na	2	70	30
CMC_S100	CMC Na	2	100	0

119

120 \*Due to their excessive fragility, it was not possible to characterize the films corresponding to the  
121 composition CMC\_S30.

## 122 **2.3. Films Characterization**

### 123 **2.3.1 Thickness**

124 The thickness of the films was measured with a hand-held digital micrometer (Mitutoyo, Japan) to  
125 an accuracy of 0.001 mm.

### 126 **2.3.2 Tensile tests**

127 Tensile tests were performed on the samples immediately after drying using a 4465 Instron  
128 dynamometer equipped with a 100 N load cell and the Series IX software package. Stress-strain  
129 curves were recorded at a crosshead speed of 5 mm/min on strip-shaped samples (20-30 mm long  
130 and 4 mm width). The Young's modulus (E), the stress at break ( $\sigma_b$ ) and the strain at break ( $\epsilon_b$ ) were  
131 evaluated. At least 10 samples were tested for each composition and the mean  $\pm$  SD are reported.

### 132 **2.3.3 Structural Characterization**

133 Fourier transform infrared spectra were recorded using a Thermo Scientific Nicolet iS10 FTIR  
134 spectrometer equipped with an ATR sampling device, using a Germanium crystal as internal  
135 reflection element. Infrared spectra were acquired at room temperature in absorbance mode from  
136 4000 to 800  $\text{cm}^{-1}$  with a resolution of 2  $\text{cm}^{-1}$ .

137 X-ray diffraction patterns were recorded using a Philips X'Celerator diffractometer equipped with a  
138 graphite monochromator in the diffracted beam. CuK $\alpha$  radiation (40 mA, 40 kV, 1.54 Å) was used.  
139 The 2 $\theta$  range was from 4° to 40° with a step size of 0,1337° and time/step of 40s.

### 140 **2.3.4 Tack test**



141 The adhesive strength of the films was evaluated by means of Antoon Paar modular compact  
142 Rheometer MCR102 with the Rheo Compass software, adapting the method reported in literature  
143 (Duncan, Abbott & Roberts, 1999). Glass and aluminum supports were used for the test. Films were  
144 cut in 3 cm-diameter circles and allowed to adhere to the two different supports by wetting them  
145 with 10 µL of distilled water and applying a gentle finger pressure. The upper plunger of the  
146 instrument was covered with double-sided tape (3M) and was lowered until a force of 5 N was  
147 applied to the film. After 30 seconds, the plunger was raised up at a speed of 1 mm/s, collecting the  
148 peak detachment force and the work of adhesion of the film from the support. Each formulation  
149 was analyzed in triplicate and the mean ± SD was reported.

### 150 **2.3.5 Barrier properties**

#### 151 **Water Vapor Permeability (WVP)**

152 WVP is the water vapor transmission rate through a flat film area induced by a vapor pressure  
153 between two surfaces under specific conditions of moisture and temperature and was measured  
154 using the ASTM E96-93 method (ASTM, 1993), slightly modified as reported in literature (Bozdemir  
155 & Tutas, 2003).

156 Films circles (2 cm-diameter) were glued with silicon on the opening of glass vials containing 2 g of  
157 anhydrous CaCl<sub>2</sub>. Vials were weighted and placed in a glass desiccator containing saturated  
158 Mg(NO<sub>3</sub>)<sub>2</sub>·6H<sub>2</sub>O solution (75% RH at 25°C). The vials were weighted every day until constant weights  
159 were achieved. WVP was calculated as follows:

$$160 \text{ WVP (gs}^{-1}\text{m}^{-1}\text{Pa}^{-1}\text{)} = \Delta W \chi / \Delta t A \Delta P \quad (1)$$

161  
162 where  $\Delta W/\Delta t$  is the amount of water gained per unit time of transfer, A is the exposed area of the  
163 samples (0.00020 m<sup>2</sup>),  $\Delta P$  is water vapor pressure difference between both sides of the film (1670  
164 Pa at 25°C, table value) and  $\chi$  is the film thickness. Samples were tested in triplicate.

#### 165 **Moisture sorption**

166 The weighed films were placed inside glass dryers at room temperature containing different  
167 saturated solutions, thus providing different environments with constant relative humidity (RH)  
168 between 38 and 98%. Samples were weighed after predetermined periods of time until 3 different  
169 consecutive measures gave the same results. The moisture content at equilibrium was calculated  
170 on dry samples, preconditioned in stove at 40°C, from which the final isotherms were obtained  
171 (Bajpai, Chand, & Chaurasia, 2010).

#### 172 **2.3.6 Film solubilization and swelling ability**

173 Square- shaped (1cm×1cm) cellulose films were weighted and immersed into 5 mL of distilled water.  
174 After predetermined periods of time, ranging from 2 minutes to 24 hours, wet samples were  
175 removed from water, wiped with filter paper to absorb excess liquid, weighted and put into water  
176 again. The extent of swelling was calculated as follows:

$$177 \text{ Swelling (\%)} = \frac{W_w - W_d}{W_d} \cdot 100 \quad (2)$$

178 where  $W_w$  and  $W_d$  are the weights of the wet and the air-dried sample, respectively.

179 After 24 hours the samples not completely dissolved were removed from water and dried until a  
180 constant weight was obtained. Solubilization as a consequence of the water uptake and dissolution  
181 of the film, as reported by Hosseini et al, (Hosseini, Rezaei, Zandi & Farahmandghavi, 2015) was  
182 calculated as follows:

$$183 \text{ Solubilization (\%)} = \frac{W_i - W_f}{W_i} \cdot 100 \quad (3)$$

184 Solubility tests at longer times (7 and 14 days) were performed in the same way only on the samples  
185 not completely dissolved after 24 hours.

#### 186 **2.3.7 UV-Vis Spectroscopy**

187 In order to evaluate the barrier properties of the films against UV-Vis light, films were cut into 1 cm  
188 wide rectangular strips, which were inserted into the sample holder of the Cary 60 Uv-Vis

189 spectrophotometer. Spectra were acquired in transmittance mode from 200 to 800 nm. The  
190 transparency of the films was evaluated from transmittance at 600 nm, by the following equation

$$191 \quad \text{Transparency} = \frac{-\log T_{600}}{X} \quad (4)$$

192 where  $T_{600}$  is the fractional transmittance at 600 nm and X is the thickness of the film (mm). The  
193 analysis were done in triplicate.

#### 194 **2.3.8 Antibacterial tests**

195 Cellulose-based films were tested *in vitro* for the evaluation of antibacterial activity by a  
196 standardized Kirby-Bauer (KB) diffusion test on Mueller-Hinton agar plate (EUCAST, 2016). For the  
197 analysis, a panel of Gram positive and Gram negative reference bacterial strains were selected:  
198 *Staphylococcus aureus* (ATCC 25923), *Staphylococcus epidermidis* (ATCC 12228), *Enterococcus*  
199 *faecalis* (ATCC 29212), *Escherichia coli* (ATCC 25922), *Pseudomonas aeruginosa* (ATCC 27853), and  
200 *Klebsiella pneumoniae* (ATCC 9591). The effectiveness of the disk-shaped cellulose films ( $\varnothing = 6$  mm)  
201 to inhibit bacterial growth was determined by measuring the diameter of the bacterial-free zone  
202 around the sample after 24 hours of incubation at 37°C. In compliance with the International  
203 guidance documents in susceptibility testing, disks containing gentamicin (GMN 10 µg) and/or  
204 imipenem (IPM 10 µg) (Oxoid SpA, Italy) were included as reference controls (CLSI, 2015). All  
205 experiments were performed in duplicate and in different days.

#### 206 **2.3.9 Biodegradation test**

207 Biodegradation tests of cellulose films were conducted in soil referring to the literature (Zhao, Lyu,  
208 Lee, Cui, & Chem, 2019). Soil was taken from the surface layer in the garden then put in a plastic  
209 tray to a thickness of around 4 cm. The films were cut into small pieces (about 2×2cm<sup>2</sup>), dried at  
210 37°C until a constant weight was raised and then buried about 2 cm beneath soil. The average of  
211 the temperature was about 25 °C. Water was sprayed once on the soil surface to maintain the

212 moisture. The degraded samples and fragments were taken out after 2 and 4 weeks, gently cleaned  
213 from residual soil with distilled water and dried at 37°C until a constant weight was obtained. Finally,  
214 the dried samples were weighted again and the weight loss of the film degraded in soil was  
215 calculated.

#### 216 **2.3.10 Statistical analysis**

217 Statistical analysis was performed with Graph Pad Prism 4. One-way analysis of variance (ANOVA)  
218 followed by Tukey's Multiple Comparison Test was employed to assess statistical significance of the  
219 experimental conditions for Tensile Tests, Water Vapor Permeability and Adhesive strength.  
220 Statistically significant differences were determined at  $p < 0.05$ .

221

### 222 **3. RESULTS AND DISCUSSION**

#### 223 **3.1 Dissolution behaviour and swelling ability of films**

224

225 Solubilization and swelling degree measurements are of particular relevance in order to evaluate  
226 the films stability in aqueous solutions: in fact, a good resistance is needed when films are proposed  
227 for applications such as food packaging (Al-Tayyar, Youssef & Al-Hindi, 2020).

228 The results of water solubility test show that HPMC-based films are highly soluble and their  
229 solubilization in water is immediate, whatever their composition. CMC-based films display a  
230 different and more interesting trend: while in absence of snail extract the films solubilize in few  
231 minutes, CMC\_S70 and CMC\_S100 films preserve their structure for more than two weeks. In fact,  
232 after 24 hours their solubilization (calculated from Eq. 3) accounted for 30% and 45%, respectively,  
233 and these values are unchanged even after 7 and 14 days, suggesting a good resistance of the films  
234 in aqueous solution.

Moreover, both the samples reached a degree of swelling of 100% after 4 hours, with no further significant variation up to 24 hours. These results suggest that the interactions between HPMC and the snail slime are not strong enough to build a network resisting to water permeation, which resulted in an increase of the free volume in the material structure (Bertuzzi, Armada & Gottifredi, 2007).

On the contrary, the significant decrease of the dissolution of the CMC-based films with the addition of S leads to hypothesize that the protonation of the  $\text{COO}^-$  groups into  $\text{COOH}$  could occurs due to acidic pH, as reported in literature (Qiu, Shaoa, Liuc, Wanga, Lic & Zhao 2014). Since CMC is less soluble than CMC sodium salt the occurrence of this transformation might account for the reduced solubility of CMC\_S70 and CMC\_S100.

### 3.2 Structural characterization

The infrared spectra collected from E5 and E50 are reported in Figure 1 a,b. The characteristic absorption bands of HPMC, in accordance with those reported in literature (Ding, Zhang & Li, 2015), can be detected: in particular, the absorption bands at  $3500\text{ cm}^{-1}$ ,  $1060\text{ cm}^{-1}$  and around  $2915\text{ cm}^{-1}$  are due to O-H, C-O and C-H stretching vibration, respectively, whereas the absorption band around  $1457\text{ cm}^{-1}$  is characteristic of the  $\text{CH}_3$  asymmetric bending vibrations. S addition provokes the appearance of new bands, centered at  $1717$ ,  $1390$  and  $1224\text{ cm}^{-1}$ , which can be attributed to the high amount of Allantoin and glycolic acid contained into Snail Mucus extract (see Table S1 and Figure S2).

In the FTIR spectra collected from CMC-based films (Figure 1c) the bands belongings to the functional groups of CMC are well recognizable: the O-H stretching and bending vibrations occur at  $3385\text{ cm}^{-1}$  and  $1324\text{ cm}^{-1}$ , respectively, while antisymmetric and symmetric vibrations bands of  $\text{COO}^-$  are at  $1600$  and  $1413\text{ cm}^{-1}$ . The absorption band at  $1059\text{ cm}^{-1}$  arises from the asymmetric stretching of glycosidic bridge C-O-C. The low intensity band centered at around  $900\text{ cm}^{-1}$  could be

259 attributed to the  $\beta$ - glycosidic linkages between sugar units (Tong, Xiao & Lim, 2008). As observed  
260 for HPMC-based films, introduction of snail extract into CMC-based films strongly modifies the IR  
261 spectra, which in addition display an impressive broadening. Figure 1c clearly shows the strong  
262 reduction of the intensity of the band at  $1593\text{ cm}^{-1}$  (attributable to the asymmetric stretching  
263 vibration of free carboxyl groups in the salt form), and the appearance of two bands at  $1717$  and  
264  $1224\text{ cm}^{-1}$  as a consequence of S addition. The band at  $1717\text{ cm}^{-1}$  could be due both to S addition  
265 (as stated above) and to the formation of COOH groups on the side chain of CMC, as a consequence  
266 of pH lowering. The reduction of the charge on the side chains supports the strong effect observed  
267 on the swelling properties and on the water uptake ability of CMC\_S70 and CMC\_S100, as well as  
268 the decrease in solubility.

269 The X-ray patterns recorded on cellulose-based films are reported in Figure 2a-c. It is known that  
270 crystallinity of cellulose is associated with strong hydrogen bonding interaction of cellulose  
271 (intermolecular and intramolecular) and Van der Waals forces between adjacent molecules. During  
272 the processing of cellulose, reactions of methylation and carboxymethylation result in the extending  
273 the distance between cellulose molecules, thus disrupting hydrogen bonds and hence lowering the  
274 crystallinity of the polymers (Sunardi, & Ahmad 2017). As a matter of fact, the reference films show  
275 the characteristic diffraction patterns of a poorly crystalline material, with two broad reflections at  
276  $9.5\text{-}12^\circ/2\theta$  and  $20.0\text{-}21.5^\circ/2\theta$  characteristic of cellulose II (Kamide et al., 1985), which is  
277 obtained by means of chemical and physical treatments of Cellulose I, the most abundant form  
278 found in nature (O'sullivan, 1997; Pérez & Mazeau, 2005).

279 The broadness of the reflections increases on increasing slime content, in agreement with a  
280 decrease of crystallinity. This effect, even more evident in the patterns of CMC films where the signal  
281 between  $9.5$  and  $12^\circ/2\theta$  is no longer appreciable, might be attributable to the presence of S  
282 interlaid between the polymer chains.

### 283 **3.3 Barrier properties**

284 The barrier properties of a polymeric film are crucial features to predict the behaviour of the  
285 material as well as the shelf-life of the product when used as a food packaging (Siracusa, Rocculi,  
286 Romani & Rosa, 2008) and they derive mainly from the permeability of the film to gases and vapors,  
287 that are noxious to the quality of the product (Zeman & Kubik, 2007).

288 The thickness measurements of the films are reported in Table 2: thickness ranged from 29 to 171  
289 microns. For every type of cellulose, the thickness increased on increasing the S amount. As all the  
290 films were prepared by casting the same amount of solution into Petri dishes with 8.5 cm in  
291 diameter, the observed trend could be due to the increasing of the dry matter. In fact, the snail  
292 extract contains approximately a 5% w/v of dry matter, and so, on increasing the S content the total  
293 amount of dry matter also increases and the thickness is greater. As thickness influences mechanical  
294 and barrier properties, all the values are normalized with respect to thickness.

#### 295 **Water Vapor Permeability (WVP)**

296 Prevention of moisture transfer between food and the surrounding atmosphere or between two  
297 different food products is a main requirement of packaging films (Bajpai, Chand, & Chaurasia, 2010).  
298 Water vapor permeability (WVP) was used to test whether moisture can easily penetrate and pass  
299 through a substance and the results for the different film compositions are reported in Figure 3.

300 The data show that the introduction of snail extract into film composition strongly influences the  
301 WVP, which decreases by one or two orders of magnitude as a function of S content: for example,  
302 the WVP values vary from  $1.3 \cdot 10^{-10}$  to  $6.5 \cdot 10^{-12}$  g·m/s·m<sup>2</sup>·Pa when measured on E5 and E5\_S100,  
303 respectively.

304 This trend could be explained by the formation of a polymeric network within the films: the different  
305 internal structure could lead to the creation of fewer empty spaces, preventing or hindering the  
306 diffusion of water molecules through the films. Greater S contents (S70 and S100) provoke a greater

307 barrier effect, independently from the HPMC molecular weight (E5 and E50). In addition, the  
308 decrease in WVP of CMC-based films could also be due to a decrease in the hydrophilicity and  
309 solubility of the films as the Solution content increases (Muppalla, Kanatt, Chawla & Sharma, 2014).

#### 310 ***Moisture sorption***

311 The water sorption isotherm represents the relationship between equilibrium moisture content and  
312 water activity at a given temperature and it is the major tool to describe and predict the water  
313 mobility in the films at different environments (Cazon, Velazquez & Vázquez, 2020). Water sorption  
314 isotherms of HPMC based films (see Figure S3 a) showed an initial slight increase in moisture content  
315 at lower RH values, while a rapid increase in superficial water adsorption was observed at RH values  
316 higher than 60%, a typical behaviour of hydrophilic materials (Enrione, Hill & Mitchell, 2007;  
317 Gontard, Guilbert & Cuq, 1993; Villalobos, Hernández-Muñoz & Chiralt, 2006).

318 For HPMC films (E5 and E50) the snail slime addition implies an increase in the moisture absorption,  
319 which could be due to the creation of additional hydrogen bonds between the exposed groups on  
320 the surface and the water molecules (Enrione, Hill & Mitchell, 2007). The increasing of moisture  
321 adsorption matched the increasing and broadening of the band centered at about  $3400\text{ cm}^{-1}$  in the  
322 infrared spectra reported in Figure 1 a-b.

323 On the other hand, the observed decrease in the moisture absorption of CMC films on increasing S  
324 content (see Figure S3 b) suggests that the formation of an insoluble network reduces the number  
325 of the surface groups which can form hydrogen bond.

#### 326 ***UV barrier, light transmittance and transparency value***

327  
328 Transparency of films for food packaging applications is one of the main requirements in the  
329 packaging industry (Haghighi et al., 2019a), as well as the UV barrier properties are a key feature to  
330 prevent chemical reactions induced by UV light in food (Wu, Sun, Guo, Ge & Zhang, 2017). In fact,  
331 the UV-radiations are responsible for the activation of reactions such as lipid oxidation, vitamins



332 oxidation or loss of colour which result in a loss of the quality of packaged foods (Guo, Ge, Li, Mu &  
333 Li, 2014). However, the mechanisms that allow films to acquire the UV light barrier properties can  
334 affect their transparency. The spectra acquired in transmittance mode on the cellulose-based films  
335 in the UV-visible region are shown in Figure 4. In the UV region (200-280 nm) samples E5, E50 and  
336 CMC show high transmittance values (see Figure 4a, 4b and 4c, respectively), which rapidly fall to  
337 zero after S addition. As reported in literature (Haghighi et al., 2019b), a transmittance value below  
338 10% at 280 nm indicates that the films have effective UV barrier properties. It can be concluded that  
339 S addition confers excellent UV barrier properties both to HPMC and CMC based films (see Figure 4  
340 a-c), regardless the S content.

341 By comparing spectra in figure 4 a-c it is worth of note that S-containing films showed also a lower  
342 transmittance in the visible range (400-800 nm) than the control films, indicating that the  
343 incorporation of snail extract into the film composition had a strong effect on the barrier properties  
344 also against visible light. A quantitative evaluation of this important feature is obtained by using  
345 equation 4 and the obtained transparency values are reported in Table 2. According to this value,  
346 the greater is the transparency value the lower is the transparency of the film (Nur Hazirah, Isa &  
347 Sarbon, 2016, Guo et al. 2014, Theerawitayaart, Prodpran, Benjakul, & Sookchoo, 2019). For E50 and  
348 CMC based films, the transparency decreases on increasing the amount of S, while S addition has a  
349 minor effect on E5-based films. Anyway, all the transparency values are lower than 5, and hence the  
350 films can be considered transparent, as reported in literature. The mechanism of action of the snail  
351 mucus could be due to the combination of two elements: the absorption of UV rays thanks to the  
352 presence of proteins containing aromatic amino acids and the decrease in transparency that  
353 prevents the visible light passing through the films (Hamaguchi, WuYin & Tanaka, 2007) that can be  
354 attributed to the effect of the macromolecular components of S dispersed into the biopolymers

355 matrix, which could change the optical properties of the material. This effect is more pronounced  
 356 for E50 and CMC -based films probably due to the higher viscosity of these solutions.

357 Table 2. Thicknesses and transparency values of the obtained cellulose-based films.

Labels	Thickness ( $\mu\text{m}$ )	$T_{600}/100$	Transparency Values
E5	84	0.90	0.545
E5_S30	113	0.87	0.535
E5_S70	158	0.85	0.447
E5_S100	171	0.84	0.443
E50	59	0.90	0.776
E50_S30	85	0.76	1.402
E50_S70	126	0.65	1.485
E50_S100	137	0.50	2.197
CMC	29	0.92	1.249
CMC_S70	60	0.77	1.892
CMC_S100	91	0.40	4.373

358  
 359

### 360 3.4 Mechanical properties

361 The tensile strength at break ( $\sigma_b$ ), the elastic modulus (E) and the deformation at break ( $\epsilon_b$ ) are  
 362 reported in Table 3, while the stress-strain curves are shown in Figure 5.

363 Table 3. Effect of S incorporation on thicknesses and tensile properties of cellulose -based films.  
 364

Labels	$\sigma_b$ (MPa)	$\epsilon_b$ (%)	E (MPa)
E5	$50 \pm 6$	$16 \pm 8$	$1760 \pm 400$
E5_S30	$31 \pm 3^{**}$	$13 \pm 5$	$1220 \pm 90$
E5_S70	$18 \pm 1^{***}$	$11 \pm 3$	$720 \pm 100^{**}$
E5_S100	$3 \pm 1^{***}$	$61 \pm 8^{***}$	$72 \pm 10^{***}$
E50	$54 \pm 10$	$22 \pm 7$	$2020 \pm 140$
E50_S30	$49 \pm 10$	$16 \pm 7$	$1920 \pm 430$
E50_S70	$31 \pm 3^*$	$34 \pm 5$	$860 \pm 80^{**}$
E50_S100	$13 \pm 1^{***}$	$46 \pm 1^{**}$	$140 \pm 20^{***}$
CMC	$32 \pm 4$	$2 \pm 1$	$2750 \pm 600$
CMC_S70	$5 \pm 1^{***}$	$29 \pm 3^{***}$	$44 \pm 14^{***}$
CMC_S100	$2.7 \pm 0.3^{***}$	$67 \pm 6^{***}$	$2 \pm 1^{***}$

365 Each value is the mean of ten determinations and is reported with its standard deviation.  
 366 (\*p < 0,05; \*\*p < 0,01; \*\*\*p < 0,001 compared to the control)  
 367

Both HPMC and CMC films exhibit a high stress at break but they can be extended only by few units percent. Enrichment of the formulation by S addition greatly enhances films extensibility, whereas it reduces the stress at break and the elastic modulus. Furthermore, the different series of films display different mechanical behavior upon S addition as shown by the stress-strain curves reported in Figure 5: in particular, while E50\_S100 and CMC\_S100 show an elastic behavior, E5\_S100 seems to display a plastic behavior.

The effect produced by S addition to cellulose-based films is similar to that obtained on S-containing chitosan films (Di Filippo et al., 2020): probably, as a consequence of the interactions between S and the polymer chains, the intermolecular interactions between cellulose molecules are reduced, thus facilitating their sliding and improving their mobility.

According to conventional standard (Hosseini et al., 2015; Kim, Lee & Park, 1995), the tensile strength of packaging films must be higher than 3.5 MPa: in this study, all the prepared films, except E5\_S100 and CMC\_S100, meet this requirement.

### **3.5 Adhesion studies**

As reported in literature for chitosan-based films (Di Filippo et al., 2020), also cellulose-based films become sticky after the S addition: the adhesive properties, expressed in terms of force needed for film detachment (F), are reported in Figure 6. In order to evaluate a possible application of our films in the field of food packaging, adhesive studies were conducted by using aluminum and glass as support: these materials are in fact very used as domestic food container.

Cellulose-based films exhibit good adhesive performances on both the substrates, even if some differences should be remarked. In particular, the adhesion force on glass support shows a fairly linear trend on increasing the amount of S. Furthermore, films based on HPMC E5 are stickier than the others even without S, although its addition considerably increases this property. On the other

391 hand, CMC based films are not very sticky on glass, but the addition of S surprisingly increases the  
392 adhesiveness of almost 10 units.

### 393 **3.6 Preservation of apple cubes.**

394 The effectiveness of the obtained films to preserve the freshness of food and to remain attached to  
395 the container for a desirable period of time was qualitatively assessed using a fresh apple purchased  
396 in a local shop at commercial maturity and immediately used, following a method reported in  
397 literature suitably modified (Shyu, Chen, Chiang & Sung, 2008).

398 The apple was cut into cubes of regular dimensions (side about 1 cm) and arranged into a glass  
399 container. The container was covered with the film CMC, as shown in Figure 7, and then placed in  
400 the refrigerator at 4°C. As a control, apples cubes were maintained in the refrigerator without any  
401 coverage. Digital photos were acquired at time zero and after ten days of storage. After ten days of  
402 storage at 4°C, the uncovered apple cubes became dried and darker while those covered with the  
403 film showed an excellent state of preservation, allowing only a slight enzymatic browning on their  
404 surface (see Figure 7) thus demonstrating the efficacy of CMC\_S70 in the preservation of food.  
405 Moreover, it is important to underline that the film is able to immediately adhere to the glass  
406 container and to remain attached to it for the whole time of storage.

407 A further investigation was made by cutting a small slice of apple and wrapping it with the CMC\_S70  
408 film in order to check its appearance after ten days of storage in the refrigerator at 4°C.

409 The adhesiveness of the CMC\_S70 film on itself and on the surface of an apple together with its  
410 effectiveness in food preservation was evaluated from a qualitative point of view. In fact, from the  
411 comparison of the digital photos of the apple slice wrapped with CMC-\_S70 taken at time zero and  
412 after ten days of storage in the refrigerator at 4°C (Figure 8 a,b) no significant oxidative processes  
413 have damaged the state of apple conservation.

414

### 3.7 Antibacterial tests

The antibacterial activity of the different cellulose-based films was assessed *in vitro* by means of a disk agar diffusion method against both Gram positive and Gram-negative bacteria. Results are reported in Figure 9 a,b.

All cellulose films without S did not show any inhibition on the tested bacteria. However, the antimicrobial activity of S containing films on contact surface around the discs was evident, indicating the antibacterial effect of snail mucus. This is further confirmed by the increased diameter of the bacterial-free zone for the samples at the highest S content. Disks used for antibacterial assay were weighted and the S content of each composition was determined, taking into account they contain about 6% wt of residual water. Considering the weight of the cellulose-based films, and the amount of snail mucus on the different 6 mm-diameter disks, it is evident that the inhibitory activity of the samples is strictly related to S content, as reported in figure 10.

Of note, even the cellulose-based disks containing S at 30% displayed a significant inhibitory zone for *Pseudomonas aeruginosa*, one of the most versatile pathogen that is present in a variety of environments, including soil and water, and intrinsically resistant to numerous antibacterial agents. It is an opportunistic pathogen responsible for a broad spectrum of infections as respiratory tract and urinary infections, primary skin infections, ear and eye infections.

### 3.8 Biodegradability test

Biodegradation tests were conducted in soil, as described in the experimental part. After 2 weeks, only fragments of the sample CMC\_S70 were still present: a weight loss of 54% was calculated. After 4 weeks, sample CMC\_S70 was fully biodegraded. The biodegradation data meet very well to the results reported in literature (Zhao, Lyu, Lee, Cui, & Chem, 2019) and represent an added value of the snail-containing cellulose films.

#### 442 4. CONCLUSIONS

443 The results of this work demonstrate that the use of snail mucus extract in the preparation of  
444 cellulose derivatives-based films provides materials characterized by high transparency, excellent  
445 UV barrier properties and very good WVP. Addition of the snail slime results in an increase of the  
446 extensibility, together with a decrease of the stress at break and of the elastic modulus, in all the  
447 three different types of cellulose (HPCM E5, HPCM E50 and CMCNa) films. Moreover, all the films  
448 prepared in the presence of S display enhanced adhesion towards glass and aluminum and a  
449 significant antibacterial activity against both Gram positive and Gram-negative bacteria.  
450 However, the structural characterization evidenced that the snail extract establishes different  
451 interactions with the internal structures of the different types of celluloses. In fact, while immersion  
452 in water causes immediate solubilization of all HPCM based films, the addition of high amounts of S  
453 to the composition of CMC-based films makes them insoluble for more than a week, thus allowing  
454 their use for food packaging. In addition, all the prepared films are fully biodegradable in few weeks.  
455 On the basis of these results, it can be inferred that snail-enriched CMC based films might find  
456 potential applications that require direct contact with food and they are good candidates to replace  
457 synthetic polymers in the packaging industry.

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## CAPTION TO THE FIGURES

Figure 1. Infrared spectra of cellulose-based films containing different amount of S: a) E5\_based films; b) E50-based films; c) CMC-based films. The arrows indicate the most intense bands due to S.

Figure 2. X-rays diffraction patterns of cellulose-based films containing different amount of S: a) E5\_based films; b) E50-based films; c) CMC-based films.

Figure 3. Water Vapor Permeability of cellulose-based films ( $***p < 0.001$ ).

Figure 4. UV-Vis spectra collected on cellulose-based films containing different amount of S: a) E5-based films; b) E50-based films; c) CMC-based films. Photographs of (from left to right): E5\_100, E50\_100 and CMC\_100 films.

Figure 5. Stress-strain curves of films obtained by mixing different amount of S with: a) E5, b) E50 and c) CMCNa.

Figure 6. Detachment forces (N) of films from glass and aluminum supports ( $*p < 0,05$ ;  $**p < 0,01$ ).

Figure 7: Apple cubes covered with the film CMC\_S70 (left) and uncovered (right), at time 0 and after ten days of storage at 4°C.

Figure 8. Apple slice wrapped with the CMC\_S70 film a) immediately after cutting and b) after ten days of storage at 4°C.

Figure 9. Antibacterial activity of cellulose-based disks against: a) Gram-positive bacteria; b) Gram-negative bacteria. Data are the mean value of the diameter (in mm) of the clear bacterial free-zone measured around the disk samples.

Figure 10. Antimicrobial activity of the cellulose-based films. Each dot plot is the bacterial-free zone (in mm) measured around the 6 mm diameter disks, for the six reference strains tested in different independent assays. Horizontal lines indicate the median values. S content (in mg) is obtained

504 considering the weight of the film samples and the amount of snail mucus used for their  
505 preparation.

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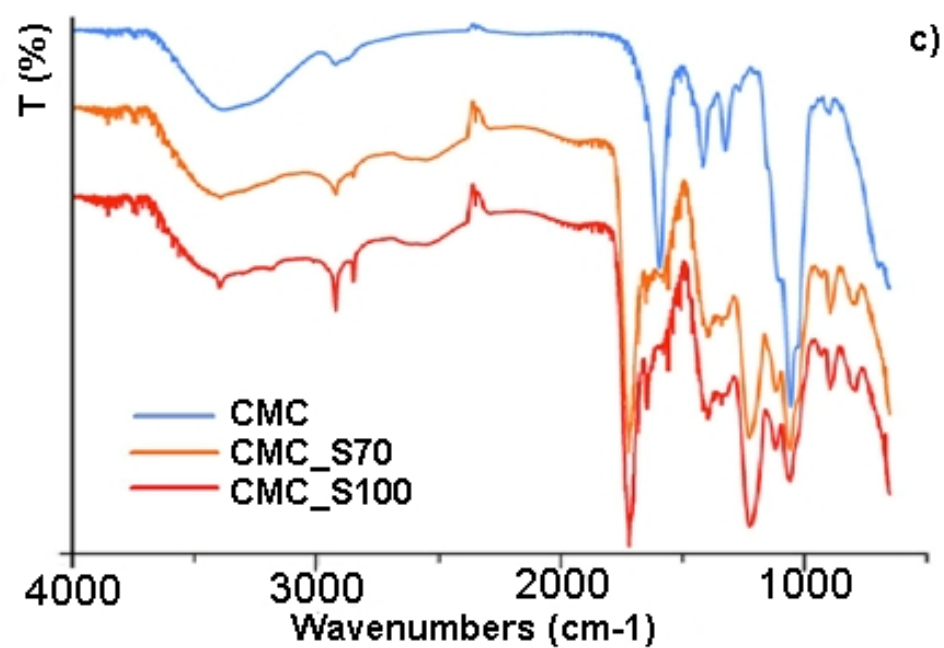
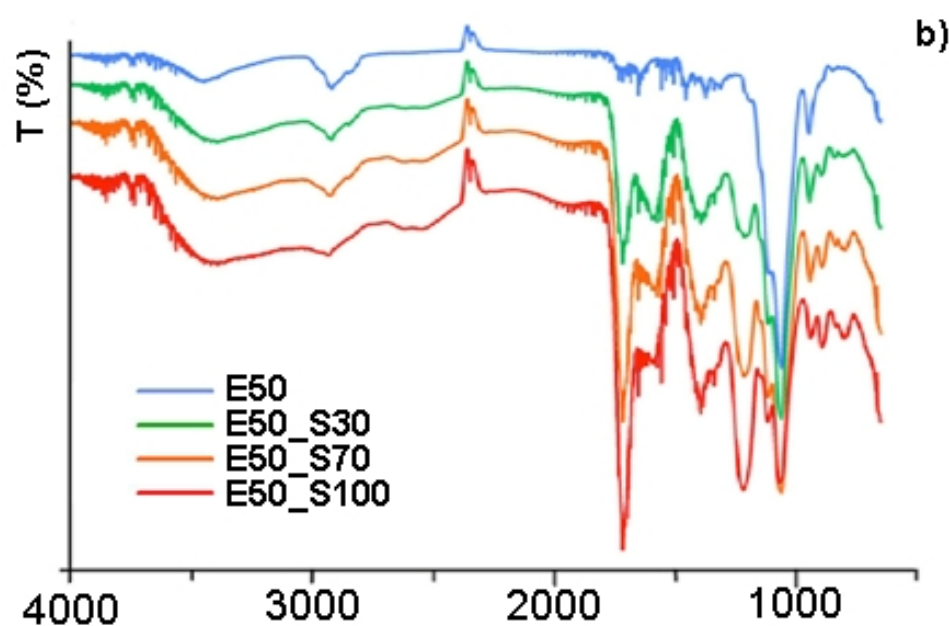
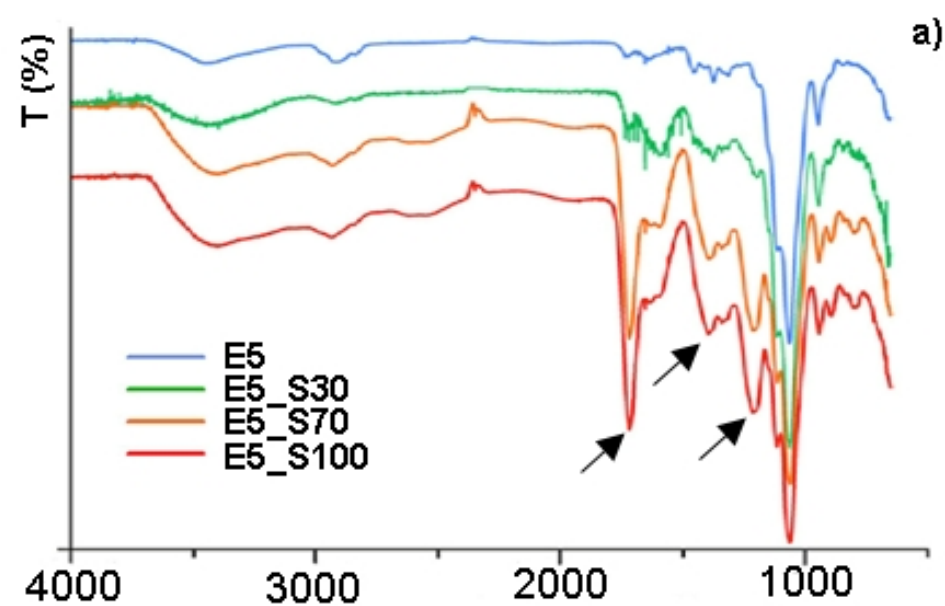
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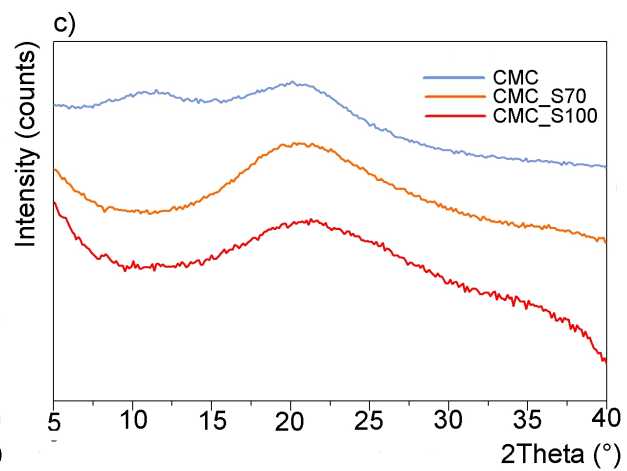
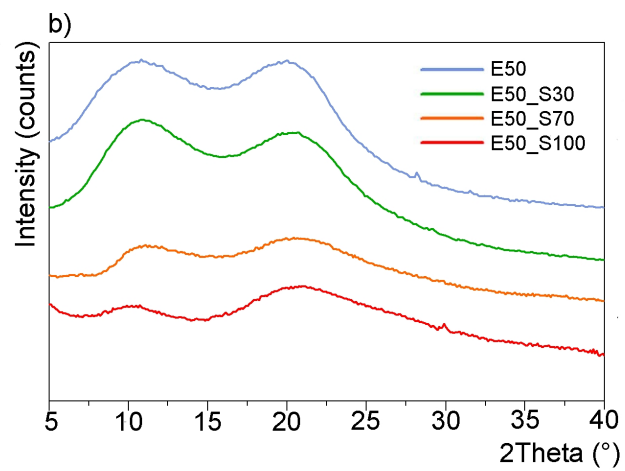
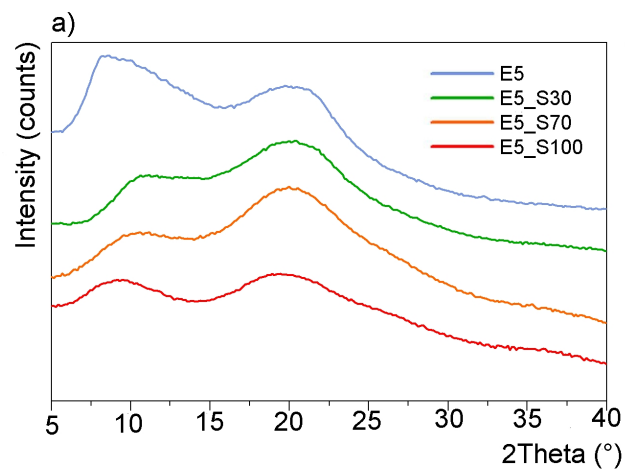
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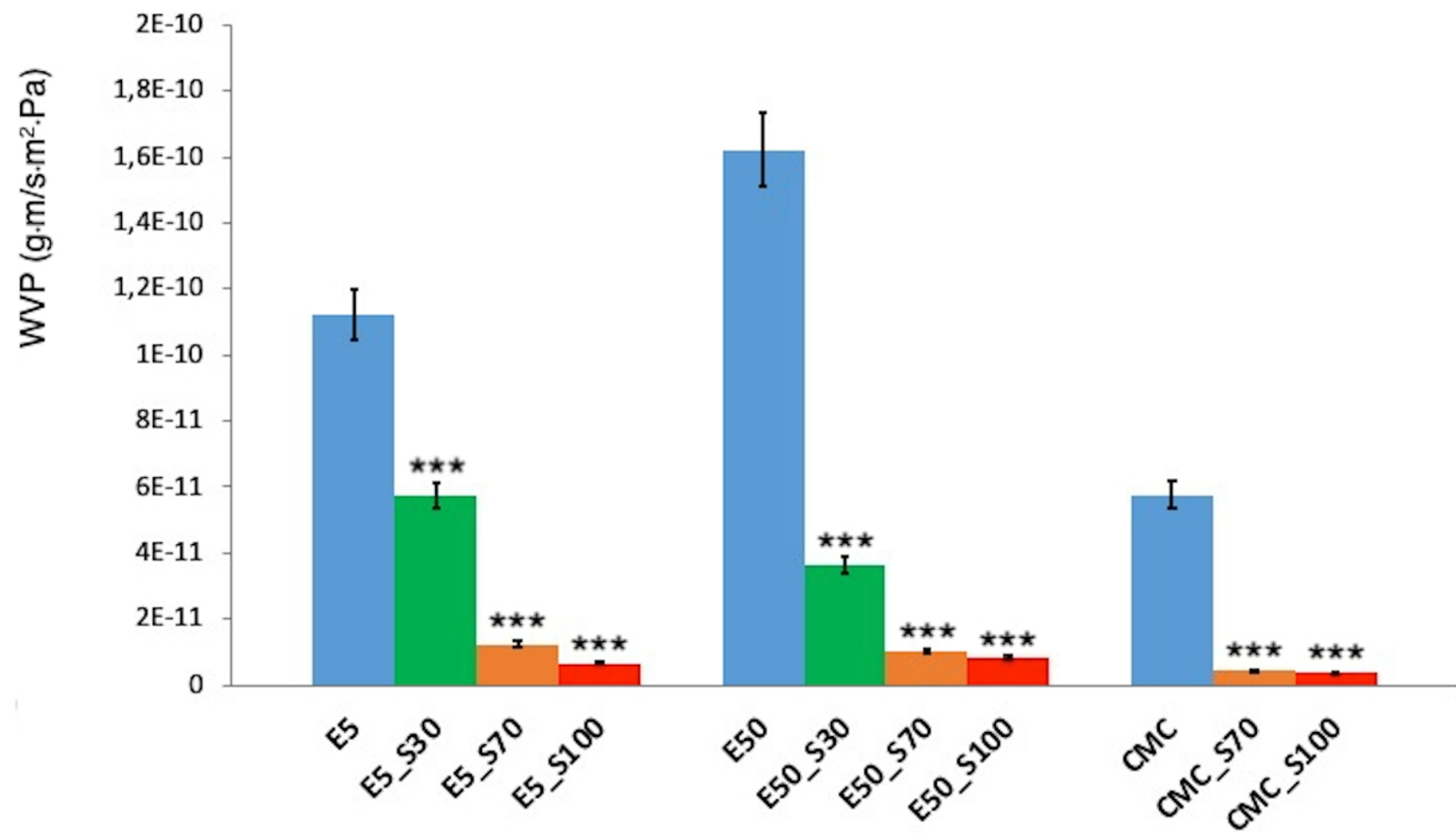
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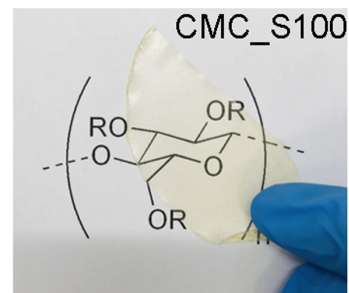
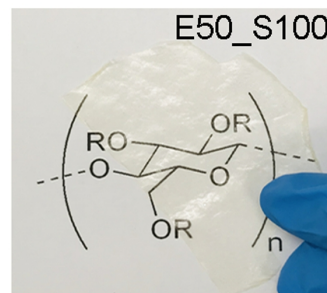
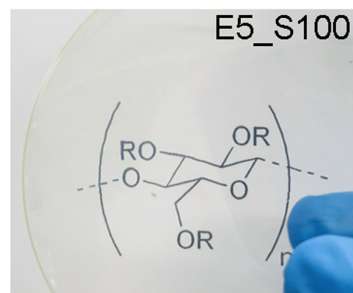
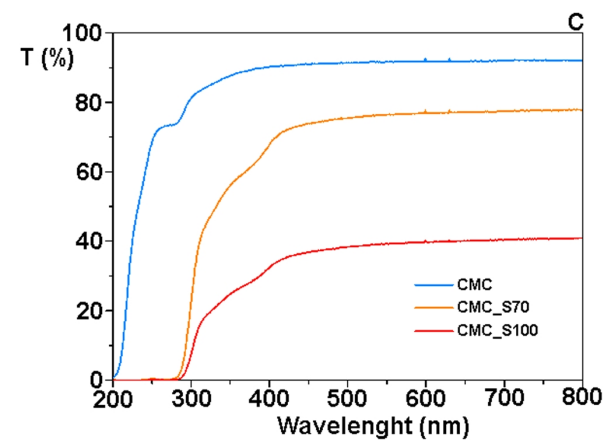
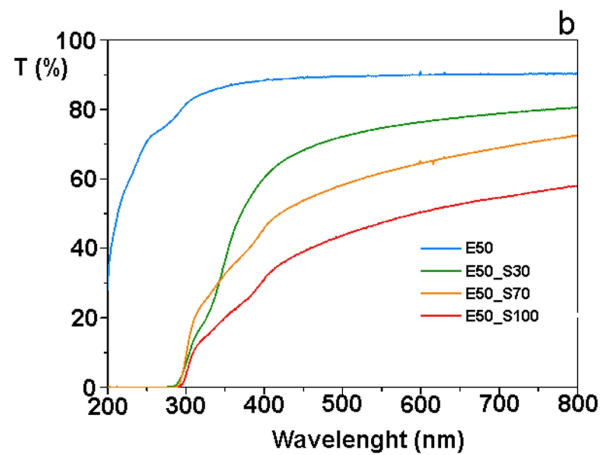
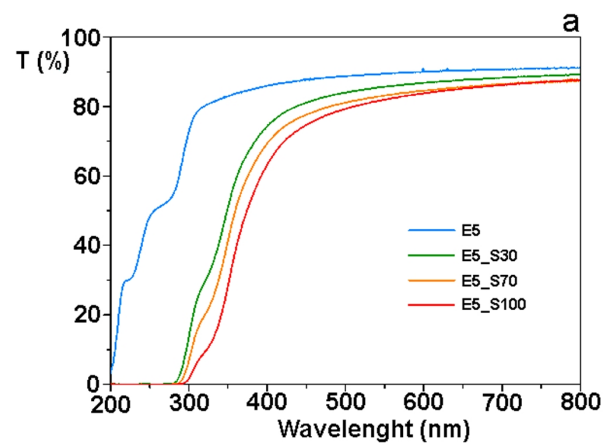
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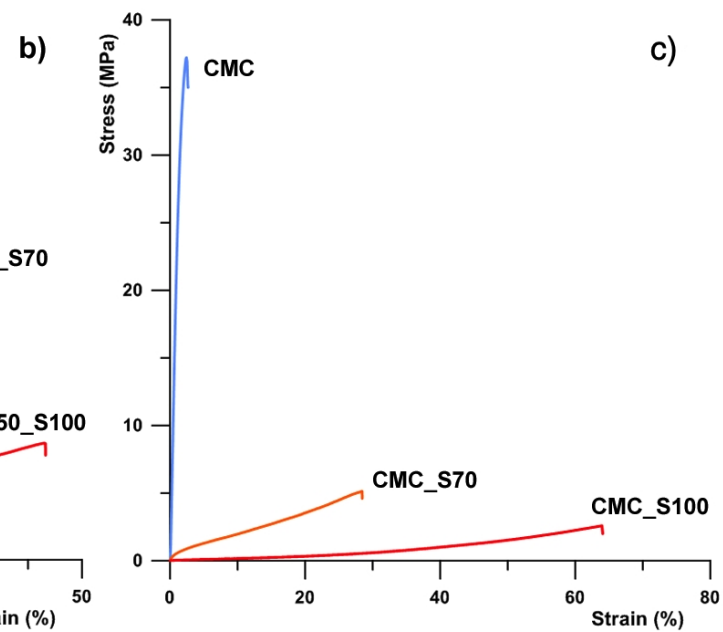
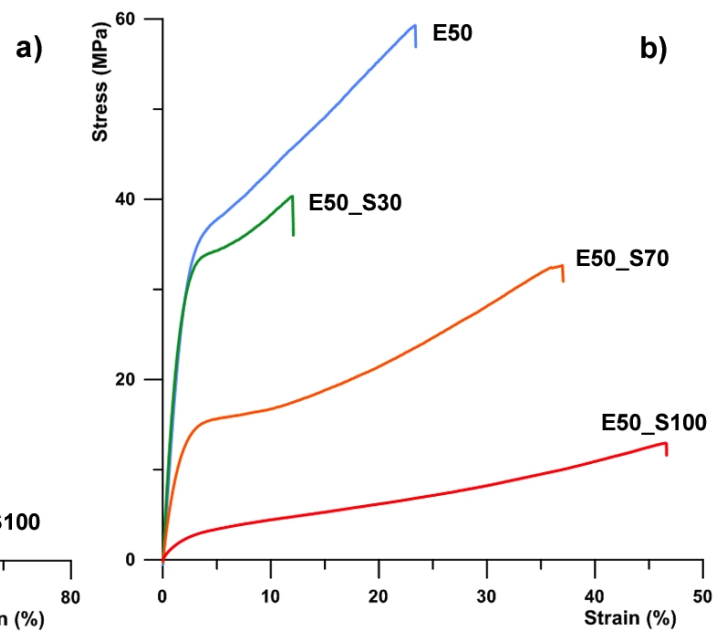
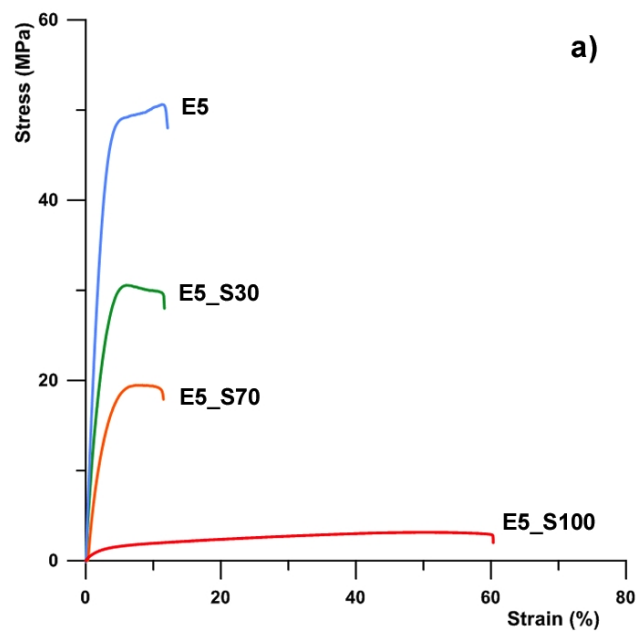


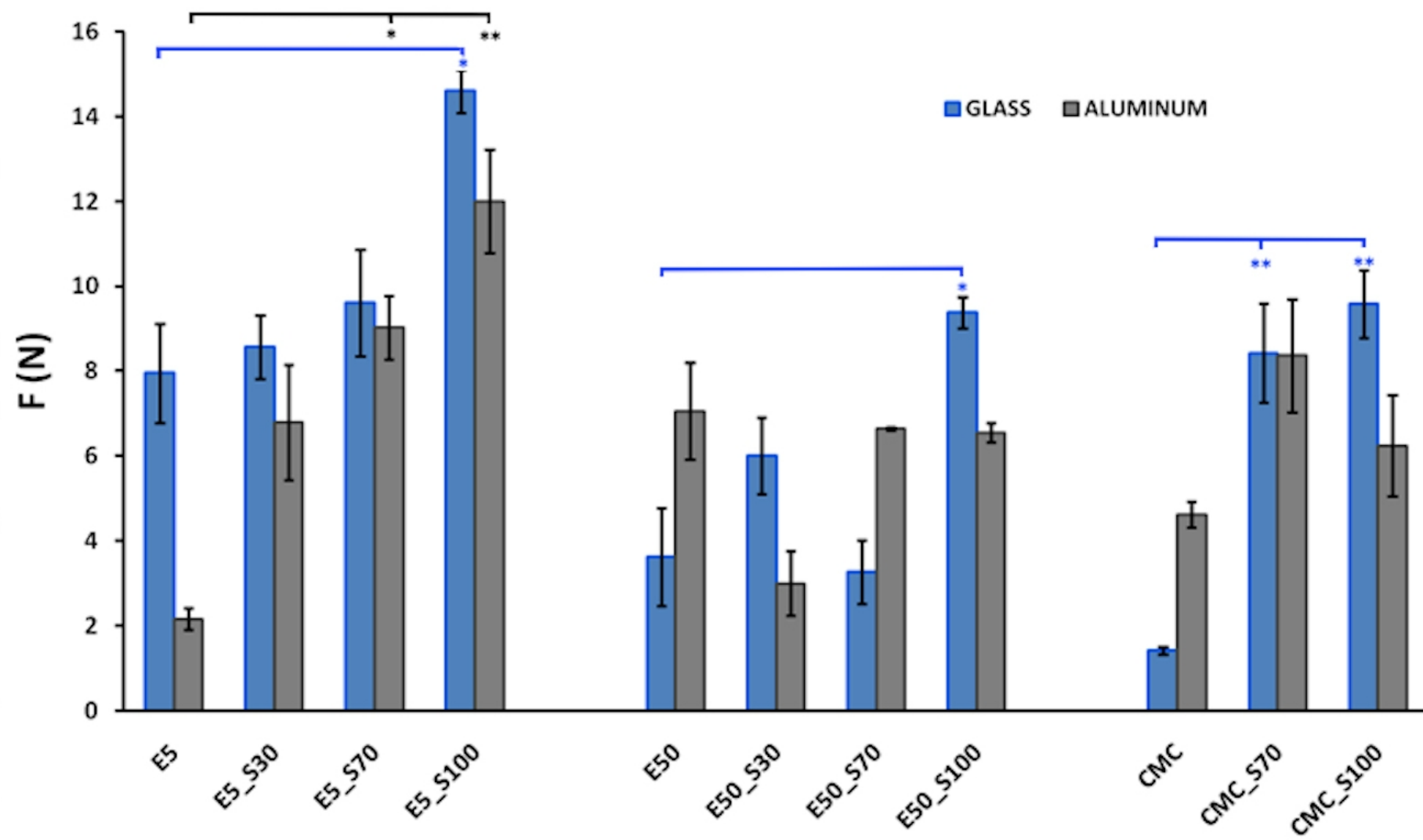








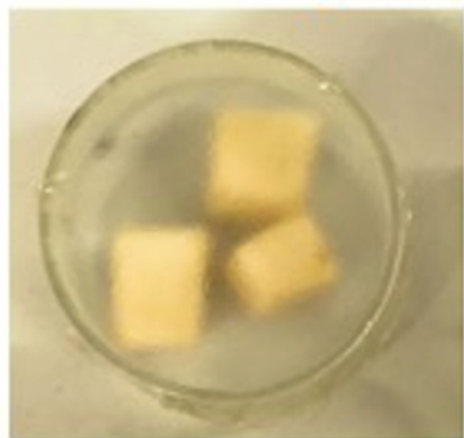


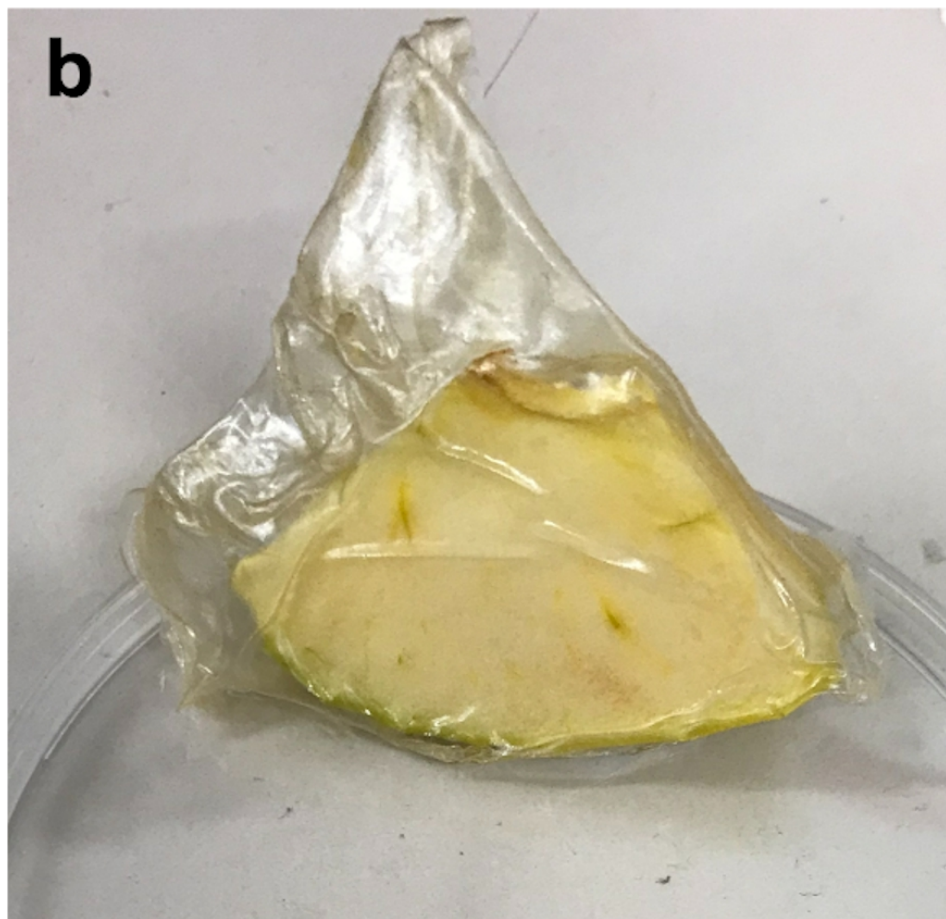
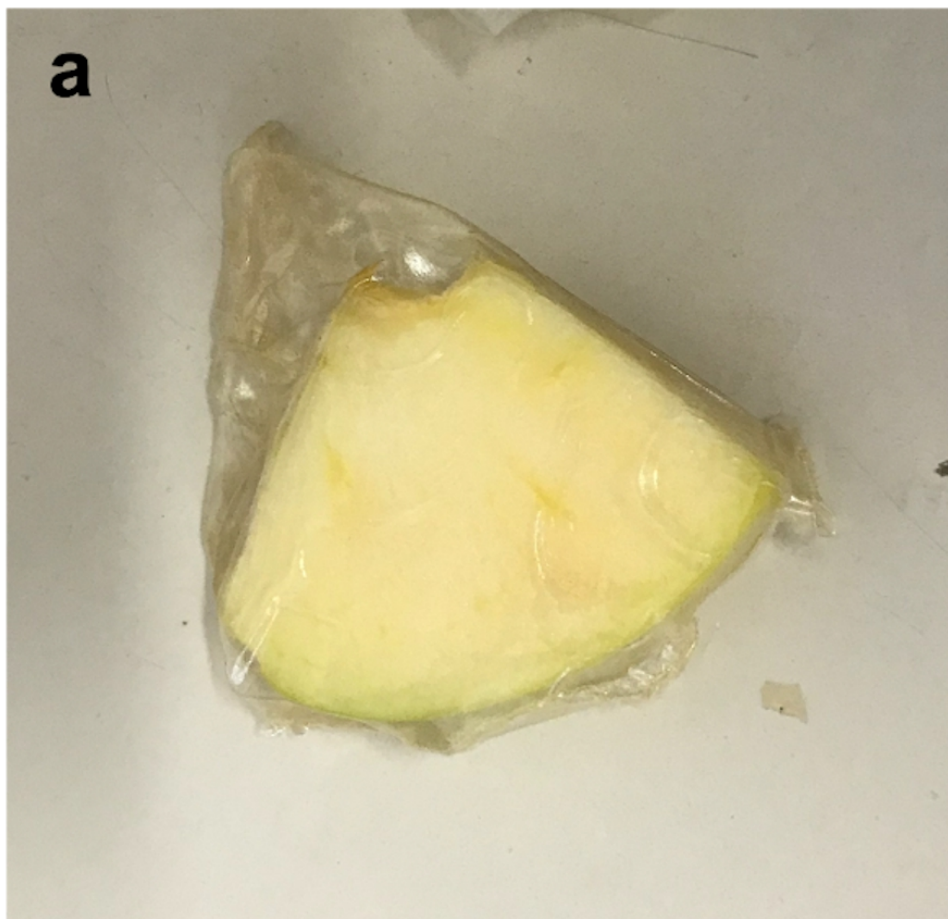


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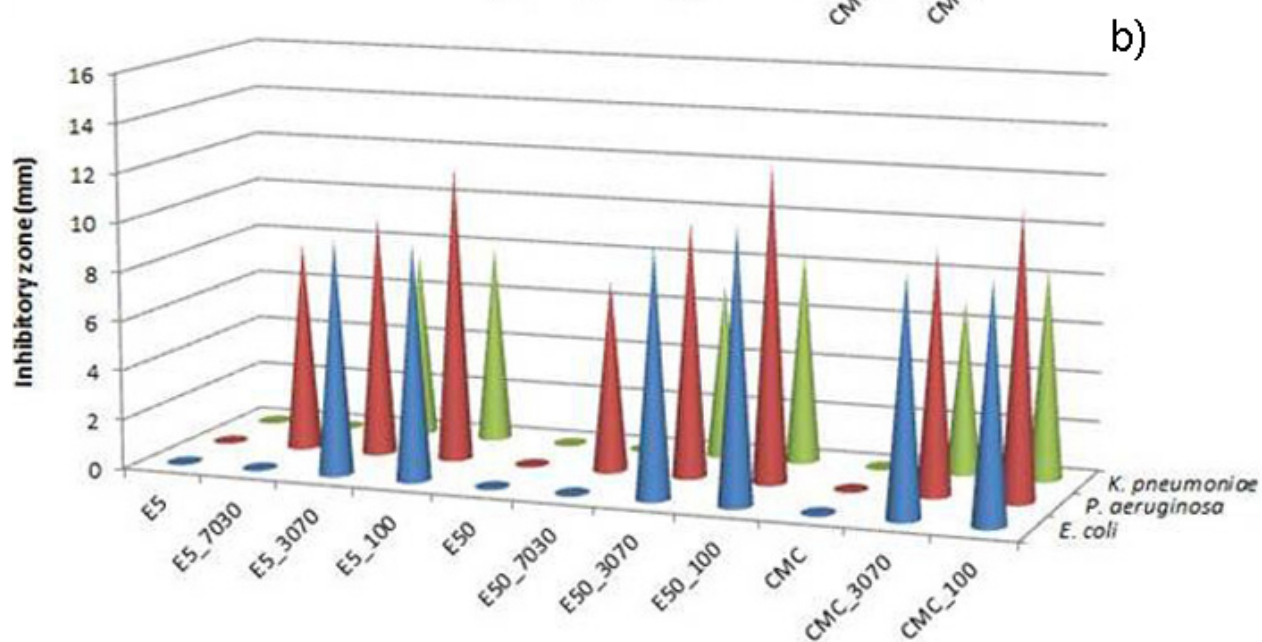
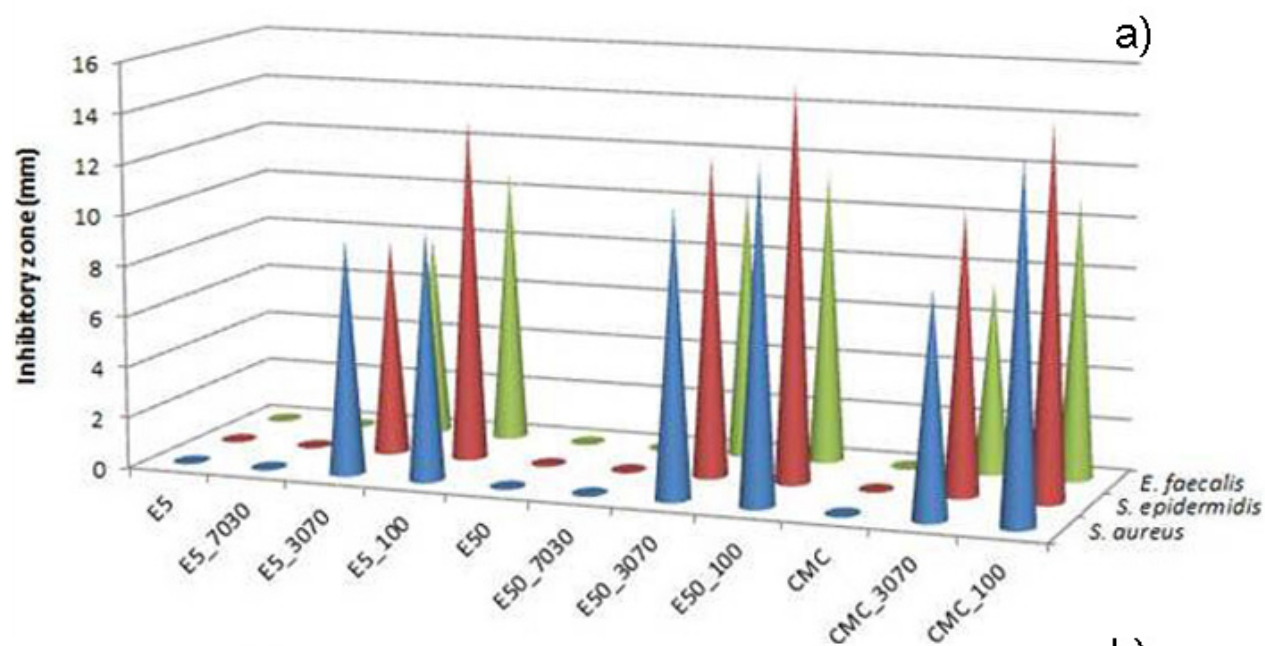


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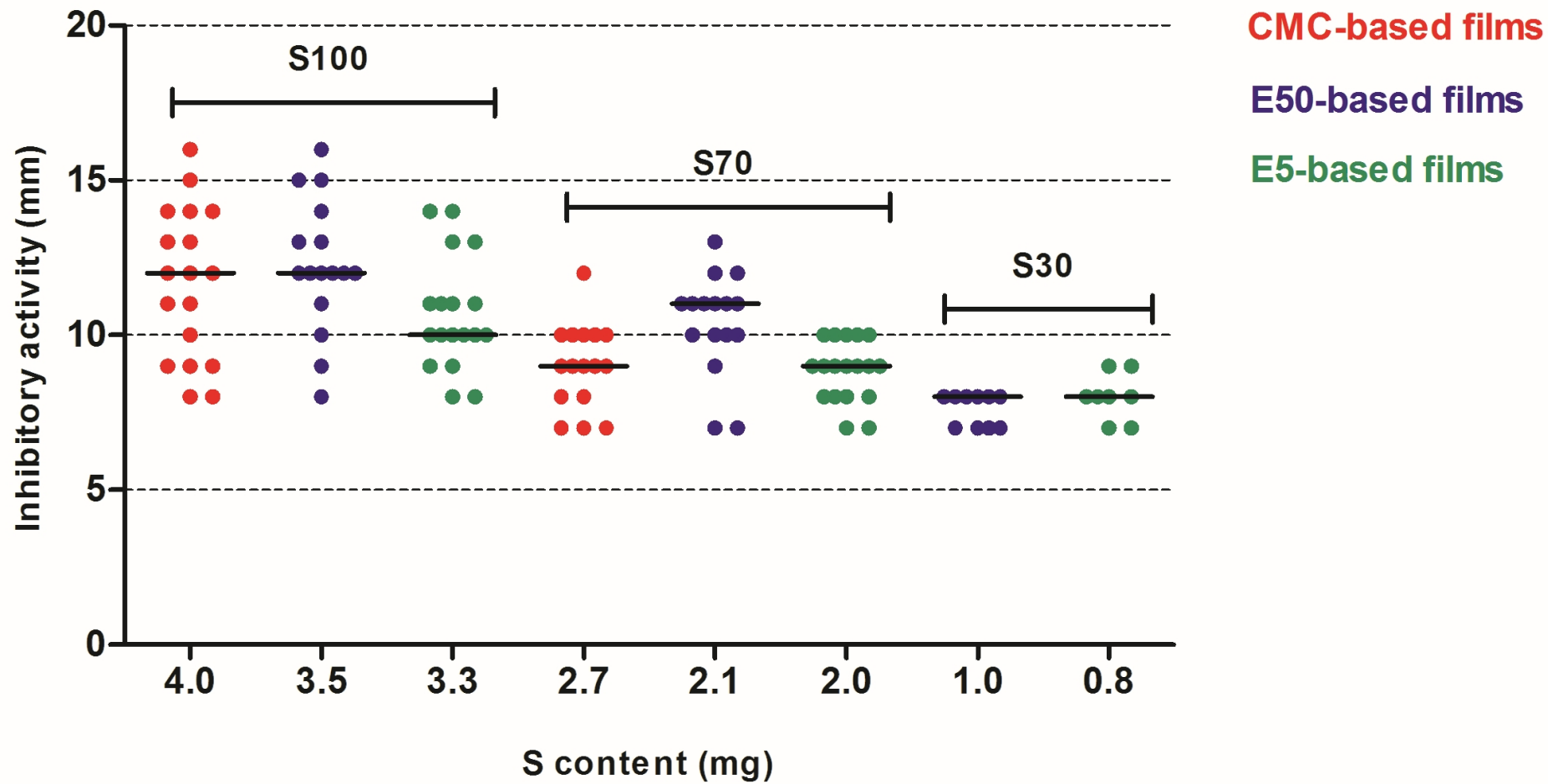












**Declaration of competing interest**

The authors declare that they have no conflict of interest in the publication of this manuscript. This study is original research that has not been published previously, and not under consideration for publication elsewhere.

## CRediT authorship contribution statement

Maria Francesca di Filippo: Conceptualization, Methodology, Writing - original draft

Luisa stella Dolci: Conceptualization, Methodology, Writing - original draft

Letizia Liccardo: Data curation, Formal analysis, Writing - original draft

Silvia Panzavolta: Supervision, Data curation, Writing - review & editing.

Beatrice Albertini: Data curation, Writing - review & editing.

Nadia Passerini: Validation, Resources

Giovanna Angela Gentilomi: Validation Francesca Bonvicini: Data curation, Investigation

Adriana Bigi: Resources, Review.

# CELLULOSE DERIVATIVES-SNAIL SLIME FILMS: NEW DISPOSABLE ECO-FRIENDLY MATERIALS FOR FOOD PACKAGING

Maria Francesca Di Filippo<sup>1§</sup>, Luisa Stella Dolci<sup>2§</sup>, Letizia Liccardo<sup>1</sup>, Adriana Bigi<sup>1</sup>, Francesca Bonvicini<sup>3</sup>,  
Giovanna Angela Gentilomi<sup>3</sup>, Nadia Passerini<sup>2</sup>, Silvia Panzavolta<sup>1\*</sup>, Beatrice Albertini<sup>2</sup>

<sup>1</sup>Department of Chemistry "G. Ciamician", University of Bologna, Via Selmi 2, 40126, Italy;

<sup>2</sup>Department of Pharmacy and BioTechnology, University of Bologna, Via S. Donato 19/2, 40127, Italy;

<sup>3</sup>Department of Pharmacy and Biotechnology, University of Bologna, Via Massarenti 9, 40138, Italy

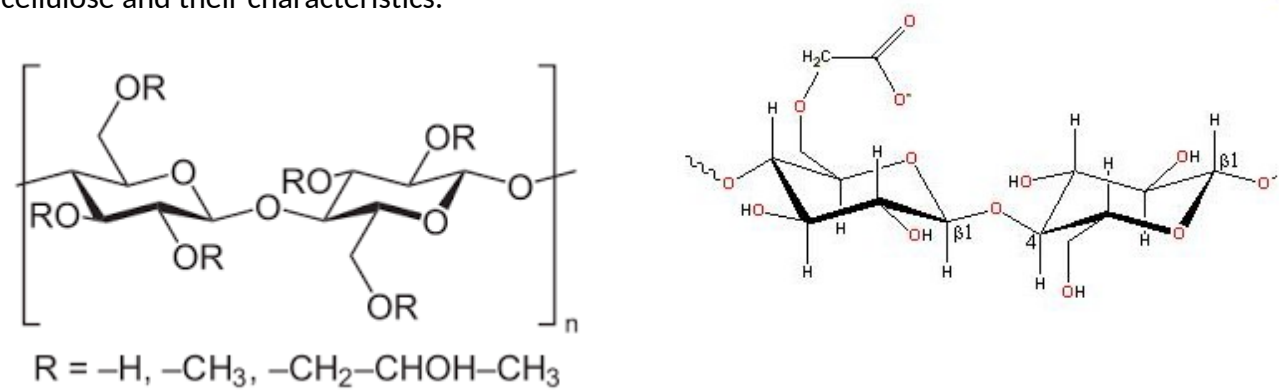
§ these Authors equally contributed to this work

\*Corresponding author: Silvia Panzavolta

Table S1: composition of Snail mucus extract as reported by the producer

Specification	Values	Measure Units	Method
Aspect	Clear		
smell	Odorless		
Color	Pale yellow		
pH	2.9		
Density	1.0-1.04	g/ml	
Dry residual	5 %	M/V	M.I.M 180305/L Rev. 0:2005
Minerals (K, Ca, Na)	538	mg/L	M.I.M 110315/C Rev. 0:2005
Heavy metals	absent		
Proteins	80 - 120	mg/L	Bradford proteins assay method
Glycolic acid	60-80	mg/L	J. Chrom. A. 1322, pp 49-53, 2013
Allantoin	100-130	mg/L	J. Chrom. A. 1322, pp 49-53, 2013
Iron	3	mg/L	M.I.M 111010/C Rev. 0:2010
Citric acid	<0.1	mg/L	M.I.M 150212/A Rev. 0:2012
Ascorbic acid	<0.1	mg/L	M.I.M 150212/A Rev. 0:2012
Antiprotease	1.3	mg/L	M.I.M 0112016/A Rev. 0:2016
D-lactic Acid	<10	mg/L	M.I.M 0112016/A Rev. 0
L-lactic Acid	<10	mg/L	M.I.M 0112016/A Rev. 0
Sodium benzoate	<0.002%	m/m	M.I.M 150212/A Rev 0:2012
Collagen	2-60	mg/L	M.I.M 0112016/H Rev. 0:2016
Gram +	<10	UFC/g	UNI-EN ISO6888-1:2004
Gram -	<10	UFC/g	ISO 16649-2:2001
Fungi	<10	UFC/g	NFV08-059:2002

Figure S1. Chemical structure of: left) hydroxypropyl methylcellulose and right) carboxymethyl cellulose and their characteristics.



Product Description	METHOCEL E5	METHOCEL E50	CMCNa
Methoxyl, %	28-30	28-30	--
Hydroxypropyl, %	7-12	7-12	--
Viscosity, 2% in water, mPa•s	4-6	40-60	850

Figure S2: Infrared spectrum of lyophilized Snail extract.

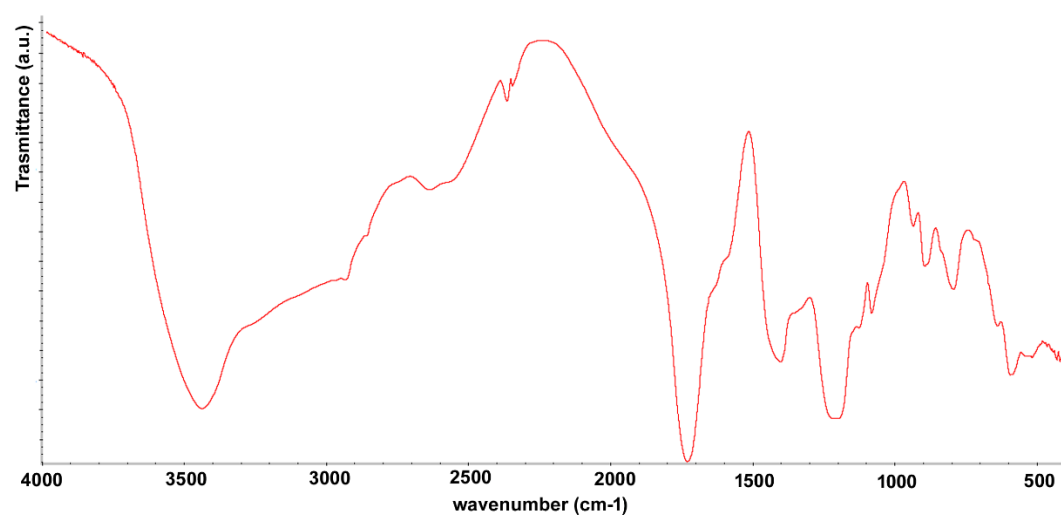


Figure S3: Moisture sorption isotherms of E5-based films (a) and CMC-based films (b).

