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(Article begins on next page)

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4 **FUNCTIONAL PROPERTIES OF CHITOSAN FILMS MODIFIED BY SNAIL**
5 **MUCUS EXTRACT**
6

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33 **Abstract**
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35 Snail mucus is an attractive natural substance, which is increasingly used in cosmetic creams and
36 syrups thanks to its emollient, moisturizing, protective and reparative properties. The aim of the
37 present study was to explore the physicochemical properties of chitosan-based films added with
38 snail mucus extracted from *Helix Aspersa Muller*. To this aim, chitosan films at different content of
39 snail mucus were fabricated by simple solvent casting technique. The results of X-ray diffraction
40 analyses, tensile mechanical tests, Infrared spectroscopy and thermogravimetry demonstrated that
41 snail mucus addition strongly modifies the properties of chitosan films. In particular, it acted like a
42 plasticizer enhancing films extensibility up to ten times and strongly improving their water barrier
43 and bioadhesion properties, with a trend depending on Snail mucus content. Furthermore, it
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provides the films with antibacterial properties and enhanced cytocompatibility, yielding materials with tailored properties for specific requirements.

Keywords

Chitosan films; snail mucus; adhesive properties

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121 **1. Introduction**
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124 The environmental problems caused by the continuous increase of plastic pollution have stimulated
125 many efforts addressed to find suitable substitutes obtained from natural and renewable sources
126 through green processes [1]. Chitosan has been proposed as an eligible material with potential
127 applications in many fields, including medicine, agriculture, food, textile, environment, and
128 bioengineering, due to its excellent properties of nontoxicity, biocompatibility, biodegradability,
129 chelating capability [2-5].
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133 In particular, chitosan ability to form films has been widely exploited for food packaging [6], wound
134 dressing and drug delivery applications [7-11]. Chitosan is a natural linear amino polysaccharide
135 made of D-glucosamine and N-acetyl-D-glucosamine units. It is obtained by deacetylation of chitin,
136 which is the structural component of the crustacean's shell (mostly shrimps and crabs), insect
137 cuticles and fungi cell walls, and represents the second most abundant natural polymer after
138 cellulose [12]. Chitosan is soluble in acid solutions due to the protonation of the -NH₂ groups on the
139 C-2 position of the D-glucosamine repeat unit: as a consequence, the polysaccharide is converted
140 to a polyelectrolyte in acidic media. DA, molecular weight and distribution of the acetyl groups along
141 the main chain are determinant factors in chitosan properties [13-15]. In particular, the positive
142 charge of the amino groups in acidic pH is considered responsible for the antimicrobial activity of
143 chitosan, through the interaction with the negatively charged cell membranes of microorganisms
144 [7].
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180 A number of studies have been focused to modify composition and properties of chitosan-based
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182 films in order to improve their performance and widen their application fields. In particular, films
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184 with improved light barrier and extra protective shield against oxidative processes were produced
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186 by incorporation of compounds of natural origin as antioxidant and/or antimicrobial agents [16-20].
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188 Addition of propolis was shown to enhance mechanical strength, antibacterial activity and
189
190 antioxidant capacity of chitosan-based materials for food packaging applications [21].
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193 Among natural-derived active substances, snail mucus is receiving a great deal of attention, as it has
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195 been recently proposed as an ingredient of several cosmetic (e.g. creams) and parapharmaceutical
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197 products for the management of wound and for the treatment of chronic bronchitis [22-23]. In fact,
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199 the mucus is an adhesive, emollient, moisturizing, lubricating and protective material, containing
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201 bioactive substances which are responsible for its unique properties not replicable in the laboratory
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203 with synthetic chemical compounds [24]. Moreover, in vitro assays on fibroblast cultures showed
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205 that snail mucus from Helix Complex does not elicit cytotoxicity, protects cells from apoptosis,
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207 promotes cell proliferation and migration, and displays antimicrobial activity [24]. However, despite
208
209 the wide use of snail mucus in commercial products, the number of studies on the characterization
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211 of this animal-derived extract is still limited [25-30] and the articles investigating the properties of
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213 snail-mucus based composite materials are even less [31].
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219 In this study we investigated the modifications of the properties of chitosan-based films induced by
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221 Snail mucus extract (S) [obtained by MullerOne extraction method](#). Recently, we patented the
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223 application [32] of snail mucus extract on different polymer-based films, demonstrating that tensile
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225 behavior and adhesive properties greatly improved by increasing the S content, thus producing
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227 materials able for applications in pharmaceutical, veterinary and cosmetics fields (patent application
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229 n. 102019000004940). To this aim, we added different amounts of Snail extract to chitosan previously
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231 solubilized in acetic acid or in lactic acid. Moreover, the acidic character of S allowed the preparation
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239 of a further series of films obtained by solubilizing chitosan directly in S solution. Films were then
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241 fully characterized for their solid-state properties, cell viability and antibacterial activity.
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244 245 **2. Materials and Methods**

246 247 **2.1 Materials**

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249 Chitosan (degree of deacetylation $\geq 93\%$, M.W.= 100 KDa) was purchased from Faravelli, Milan, Italy.
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251 Snail mucus from *Helix Aspersa Muller* (kindly offered by "I Poderi" farm, Montemerano, Italy) was
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253 extracted by MullerOne method (<http://www.mullerone.com/it/en/extraction-process>) and stored
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255 at 4°C in a sealed polyethylene bottle until use. Analysis of Snail mucus was obtained from the
256
257 supplier and reported in table S1. Selected amounts of Snail mucus were also freeze-dried for 24 h
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259 to evaluate the amount of the dispersed components and stored at 4°C.
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263 Acetic acid and lactic acid were purchased from Sigma Aldrich (Milan, Italy).
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268 269 **2.2 Preparation of Chitosan films**

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271 Chitosan films (1% w/v) were prepared by dissolving the proper amount of pure chitosan in either
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273 acetic acid (A, 1% v/v), or lactic acid (L, 1% v/v), as reported in literature, or directly in S. The mixture
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275 was kept at room temperature under stirring until complete dissolution of the polymer. A volume
276
277 corresponding to 11 mL of this solution was poured in polyethylene Petri dishes ($\emptyset= 5$ cm) and put
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279 under laminar hood at room temperature overnight. The obtained films were labeled C_A, C_L and
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281 C_S, respectively.
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286 287 **2.3 Preparation of Chitosan-Snail mucus films**

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289 For both C_A and C_L solutions, two chitosan-snail mucus volume ratios were selected: 70:30 and
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291 30:70, where the first number refers to the relative volume of chitosan acidic solution and the
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298 second one to the volume of S with respect to the total volume. For example, 100mL of chitosan-S
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300 blend 70:30 were obtained by adding 1g of chitosan to 70mL of A (1% v/v) or L (1% v/v). The mixture
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302 was stirred at room temperature until complete dissolution of the polymer and then 30mL of S were
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304 added (30% of the final volume). After 2h of stirring, 11 mL of this solution were poured in each
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306 Petri dish and put under laminar hood overnight. The obtained films were labeled CA_7030 and
307
308 CL_7030, respectively.
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311 The films obtained with the chitosan:S volume ratio of 30:70 were labeled CA_3070 and CL_3070.
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313
314 In order to obtain a chitosan-based film using the S as acidic medium, the same procedure was used:
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316 1g of Chitosan was dissolved in 70 or 30mL of S and then 30 or 70mL of distilled water were added
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318 in order to obtain the films labeled CS_7030 and CS_3070, respectively.
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321 Films were stored at room temperature between two sheets of plastic-coated aluminum closed
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323 inside PVC bags.
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325 326 **2.4 Films Characterization** 327

328 329 *2.4.1 Thickness* 330

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333 Films thickness was measured using a hand-held digital micrometer (Mitutoyo, Japan) at six
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335 different positions in each specimen to an accuracy of 0.001 mm.
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338 339 *2.4.2 Mechanical properties* 340

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342 Tensile tests were performed on strip-shaped samples (40 mm long and 4 mm width) using a 4465
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344 Instron dynamometer equipped with a 100 N load cell. Stress-strain curves were recorded at a
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346 crosshead speed of 5 mm/min by the software SERIE IX for Windows. Ten samples were tested for
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348 each composition.
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2.4.3 Infrared spectroscopy

The Fourier transform infrared spectra were recorded using a Thermo Scientific Nicolet iS10 FTIR spectrometer equipped with an ATR sampling device that uses a Germanium diamond as element for internal reflection. Spectra were acquired at room temperature in absorbance mode from 2300 to 800 cm^{-1} with a resolution of 2 cm^{-1} .

2.4.4 Water Vapor Permeability (WVP)

WVP is the water vapor transmission rate through a flat film area induced by a vapor pressure between two surfaces under specific conditions of moisture and temperature and was measured using the ASTM E96-93 method [33], slightly modified [34]. Films disks ($\varnothing=2$ cm) were glued using silicon on the opening of glass vials containing 2g of anhydrous CaCl_2 . Weighted vials were placed in a glass desiccator containing saturated $\text{Mg}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ solution (75% RH at 25°C). The vials were weighted every day until constant weights were achieved (4 days). WVP was calculated as follows:

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

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

2.4.5 X-Ray diffraction

X-ray diffraction patterns were carried out by means of a Philips X'Celerator diffractometer equipped with a graphite monochromator in the diffracted beam (2θ range from 4° to 50°, step size = 0,06680, 40s/step). $\text{CuK}\alpha$ radiation (40 mA, 40 kV, 1.54 Å) was used.

2.4.6 Thermogravimetric analysis (TGA)

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415
416 TGA was carried out using a Perkin-Elmer TGA-7. Heating was performed in a platinum crucible in
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418 air flow (20 mL/min) at a rate of 10°C/min up to 800°C. Samples weights were in the range of 5–10
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421 mg.

422 423 424 *2.4.7 Adhesive strength*

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427 The in vitro evaluation of the bioadhesive properties of the films was performed using an Anton
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429 Paar modular compact rheometer MCR102, adapting the method reported in the literature [35]. Pig
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431 rind bought in a local butcher's shop was used as support for the adhesion tests. Pig rind disks ($\varnothing=2.5$
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433 cm) were gently washed with detergent in order to remove fat and rinsed with Phosphate Buffer
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435 (PB, pH=7.4) and water repeatedly. Rind disks were glued on the disposable aluminum inferior
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437 support of the instrument. The film was allowed to adhere on the skin by wetting the pig rind with
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439 a fixed volume (40 μ L) of PB and applying a gentle finger pressure for 1 minute. Subsequently, the
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441 upper plunger of the instrument, covered with double-sided tape (3M), was lowered until a force of
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443 10 N was applied to the film for 30 seconds. After that, the plunger was raised up at a speed of 1
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445 mm/s and data were collected by using the RheoCompass Software. Work of adhesion and peak
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447 detachment force were used to evaluate the bioadhesive strength of the films. Each formulation
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449 was analyzed in triplicate and the mean \pm SD was reported.

450 451 452 453 454 455 *2.4.8 Statistical analysis*

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459 Statistical analysis was performed with Graph Pad Prism 4. One-way analysis of variance (ANOVA)
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461 followed by Tukey's Multiple Comparison Test was employed to assess statistical significance of
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463 the experimental conditions for Water Vapor Permeability and Adhesive strength; statistically
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465 significant differences were determined at $p < 0.05$.

466 467 468 469 **2.5 Cell viability bioassay**

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475 The effect of chitosan films on nonmalignant epithelial cells metabolism was assessed *in vitro* after
476 incubation of disks ($\varnothing = 6$ mm) in 1 mL of Eagle's Minimal Essential Medium (MEM) at 37°C for 24h.
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478 Then, the media were used for the analysis on African green monkey kidney cells (Vero ATCC CCL-
479 81). Briefly, cells were cultured in MEM supplemented with 10% fetal bovine serum (Carlo Erba
480 Reagents, Milan, Italy), 100 U/mL penicillin and 100 $\mu\text{g}/\text{mL}$ streptomycin at 37°C with 5% CO_2 . For
481 experiments, cells were seeded into 96-well plates at 10^4 cells/well, and incubated at 37 °C for 24
482 h; subsequently, cell monolayer was washed with PBS and incubated with 100 μL of the different
483 solutions, previously diluted twenty times in cell culture medium. The cell viability was assessed by
484 a WST8-based assay according to the manufacturer's instructions (CCK-8, Cell Counting Kit-8,
485 Dojindo Molecular Technologies, Rockville, MD, USA). After 72 h of incubation, cell monolayer was
486 washed with PBS, and 100 μL of fresh medium containing 10 μL of CCK-8 solution were added. After
487 2h at 37°C, the absorbance was measured at 450/630 nm; results were expressed as the percentage
488 of absorbance relative to the untreated controls. Experiment was carried out in triplicate.
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506 **2.6 Antibacterial activity**

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509 The *in vitro* antibacterial activity of the chitosan films was evaluated against *Staphylococcus aureus*
510 (ATCC 25923) and *Escherichia coli* (ATCC25922) selected as controls and representative strains for
511 Gram-positive and Gram-negative bacteria. The effectiveness of samples to inhibits bacterial growth
512 was assessed by a standardized Kirby-Bauer (KB) diffusion test on Mueller-Hinton agar plate and by
513 measuring the bacterial-free zone around the disk-shaped samples ($\varnothing = 6\text{mm}$) after 24h of
514 incubation at 37°C [36]. All experiments were performed on duplicate in different days.
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523 **3. Results and discussion**

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534 Films-forming solutions of chitosan in lactic or acetic acid are extensively reported in literature
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536 [37,38]. However, to the best of our knowledge, this is the first paper which reports the peculiar
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538 properties provided to chitosan films by the addition of Snail mucus obtained by MullerOne
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540 extraction. This method of extraction provides an acidic solution suitable for solubilization of
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542 chitosan, which needs pH values below 6 to dissolve. It follows that the use of S allows direct
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544 solubilization of chitosan through a 'green' procedure and provides materials where the good
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546 characteristics of chitosan are enriched by the peculiar properties of Snail mucus extract.
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550 All the chitosan-based films appeared transparent, with color gradually turning to yellow on
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552 increasing the amount of Solution (Fig. 1). The SEM images of some chitosan films containing or not
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554 Snail mucus extract are reported as example in Fig.1: all the films revealed a smooth surface without
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556 uneven areas.
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559 As a general consideration, the flexibility of the films and their adhesiveness increase with S volume,
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561 making difficult their detachment from Petri dishes.
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564 As reported in Table 1, films thickness is significantly affected by the acid used for chitosan
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566 dissolution: the thickness increases from acetic to lactic acid films can be ascribed to the increasing
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568 dimensions of the counterion [39]. A significantly greater augmentation of the values of thickness
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570 occurs on increasing the S content, most likely as a consequence of the increasing amount of dry
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572 matter (dry matter content of S after lyophilization: 5% m/V). The influence of the nature and
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574 composition of the film forming solution on thickness is clearly shown by the results obtained for
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576 the C_S samples, where water addition does not significantly affect the values of thickness [40].
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Table 1. Effect of S incorporation on chitosan-based films on thicknesses and mechanical properties.

SAMPLES	Thickness (mm)	σ_b (MPa) ^a	ϵ_b (%) ^a	E (MPa) ^a
C_L	0.076 ± 0.009	32 ± 4	4.0 ± 0.8	1200 ± 100
CL_7030	0.100 ± 0.006	7 ± 2	23 ± 8	170 ± 20
CL_3070	0.156 ± 0.007	1.4 ± 0.2	50 ± 10	46 ± 12
C_A	0.052 ± 0.020	40 ± 3	10 ± 3	1760 ± 300
CA_7030	0.096 ± 0.019	15 ± 4	13 ± 6	270 ± 100
CA_3070	0.118 ± 0.007	0.9 ± 0.1	138 ± 10	0.8 ± 0.1
C_S	0.199 ± 0.005	0,43 ± 0.2	163 ± 30	0,38 ± 0,05
CS_7030	0.168 ± 0.021	1.1 ± 0.19	88 ± 7	1.4 ± 0.22
CS_3070	0.158 ± 0.010	8.6 ± 1.5	12 ± 4	301 ± 44

^aEach value is the mean of ten determinations and is reported with its standard deviation.

The results of mechanical characterization are summarized in Table 1. Both C_L and C_A films are rigid and brittle with high values of elastic modulus (E) and stress at break (σ_b), whereas C_S films exhibit a relatively high extensibility (ϵ_b) and low values of E and σ_b . In agreement, S addition to C_L and C_A compositions greatly influences the mechanical properties of the films, as clearly shown in Fig.2: ϵ_b increases with S content while σ_b and E decrease. The same trend is observed on going from CS_3070 to CS, in agreement with the increase of S content. The effect produced by S addition is similar to that obtained by the introduction of plasticizers into the composition of chitosan films [41-43]. The increased extensibility of the films at higher S concentration can be attributed to Solution-polymer interactions, which reduce the intermolecular interactions between polymer chains, facilitating their sliding and mobility and improving the overall extensibility.

The increased mobility of the polymer chains usually promotes also water vapor permeability (WVP) [43], that is the ease of moisture for penetrating and passing through the hydrophilic portion of film [44]. However, in our samples we found a peculiar trend as a function of composition (Fig. 3) since CA_7030 and CL_7030 exhibit WVP values significantly smaller than the other films of the series.

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651
652 Interestingly, C_S films exhibit lower permeability than C_L and C_A films (*p<0.05). Moreover, the
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654 WVP values of C_S films are not significantly affected by water addition.
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657 As stated previously, the films become sticky after addition of S: the adhesive properties, expressed
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659 in terms of force needed for film detachment (F) and work of adhesion (W), are reported in Fig. 4.
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661 C_A films do not exhibit any adhesive performance. However, the addition of S induces a certain
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663 adhesiveness, requiring a force up to about 10 N (CA_3070) to detach the films. A similar trend is
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665 observed for films prepared in lactic acid: C_L exhibits an appreciable adhesive behavior, which is
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667 further enhanced by S addition. As expected, the highest adhesive properties are recorded for films
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669 prepared by direct solubilization of chitosan into S Solution. The increased flexibility and adhesion
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671 of S-containing films should improve the contact with skin, thus allowing a better penetration of
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673 film components into the tissue. Besides, the presence of polar groups into Solution, most probably
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675 belongs to glycolic acid, allantoin and proteins (see Table S1) could increase the interactions.
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681 *Structural characterization*

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683 According to the literature, a number of crystalline polymorphs are known for chitosan; the most
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685 represented ones are an anhydrous form indicated as “annealed polymorph” and two different
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687 hydrated forms named “tendon” and “Type II” [45,46].
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690 The variety of polymorphs is due to the presence of water molecules, which play an important role
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692 in the packing, conformation and mechanical properties of chitosan-based films. In hydrated forms,
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694 the chitosan structure can be stabilized by several hydrogen bonds between -N-H groups and water
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696 molecules. In addition, the crystalline structure of chitosan is strongly dependent on its processing
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698 treatment, as well as on its origin and molecular composition, such as degree of deacetylation and
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700 molecular weight [47].
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711 The X-ray diffraction patterns collected from chitosan films are reported in Fig.5: films obtained in
712 acetic acid (C_A) show two prominent reflections at about 9.2° and $12^\circ/2\theta$, together with a sharp
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716 peak at $19^\circ/2\theta$ attributed to type II hydrated polymorph of chitosan acetate [48]. Snail mucus is a
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718 complex mixture of active ingredients and it is not easy to discriminate the effectiveness and the
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720 interaction of each component with the chitosan functionalities. However, the comparison of the
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722 patterns collected from Solution-containing samples puts into evidence that the material becomes
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724 less crystalline on increasing the S content. In fact, CA_7030 films show only two broad halos,
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726 centered at about 8° and 20° of 2θ , while only a broad halo centered at 20° of 2θ can be detected
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728 when the samples contain a greater amount of solution (CA_3070). The X-ray pattern of the chitosan
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730 films obtained in lactic acid (C_L), reported in Fig. 5, evidences a poorly crystalline structure, with
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732 two broad reflections at about 6° and at about $20^\circ/2\theta$.

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735 S addition provokes an overall decrease of the intensity of the diffraction patterns. It can be
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737 hypothesized that, on S addition, chitosan-Solution interactions outweigh chitosan-chitosan
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739 interactions, leading to loss of structural order and, consequently, to the observed significant
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741 reduction in crystallinity. In agreement, the XRD patterns of all the samples of the C_S series display
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743 just a very broad halo centered at about 20° of 2θ .

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745
746 In agreement with the X-rays patterns, the infrared absorption spectrum of C_A films displays a
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748 number of bands which can be ascribed to the hydrated polymorph of chitosan [49]. In particular,
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750 the absorption band at about 1640 cm^{-1} can be assigned to the C=O stretching (amide I), whereas
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752 those centered at about 1540 and 1390 cm^{-1} can be attributed to N-H bending (amide II) and C-N
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754 stretching, respectively [10]. Addition of S solution provokes a general broadening of the spectra,
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756 which assume characteristic features of the spectrum of S powder (see Fig.5), where an intense
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758 absorption peak, probably due to the high content of allantoin and glycolic acid in the solution (see
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760 Table S1), appears at around 1712 cm^{-1} . This absorption band also appears in the spectra of
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770 **composite films** and its intensity increases on increasing S content. The resolution of the absorption
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772 bands centered at about 1072 cm⁻¹, associated to C-O stretching, decreases on increasing S content,
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774 suggesting interactions between the hydroxyl groups of chitosan and polar groups of S through
775
776 hydrogen bonds [10]. **By comparing IR spectrum of S with that reported in literature [24] it is clear**
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778 **that Snail extract obtained by MullerOne contains a minor amount of proteins (see also table S1)**
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780 **with respect to that obtained by a different method of extraction. We hypothesize that the use of**
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782 **ozone during snail stimulation should induce a partial protein degradation, thus lowering the proteic**
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784 **component of the final extract.**
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788 In agreement with XRD results, the infrared absorption spectrum of C_L films is quite different and
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790 resembles those reported in literature for chitosan films prepared in lactic acid [50]. Addition of S
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792 to C_L films has a similar effect to that observed on C_A films, and the spectra are similar to those
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794 recorded for the C_S series.
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796

797 The thermal stability of chitosan films was assessed by TGA analysis in air. Results obtained for the
798
799 different films are reported in Figure 6 together with the thermal behavior of lyophilized S. C_A films
800
801 display three steps of thermo-oxidative degradation [51]. The first one, in the temperature range
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803 35–160°C, is attributed to the loss of absorbed water. The second one, between 160°C and 460°C
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805 and centered around 310°C, corresponds to the chemical degradation and deacetylation of chitosan
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807 [52], while the third step, in the temperature range 460–700°C, can be associated with the oxidative
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809 degradation of the carbonaceous residue formed during the second step. The thermogravimetric
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811 plot of C_L differs from that of C_A in the first region, which shows two distinct weight losses in the
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813 range 37-240°C, in agreement with the different structures evidenced by XRD and FT-IR data. The
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815 derivative plot of TGA (DTG) of freeze-dried S (Fig. 6) displays a weight loss centered at 190°C, which
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817 accounts for about 70% wt of weight loss, and further degradation steps between 300 and 800°C,
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819 probably due to the degradation of residues. A very similar thermogravimetric plot is shown by C_S,
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829 with just some shift of the degradation steps to higher temperatures. Water addition (CS_7030 and
830 CS_3070) causes just a reduction of the relative amount of the first weight loss. When S is added to
831 the composition of C_A and C_L, all the films display similar thermogravimetric plots to that of C_S
832 series: in particular, the thermal degradation starts at a temperature lower than that of pure
833 chitosan films and the first mass loss, determined between 37°C and 300°C, accounts for about 35%
834 wt and 48% wt for the 7030 and 3070 compositions, respectively. Moreover, no water loss was
835 observed between 35°C and 160°C.
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846 *Biological properties*

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849 In our experimental conditions C_L and C_A films did not interfere with Vero cells metabolism after
850 72h of incubation (93.7% and 103.3 %, respectively, and relative to untreated control cells) (Fig. 7).
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852 Addition of snail mucus components to these films induced an improvement in cell viability,
853 especially for the samples of CA series. These samples exhibit a dose dependent increase in Vero
854 viability as function of S content. The lowest cell viability was detected for C_S films (72.7%);
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856 nevertheless, as a material is considered cytotoxic when its viability is less than 70% in comparison
857 to untreated controls [53], all samples displayed a promising safety profile.
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865 The antibacterial properties of the different chitosan films were evaluated *in vitro* by means of a
866 disk agar diffusion method where inhibition of bacterial growth is demonstrated by the clear
867 bacterial-free zone around sample disks following a 24 h-incubation. Results are reported in Table 2.
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872 There was no difference between the antibacterial effects on the different microbial species.

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874 Although it is generally recognized that chitosan solutions have strong antibacterial activities [54],
875 chitosan films did not inhibit bacterial growth in agar diffusion tests because chitosan in a film form
876 is unable to diffuse through the surrounding agar media [55]. the present results confirm this feature
877 since both C_A and C_L samples did not inhibit bacterial growth. As a consequence, the bacterial-
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free zones observed for S-containing films could be definitely ascribed to snail mucus addition. Chitosan films prepared in acetic acid and directly in S showed antibacterial activity at the highest S content (CA_3070, C_S and CS_7030) confirming the inhibitory role of snail mucus. On the contrary, films prepared in lactic acid did not display antibacterial properties irrespective to S content.

Table 2. Ranges of the inhibition zone diameters (mm) measured for the chitosan films

Sample	<i>S. aureus</i> (ATCC 25923)	<i>E. coli</i> (ATCC 25922)
C_L	NA*	NA
CL_7030	NA	NA
CL_3070	NA	NA
C_A	NA	NA
CA_7030	NA	NA
CA_3070	12-13	11-12
C_S	15-22	11-14
CS_7030	15-16	12-13
CS_3070	NA	NA
GMN 10 µg	18-19	18-19

*NA; not appearing

4. Conclusion

New and highly versatile materials containing different amounts of snail mucus, obtained by MullerOne extraction method, and chitosan have been obtained by simple solvent casting. The results of this work show that snail mucus can be added to chitosan previously solubilized in acetic or lactic acid, or it can also be used directly to dissolve chitosan through a greener route. Tensile tests revealed that composite films can be stretched up more than ten times with respect to chitosan films, demonstrating that S addition displays a plasticizing effect on the films. Moreover, snail mucus also enhances water barrier properties and bioadhesion. Structural characterizations indicate that the interactions between snail mucus and chitosan chains involve hydrogen bonds between the hydroxyl groups of chitosan and polar groups of S, even if the complexity of the extract composition requires more detailed analysis. Thanks to the presence of S, composite films display enhanced cytocompatibility and significant antibacterial activity towards both Gram-positive and Gram-

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947 negative bacteria. These results demonstrate that variations in composition can be utilized to
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949 modulate the properties of these materials for possible applications including those for the
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951 biomedical field or as edible coating for food packaging. However, due to the influence of the
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953 extraction methods on the snail extract properties, composite films made with different snail extract
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955 should be useful to get more information about the interactions between the components and to
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957 highlight the peculiar extract effect.
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965 This research did not receive any specific grant from funding agencies in the public, commercial, or
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967 not-for-profit sectors.
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Figure captions:

Fig. 1. Appearance of the prepared films. On the right some selected scanning electron micrographs are reported. Bar = 100 micron.

Fig. 2. Stress-strain curves recorded on chitosan films prepared in: a) lactic acid; b) acetic acid; c) S.

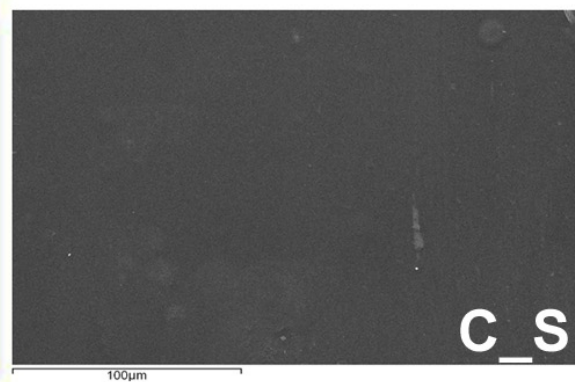
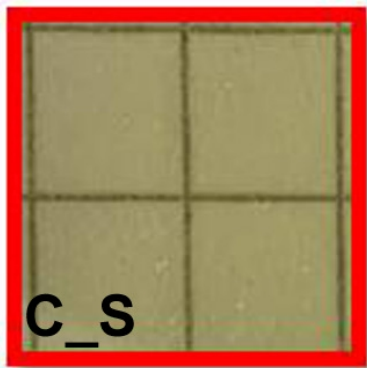
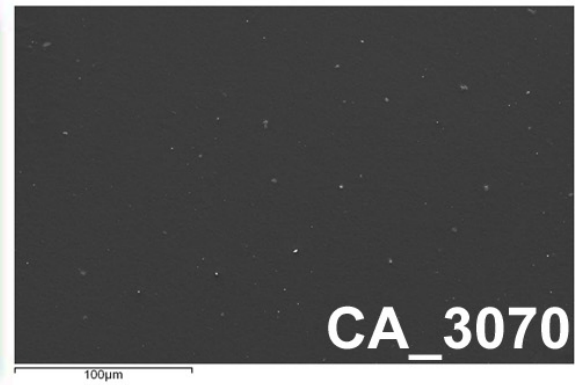
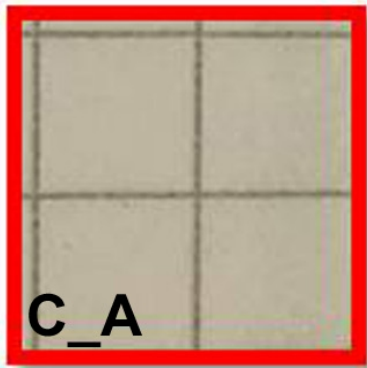
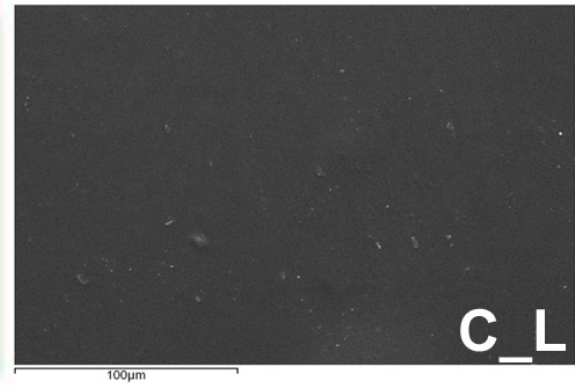
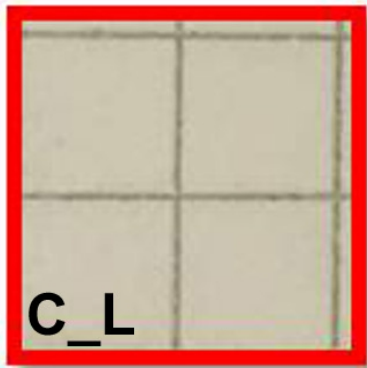
Fig. 3. Water Vapor Permeability (** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$)

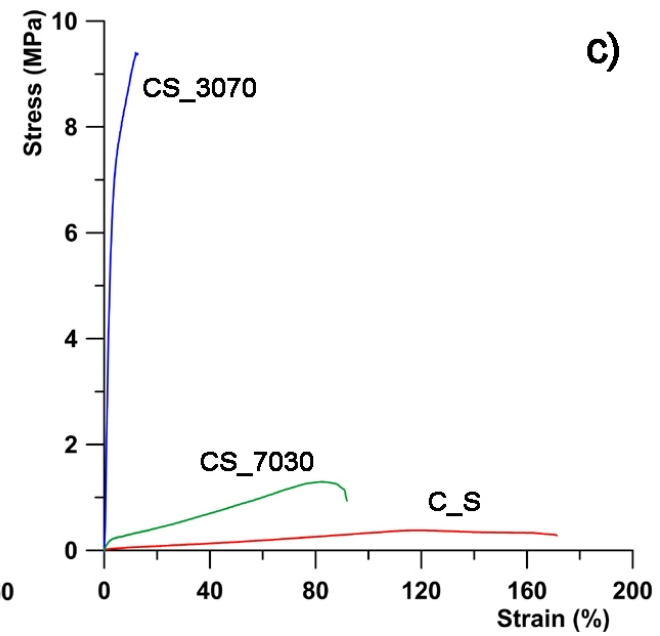
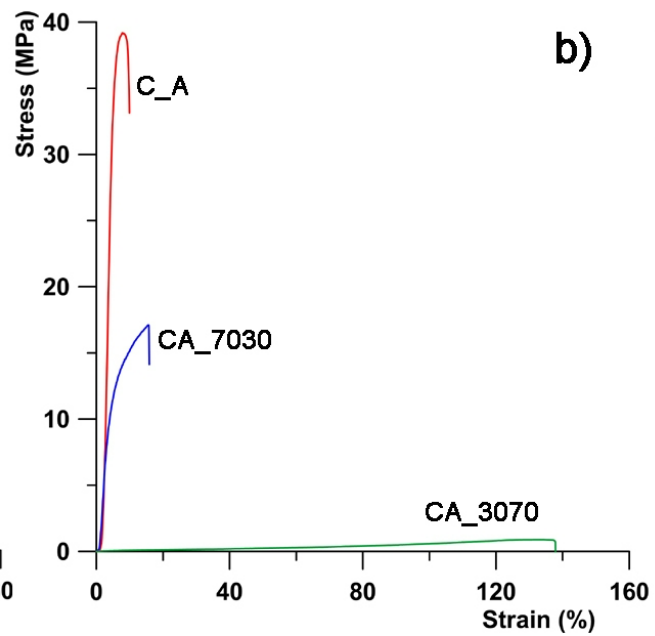
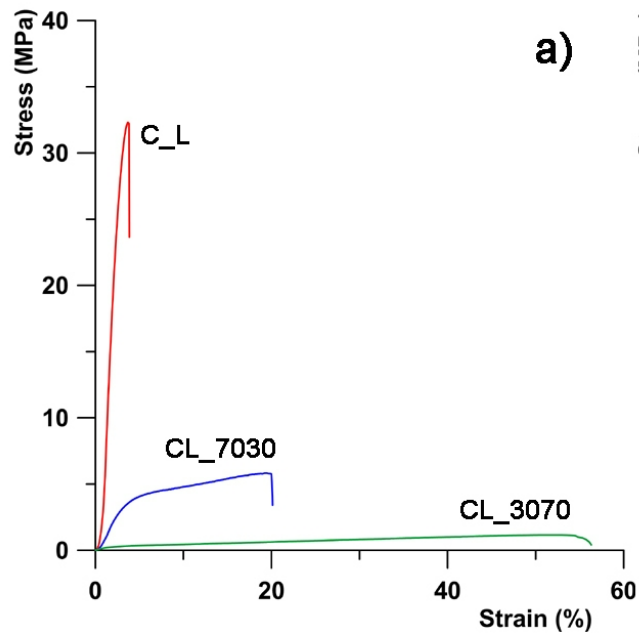
Fig. 4. Adhesive properties of chitosan-based films(** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$)

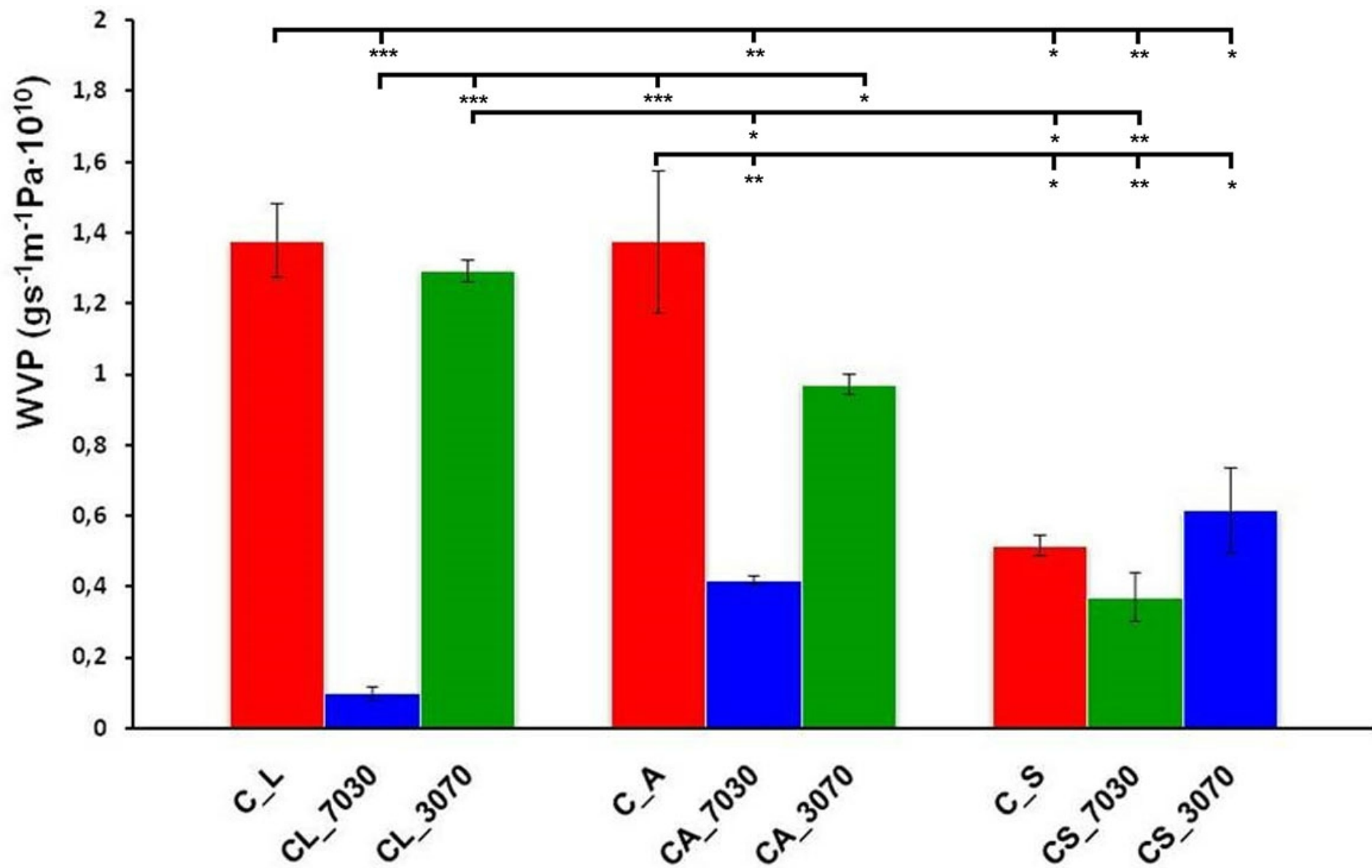
Fig. 5. X-rays diffraction patterns (left) and corresponding IR spectra (right) of chitosan films and lyophilized solution.

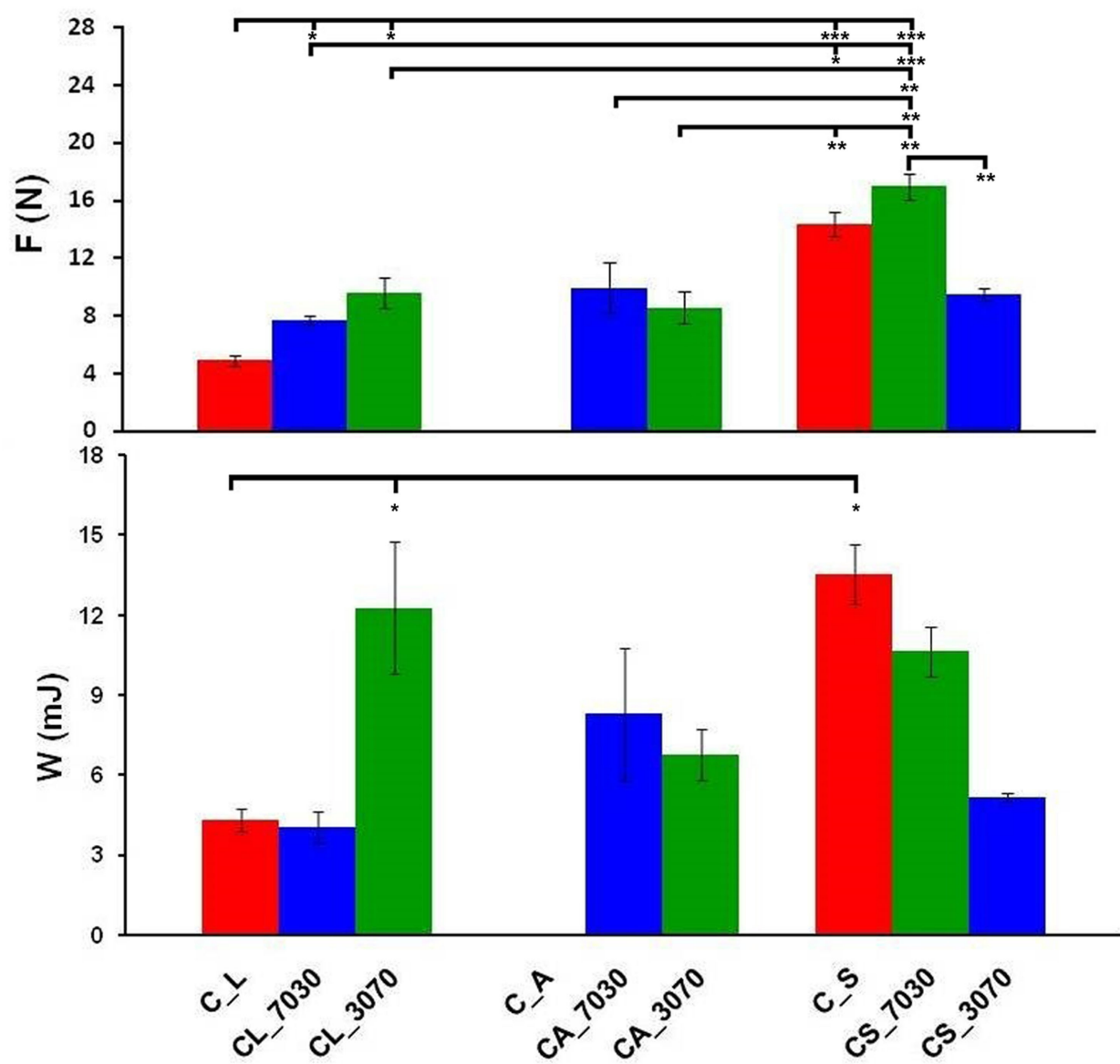
Fig. 6. DTG plots recorded on (from top to bottom): films in L, films in A, films in S and lyophilized S.

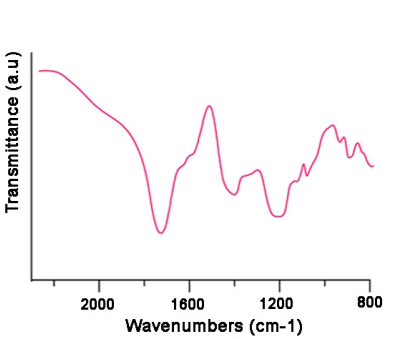
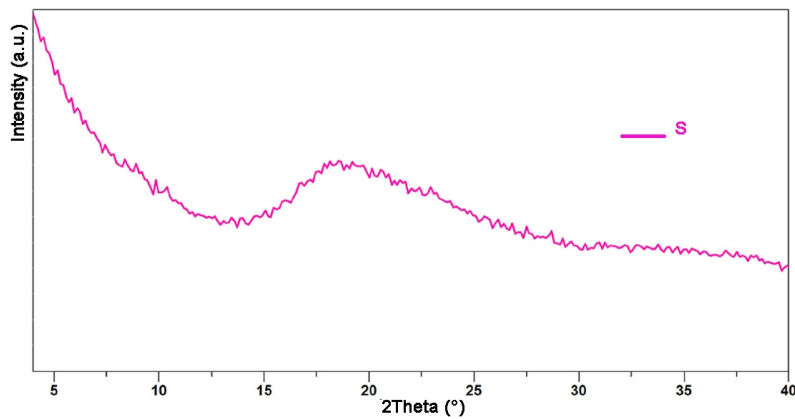
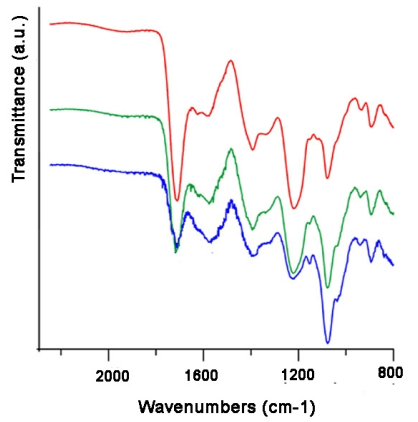
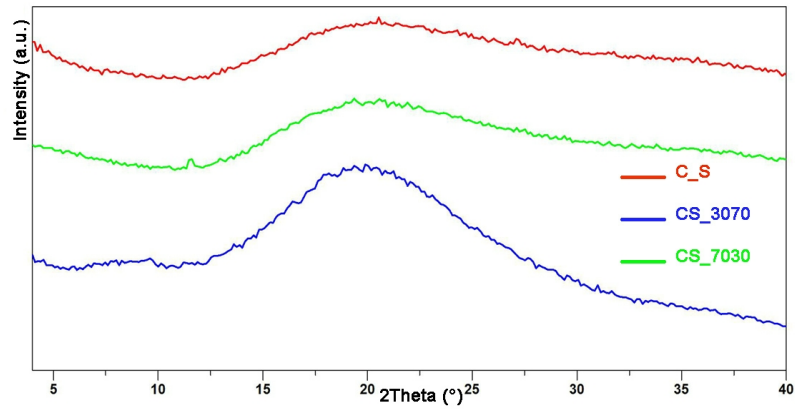
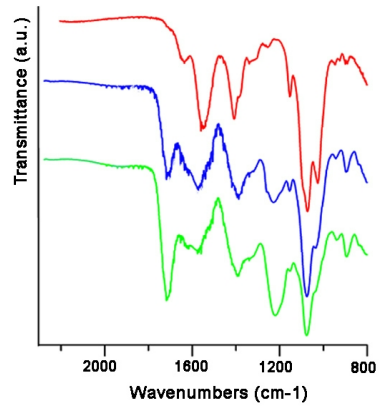
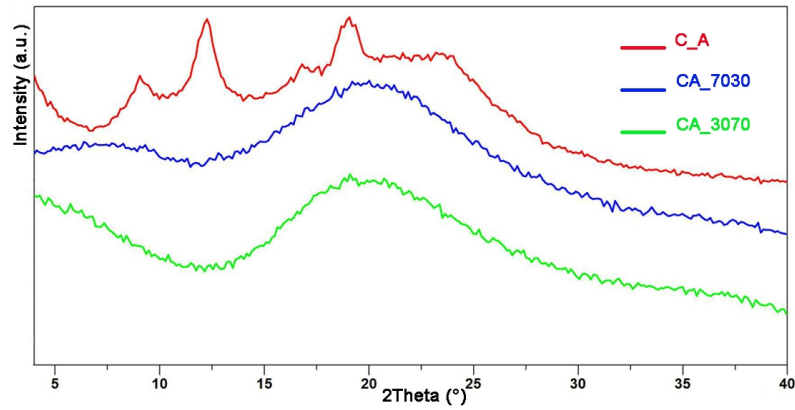
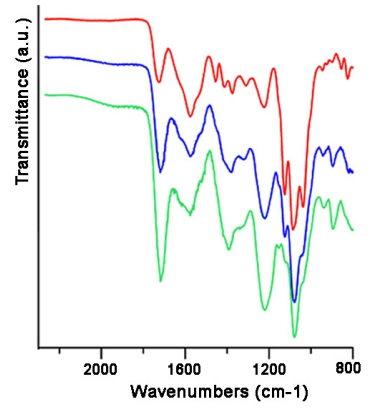
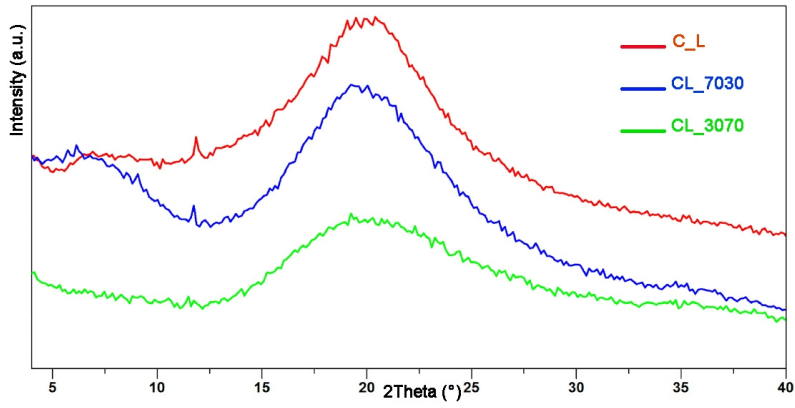
Fig. 7. Vero cell viability after 48h of incubation with the media containing film components following disks dissolution. Data (mean values \pm SD) are relative to the untreated control (set to 100%).

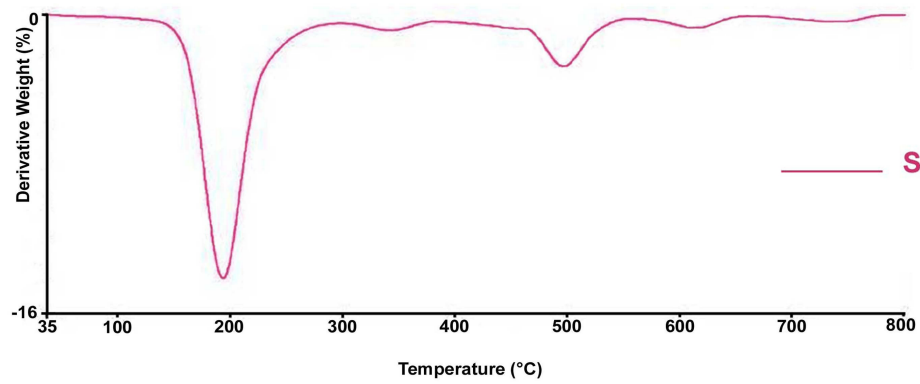
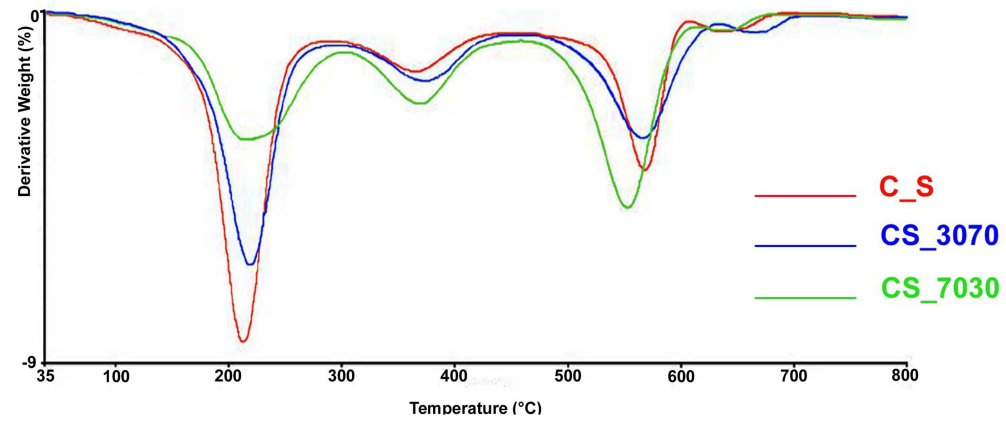
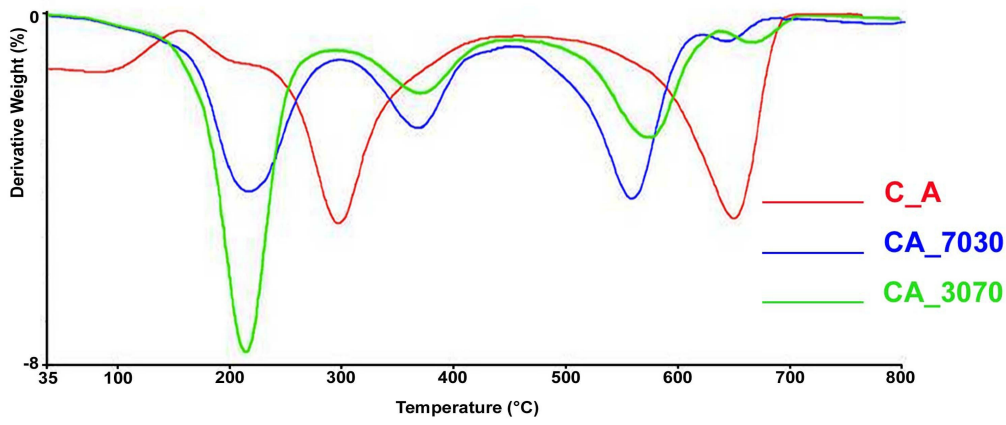
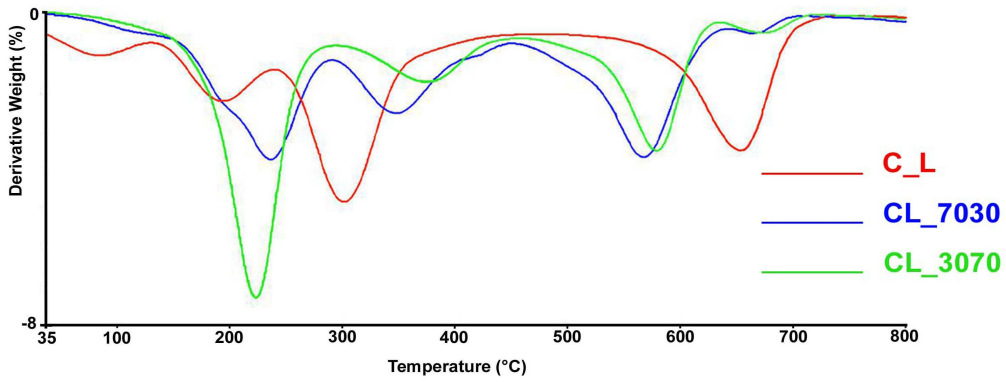


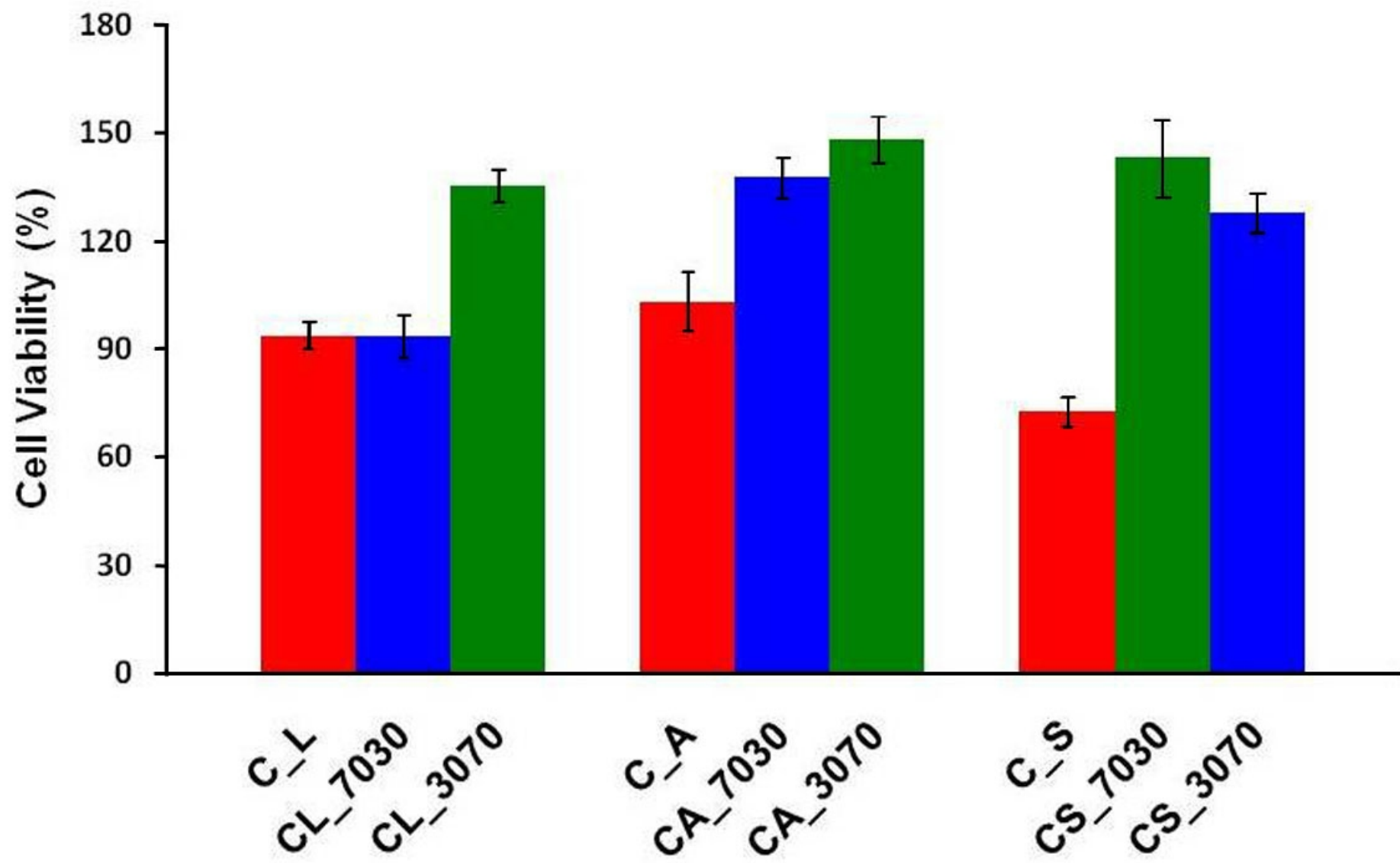












Author Contribution Statement

Maria Francesca Di Filippo: Data curation, Formal analysis, Investigation, Roles/Writing – original draft;

Silvia Panzavolta: Conceptualization, Methodology, Project administration, Supervision, Roles/Writing – original draft, Writing – review & editing;

Beatrice Albertini: Methodology, Roles/Writing – original draft, Writing – review & editing;

Francesca Bonvicini: Data curation, Formal analysis, Investigation;

Giovanna Gentilomi: Resources

Ramona Orlacchio: Data curation, Formal analysis, Investigation;

Nadia Passerini: Resources, Supervision;

Adriana Bigi: Supervision, Writing – review & editing;

Luisa Stella Dolci: Conceptualization, Resources, Validation.

FUNCTIONAL PROPERTIES OF CHITOSAN FILMS MODIFIED BY SNAIL MUCUS EXTRACT

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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

Table S1: Characterization of snail mucus obtained from *Helix Aspersa Muller* by means of MullerOne technology. Analyses were obtained by the supplier. The chemical and microbiological parameters evaluated are among those suggested for snail mucus characterization, as reported in <http://www.mullerone.com/it/en/our-slime-helix>.