Da: International Journal of Biological Macromolecules <em@editorialmanager.com>
Data: 21 agosto 2020 15:20:31 CEST
A: Daniela Giacomazza <daniela.giacomazza@cnr.it>
Oggetto: Decision on submission to International Journal of Biological Macromolecules
Rispondi a: "International Journal of Biological Macromolecules" <biomac.journal@elsevier.com>

Manuscript Number: IJBIOMAC-D-20-03419R2

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Agarose/κ-carrageenan-based hydrogel film enriched with natural plant extracts for the treatment of cutaneous wounds

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Abstract: Hydrogels for complex and chronic wound dressings must be conformable, absorb and retain wound exudates and maintain hydration. They can incorporate and release bioactive molecules that can accelerate the healing process. Wound dressings have to be in contact with the wound and epidermis, even for long periods, without causing adverse effects. Hydrogel dressing formulations based on biopolymers derived from terrestrial or marine flora can be relatively inexpensive and well tolerated. In the present article hydrogel films composed by agarose (1.0 wt%), κ-carrageenan at three different concentrations (0.5, 1.0 and 1.5 wt%) and glycerol (3.0 wt%) were prepared without recourse to crosslinking agents, and characterized for their mechanical properties, morphology, swelling and erosion behavior. The films resulted highly elastic and able to absorb and retain large amounts of fluids without losing their integrity. One of the films was loaded with the aqueous extract from *Cryphaea heteromalla* (Hedw.) D. Mohr for its antioxidant properties. Absence of cytotoxicity and ability

to reduce the oxidative stress were demonstrated on NIH-3T3 fibroblast cell cultures. These results encourage further biological evaluations to assess their impact on the healing process.

Keywords: Agarose/κ-carrageenan lend; *Cryphaea heteromalla* bryophyte; Wound healing

1. Introduction

Modern wound dressings are required to have an active role in promoting healing. Wound healing is a complex, dynamic mechanism structured in four different consecutive and partially overlapping phases: i) coagulation and hemostasis; ii) inflammation, during which exudate formation happens; iii) proliferation, where rebuilt of new tissue, collagen, extracellular matrix and network of blood vessels occur, and iv) maturation, that is the final phase of the whole process involving the remodeling of the new tissue and the transition from type III to type I collagen [1, 2].

Many different materials proposed for wound dressing are specifically designed to prevent microorganism contamination and provide protection against the external environment [3]. They also accomplish additional functions, such as absorbing exudates and debris, maintaining a moist environment, allowing oxygen permeation and preventing odor formation and release bioactive molecules [4]. These materials also need to be mechanically resilient, compliant, conformable to various body parts and, most importantly, biocompatible [5-7]. Traditional wound dressing materials, both natural (cotton, wool) or synthetic (rayon and polyester), are dry, require frequent changes and, due to excessive drainage, strongly adhere to the skin [6].

Modern wound dressings have been designed to be easily removable from the damaged area without causing injury to the newly formed tissue and pain to the patient [8]. In addition, novel wound devices can incorporate diagnostic tools, such as biosensors, to detect physiological parameters and remotely transmit them to a monitoring device [9, 10] and/or drugs and growth factor to accelerate the healing process [11].

Hydrogel films made of selected natural and/or synthetic polymers possess all the above requisites and are ideal candidates as wound dressing materials. They can also be biodegradable and transparent, allowing visual inspection of the wound through the dressing. A meta-analysis study, performed on 1473 clinical subjects, concluded that the use of hydrogels in cutaneous wounds significantly reduces the healing time and patient pain if compared with traditional non-hydrogel dressings [12]. Furthermore, results indicated they are more effective and safer in the wound management [12]. An updated and exhaustive roundup of commercial hydrogels for wound dressing can be found in Aswathy and coworkers [13].

Agarose is a well-known biocompatible polysaccharide derived from seaweed, very abundant and inexpensive, composed by D-galactose and 3,6-anhydro-L-galactopyranose units. It is able to aggregate in aqueous solution to form a fibrous network without addition of crosslinking agents [14].

κ-Carrageenan is a sulfated polygalactan (from 15% to 40%), constituted by d-galactose and 3,6-anhydro-galactose joined by α-1,3 and β-1,4-glycosidic linkages and molecular mass ranging from 100 kDa to 1000 kDa [15]. It is obtained by red seaweeds and is widely used as thickening, gelling and stabilizing agent in the cosmetic, pharmaceutical and food industries [16]. It forms gels in presence of mono- and di-valent ions, with a clear preference for potassium ions [17-19].

The use of algal polysaccharides is due to the plethora of the beneficial biological effects they exert. In fact, they are active i) against viral, bacterial and fungal agents; ii) in the coagulation and hemostasis; iii) in the reduction of oxidative stress and inflammation; iv) in the immune response and angiogenic processes [20]. As drug delivery systems, hydrogels obtained by algal polysaccharide have found numerous applications. Formulations of cross-linked k-carrageenan/starch and k-carrageenan/gelatin were successfully prepared for the extended release of zaltoprofen, a non-steroidal anti-inflammatory drug [21, 22]. Agarose nanoparticles were developed and characterized as injectable vehicles for the delivery of proteins and peptides [23]. The development of wound care medical devices had recently a sudden rise due to several formulations based on the seaweed hydrogels. A pH responsive film, working in the pH range 5.0 to 9.0, obtained from k-carrageenan and locust bean gum, loaded with cranberry extract has been recently proposed for monitoring bacterial infections [24]. Alginate blended with chitosan hydrogel films were successfully designed as wound healing medical device with antimicrobial and sustained release properties [25].

In the present work, agarose (AGA, 1.0 wt%) and κ-carrageenan (κ-CAR) at three different concentrations (0.5, 1.0 and 1.5 wt%) were used to design a natural wound dressing medical device. During preparation, glycerol (GRO, 3 wt%) was added as plasticizer, to obtain films with suitable viscoelastic properties. The bryophyte *Cryphaea heteromalla* (Hedw.) D. Mohr aqueous extract (*Ch*AE), recently characterized by HPLC-TOF-MS technique [26], was added to the film. Bryophyte group consists of small plants, that, since ancient times, have been used for many applications from clothing fabrication [27] to animal feeding [28] and traditional medicine [29] possessing a strong antioxidant machinery that makes them able to survive in unfavorable environments.

All films were characterized for their mechanical properties and absorption capacity, once immersed in pure water, physiological phosphate buffer (PBS) or bovine calf serum (BCS) at 25 °C. The film containing Agarose 1.0 wt%, κ-Carrageenan 1.0 wt% and Glycerol 3.0 wt% was selected on the basis of its mechanical properties and loaded with either a model molecule, the fluorescent probe Atto633, or *Ch*AE extract. The release of the Atto 633 and of the biophenols present in the *Ch*AE extract from the corresponding loaded films was investigated in pure water, PBS or BCS at 25 °C. The hydrogel film was freeze-dried and characterized for its morphology by scanning electron microscopy (SEM), soon after being produced ("as-prepared") and upon 3 h and 48 h immersion in pure water, PBS or BCS.

The cytotoxicity of the film was verified *in vitro,* using the NIH-3T3 fibroblast cell culture, conducting tests in accordance to the International Standard for testing of medical devices [25]. Furthermore, the antioxidant power of the compounds released by the *Ch*AE-loaded film was validated by Oxygen Radical Absorbance Capacity (ORAC) test. Finally, its ability in reducing the oxidative stress generated by tert-butyl-hydroperoxyde (TBH) in NIH-3T3 fibroblast cell culture was evaluated. At the best of our knowledge, this article presents, for the first time, the application of aqueous extract from bryophyte species as an active component of wound dressing film obtained from bio-based polymers.

Hereafter samples will be indicated by the code AGA1_κ-CARX_GRO3, where AGA1 indicates the agarose at the concentration of 1 wt%, κ-CARX the concentration of κ-carrageenan where X=0.5, 1.0 or 1.5 wt%, and GRO3 the glycerol at the concentration of 3 wt%.

2. Materials and methods

2.1 Materials

AGA and κ-CAR were purchased from Seakem Ltd. (High Gelling Temperature, HGT, MW~120,000) and Copenhagen Pectin Ltd., Denmark (MW~800,000), respectively. Glycerol (GRO), amino-functionalized Atto633, Folin-Ciocalteau solution, sodium carbonate, fluorescein, 2,2′-azobis(2-amidinopropane), dihydrochloride (AAPH), (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (trolox), gallic acid, methanol (HPLC grade), acetonitrile (LC-MS grade), phosphoric acid 85%, Luperox® TBH70X tert-butyl hydroperoxide solution (TBH), 2',7'-di-chlorodihydrofluorescein diacetate (DCFH-DA), sodium-phospate di-basic, potassium phospate monobasic and NaCl were purchased from Sigma-Aldrich (Milan, Italy). CellTiter 96® Aqueous One Solution Assay (MTS) was purchased from Promega (Milan, Italy). Bovine calf serum (BCS) was purchased from Sigma Aldrich and used diluted 1:10 with

DMEM culture medium (hereafter simply reported as BCS). The water used in all experiments was Millipore Milli-Q (pure water).

2.2 Preparation of Cryphaea heteromalla aqueous extracts (ChAE)

Cryphaea heteromalla fresh plants were collected on January 2017 in the Bonifato mountain in the Trapani area (Sicily, Italy), macro- and microscopically authenticated by an expert botanist of the University of Palermo and treated as described in [26]. Briefly, plants were repeatedly washed with tap water and lastly three times with distilled water. Then, they were air dried for 10 days up to the achievement of a constant weight and crushed in a mortar with liquid nitrogen to obtain a fine powder. Later, 0.3 g of the powder were mixed with 7.5 mL of pure water and incubated under magnetic stirring at 20 °C for 3 days, then sonicated for 20 minutes into an ultrasonic bath at 59 kHz and 198 W. The last step allowed a lower degree of antioxidant compound degradation with respect to other techniques [30]. The aqueous extract was centrifuged at 5000 rpm for 25 minutes at 4 °C. The supernatant was lyophilized and the obtained powder was stored at -20 °C.

2.3 Determination of total biophenol content

The Folin-Ciocalteu (F-C) assay was performed in agreement with [31] with a few minor modifications. In brief, 0.2 mL of sample were added to 4.8 mL of Milli Q water, followed by the addition of 0.5 mL Folin-Ciocalteu reagent. After 3 min, 1mL sodium carbonate solution (20%, w/v) was added to the reaction mixture, finally mixed and diluted with Milli Q water to 10 mL final volume. The absorbance of the mixtures was measured after 2 hours against a blank sample on a Shimadzu Spectrophotometer at the wavelength of 765 nm. A gallic acid calibration curve was used as reference (linear range 62.5÷250 mg/mL). The total biophenol content was expressed as mg of gallic acid equivalents per gram of dried sample.

2.4 Film preparation

Agarose (1 wt%) powder was dissolved in cold Milli Q water together with κ-carrageenan at the concentration of 0.5, 1.0 or 1.5 wt%. The suspension was put in hot bath (100 °C) under stirring for 20 minutes, during which the sample bottle was opened, shaken and reclosed three times to avoid air bubble entrapment. Then, the glycerol (3 wt%) was added and the suspension kept at 100 °C for further 10 minutes. The hot solution was poured in a 50 mm Petri dish or in a 12-well plate placed in a laminar flow hood (344 m^3/h) at room temperature (ca. 20°C), to maintain sterile conditions, and allowed to dehydrate for two days up to the attainment of a

constant weight. The residual water content in the films was calculated from the difference between the initial and final weight of the samples.

2.5 Rheological measurements

Dynamic-mechanical measurements were performed on films using a stress-controlled Rheometer AR G2 (TA Instruments) with a peek plate (ø 40 mm) at the temperature of 25 ± 0.1 °C, controlled by a built-in Peltier system. 40 mm diameter disks were obtained from the 50 mm diameter films by using a sharp punch. The thickness of the as prepared films was about 2 mm. Then, the disks were immersed in water, physiological PBS at pH 7.4, or BCS at 25°C for 3 hours or 48 hours. The swollen samples showed a significant thickness increase (4 - 7 mm). The gap was adjusted to the disk thickness. All the mechanical spectra were recorded in the frequency range 0.1–10 Hz at the strain of $8x10^{-3}$. This strain value was chosen within the linear viscoelastic region of the systems, previously determined by strain sweep tests performed at the frequency of 1 Hz at 25 °C. For each film, three different preparations were done and measurements were performed, at least, in triplicate.

2.6 Swelling and erosion

The swelling and erosion behavior of the films was evaluated by immersing them in Milli Q water, physiological PBS at pH 7.4, or BCS. Samples were weighed at predetermined time intervals, then the excess of water was removed by placing them on a filter paper.
The swelling degree (*SD*, %) was calculated as:

$$SD(\%) = \frac{(W_t - W_i)}{W_i} \text{ x } 100 \qquad [1]$$

where W_t and W_i correspond to the weight of the swollen sample at the time t and the initial weight, respectively. For each film, three independent preparations were done and each measurement was performed, at least, in duplicate.

2.7 Scanning Electron Microscopy (SEM)

Surface morphology was imaged by a Field Emission Scanning Electron Microscope (FESEM-JEOL) at an accelerating voltage of 10 kV. The films were washed to extract glycerol and other soluble fractions, quenched with liquid nitrogen, freeze-dried, mounted on SEM aluminum stubs by means of a graphite adhesive layer and gold coated by JFC-1300 gold coater (JEOL) for 90 s at 30 mA before scanning.

2.8 Atto633 and ChAE release measurements

For the preparation of Atto633- or *Ch*AE-loaded films, 80 μg of Atto633 per gram of film or *Ch*AE extract containing ca. 62.5 μg of biophenols per gram of film, were added to the hot suspension and vortexed just before pouring it into a 12-well plate in the laminar flow hood. The concentration of the loaded *Ch*AE biophenols was chosen to guarantee significant antioxidant activity without inducing cytotoxicity in the hypothesis of its complete release [26]. For each release measurement, six small circular disks (each one of 0.2 cm^2 with an average weight of about 0.02 g) were placed in 5 mL of pure water, physiological PBS or BCS medium. The release study was performed over a period of 48 h by collecting 800 μL of the external medium (pure water, physiological PBS or BCS) and replacing it with an equivalent amount of the same medium. The amount of Atto633 released in the external media was spectrophotometrically measured using a Shimadzu UV-Vis 2401-PC spectrophotometer by reading the absorbance intensity at the wavelength of 633 nm.
The release of biophenols by the *Ch*AE-loaded film was evaluated measuring the concentration of biophenols the external media by Folin-Ciocalteu assay, as described in the paragraph *2.3.*

2.9 Cell culture

Mouse embryonic fibroblast cell line NIH-3T3 (SIGMA-ALDRICH) was cultured in Dulbecco's Modified Eagle Medium (DMEM)-high glucose supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 10% bovine calf serum (culture medium) at 37°C in 5% CO_2 humidified atmosphere.

2.10 Cell viability assay

The biocompatibility of the AGA1_κ-CAR1.0_GRO3 film was evaluated on NIH-3T3 cell line by extract and direct contact tests according to International Standard recommendations for biological evaluation of medical devices [32].
In the extract test, the potential cytotoxicity of any released component from the AGA1_κ-CAR1.0_GRO3 film was evaluated on NIH-3T3 cell line by MTS assay. The AGA1_κ-CAR1.0_GRO3 film was extracted aseptically in culture medium (extraction vehicle) for 24 h at 37°C in 5% CO_2. The ratio of the AGA1_κ-CAR1.0_GRO3 film to extraction vehicle was 1.25 cm^2/ml. NIH-3T3 cells (10^4 cells/well) were seeded in 96 well plate at 37°C in a humidified incubator (5% CO_2, 95% air) for 24 h in 100 μl of culture medium. The medium was discarded on the next day and replaced with 100 μl of extraction vehicle (conditioned medium) mixed 1:1 with fresh culture medium and cells incubated for 24 and 48 h at 37 °C in 5% CO_2. At the end of each treatment time, cells were microscopically examined and then 20 μl of reagent solution of

CellTiter 96 Aqueous One Solution Cell Proliferation Assay Kit (PROMEGA) were added into each well and the plates were incubated for 4 h at 37 °C in 5% CO_2. Absorbance was read at the wavelength of 490 nm with the iMark Multiplate reader (BioRad). Untreated NIH-3T3 cells were used as control, under the same experimental conditions. Images of untreated and treated cells were taken at the different incubation times using an optical inverted microscope (Leica DM IL).

In the direct contact test, the potential cytotoxicity of the AGA1_κ-CAR1.0_GRO3 film was evaluated by a physical interaction of the film with NIH-3T3 cells. NIH-3T3 fibroblasts ($3x10^4$ cells/well) were seeded in 24 well plates at 37°C in a humidified incubator (5% CO_2, 95% air) for 24 h in 500 µl of culture medium. Afterwards, AGA1-κ-CAR1.0-GRO3 film (0.2 cm^2 with an average molecular weight of 0.02 g) was carefully placed onto the cell layer. Cells were kept in contact with the film for 24 and 48 h and analyzed in comparison with cells grown in absence of film. Cell morphology was assessed using an optical inverted microscope (Leica DM IL) and quantitative analysis was performed by MTS assay. In brief, after each treatment, 100 µl of reagent solution were added into each well and the plates were incubated for 4 h at 37°C in 5% CO_2. Finally, 100 µl were moved from each well to a 96 well plate and the absorbance was read at the wavelength of 490 nm with the iMark Multiplate reader (BioRad).

The effects of AGA1_κ-CAR1.0_GRO3 loaded with *Ch*AE extract containing ca. 62.5 µg of biophenols per gram of film were analyzed on NIH-3T3 fibroblasts by direct contact for 24 h. Cell morphology was assessed using an optical inverted microscope (Leica DM IL) and quantitative analysis was performed by MTS assay as described above.

For all tests, cell viability was quantified as the percentage relatively to untreated cells (Control). Three replicates for test samples and controls were performed.

2.11 Antioxidant activity assays

The antioxidant properties of the unloaded and *Ch*AE-loaded AGA1_κ-CAR1.0_GRO3 films were evaluated by DCFH-DA assay on the murine cellular model by using the indirect method. In DCFH-DA assay, the antioxidant properties of both films (unloaded and *Ch*AE-loaded) were evaluated for their ability to scavenge the reactive oxygen species (ROS) induced by TBH in NIH-3T3 murine cell line, previously treated with the film components released in the culture medium (conditioned medium). In brief, each film sample (0.2 cm^2 with an average weight of about 0.02 g) was independently soaked in 500 µl of culture medium without phenol red for 24 h at 37°C in 5% CO_2 in a 24 well plate. Meanwhile, $2.2x10^4$ cells were seeded in 8 well chamber slide (Nunc™ Lab-Tek™ II) with 200 µl of culture medium without phenol red. After 24 h, 100

µl of culture medium were removed and replaced with 100 µl of conditioned medium. Then, the cells were treated with DCFH-DA and TBH as described in [26]. Cells treated or not with 0.5 µg/ml of biophenols from *Cryphaea heteromalla* aqueous extract were used as positive or negative control, respectively. The ROS presence within the cell cytosol was evaluated by the fluorescence signal of DCF (2'-7'-diclorodihydrolfuorescein), a high fluorescent molecule obtained by the DCFH-DA oxidation caused by the ROS presence. The nuclei were stained with Hoechst 33342 fluorescent DNA-binding dye at 0.01 mg/ml. DAPI filter (blue emission) and FITC filter (green emission) were used to detect nuclei and intracellular ROS, respectively, on a Nikon Eclipse 80i microscope equipped for epifluorescence and recorded by a digital camera system.

2.12 Oxygen Radical Absorbance Capacity (ORAC) assay

The ORAC assay was performed to quantify the antioxidant capacity of unloaded and *Ch*AE-loaded AGA1_κ-CAR1.0_GRO3 films. Three film samples (0.2 cm^2 with an average weight of about 0.02 g) were independently soaked in 500 µl of PBS for 24 h at 37°C in 5% CO_2. Then, 100 µl were collected and diluted 1:1 with fresh PBS. Then, 40 µl were further diluted with 140 µl of fluorescein 0.04 µM in Na-K phosphate buffer 0.075 M (pH 7.0) and 20 µl of AAPH 40 mM to obtain a final volume of 200 µl. 40 µl of PBS and 40 µl of 100 µM Trolox were used as blank and standard reference, respectively. The assay was performed as reported by Provenzano and coworkers [26], and the antioxidant capacity of films was expressed as µmol of Trolox Equivalents (TE)/g of film, by using the following equation:

$$ORAC\ value\ (\mu mol\ TEg^{-1}) = a \cdot h\ \left[\frac{(S_{sample} - S_{blank})}{(S_{Trolox} - S_{blank})}\right] \quad [2]$$

where a is the ratio between the extraction volume (liters) and the film grams; h is the final concentration of Trolox expressed as µmol/l, S is the area under the curve of fluorescein in the presence of sample, Trolox or extraction buffer. At least three replicates of each sample were performed.

2.13 Statistical analysis

All values reported were obtained as the mean of at least three independent experiments ± standard errors (SE). Results were compared using one-way analysis of variance with pairwise comparisons among treatments made using T test. The analyses were performed using the

SigmaPlot 11.0 statistical program (Systat Software Inc. U.K.). Results were considered statistically significant at $p < 0.05$.

3. Results and discussion

3.1 Preparation and mechanical properties of the films

Transparency of films designed for wound healing, as those ones presented here, is an important aspect of modern dressings because it allows the visual inspection of the status of the wound without removal of the bandage.

Three formulations have been designed by using two different, inexpensive and easily accessible, algal polysaccharides: agarose and κ-carrageenan. The formulations contained also glycerol as plasticizer, whose function is to reduce the internal friction among the polymer chains and their resistance to deformation, with the aim of making the film more pliable and malleable, thus better fitting with joint movements [6].

In Table 1 the final concentrations of the film components after the attainment of the steady weight ("as-prepared" films) are reported, calculated from the difference between the initial and the final weight of the samples. It can be noted that dehydration is only partial, and water still remains the main component of the films, being in the range 50-60% of the total weight, in agreement with the wound dressing hydrogel definition by FDA [33].

In Fig. 1 viscoelastic moduli of the as-prepared films as a function of frequency are reported. It can be detected that all films behaved as strong gels, being both moduli almost independent on the frequency, with G' always one order of magnitude higher than G''. As expected, an increase of both moduli at increasing κ-CAR concentration, hence total polymer concentration, is observed.

3.2 Swelling/erosion and mechanical properties of the hydrated films

Fig. 2 reports the swelling behavior of the as-prepared films as a function of the time in pure water, PBS and BCS. Reported data are the mean of the results obtained by three independent preparations (± SE). Each measurement was performed, at least, in duplicate.

In all the external media the swelling degree increases with increasing κ-CAR concentration, and hence also total polymer concentration. This apparently counterintuitive behavior can be rationalized taking into account the ionic nature of the κ-

carrageenan, that increases the osmotic pressure difference between the external medium and the gel. Changing the swelling medium from pure water to PBS and BCS, the swelling degree at plateau decreases from 1000% (in pure water) to 200% (in BCS). This behavior can be explained considering the reduction of the osmotic pressure due to the augmented osmolarity of the external medium. It is worth noticing that all swollen films maintain their aspect and dimensions also after two-month immersion in the three media. The swelling profiles have been fitted with the Weibull equation:

$$SD = SD_{\infty}\left(1 - exp\left(-\left(\frac{t}{t_{c,s}}\right)^{n_s}\right)\right) \quad [3]$$

where SD_{∞} represents the asymptotic swelling degree at infinite time, while $t_{C,S}$ and n_S are fitting parameters.

This empirical stretched exponential provides high quality fits to the experimental data and was also successfully applied to fit the release data that will be presented and discussed below, alongside with the limitations of the model.

Mechanical spectra were performed on samples swollen for 3 hours and 48 hours in pure water, PBS or BCS. As in the case of the as-prepared films, the viscoelastic moduli did not show any significant dependence on frequency, maintaining the typical character of strong gels. Thus, the mechanical properties of the swollen samples are fully defined by the only two parameters, the average G' and tgδ values, reported in Table 2. The storage modulus is related to the crosslinking density in the films, whereas the loss tangent measures the balance between viscous and elastic components.

As expected, solvent absorption causes a reduction of the elastic modulus with respect to the one of the as-prepared films, and such an effect is more pronounced for pure water as swelling medium and for films with lower κ-CAR concentrations. The partial loss of glycerol, after immersion in the external media, causes a tgδ reduction that is higher in the as-prepared films due to its prevalently viscous contribution. The system produced with the highest polymer concentration, despite of the highest swelling degree, yet exhibits the strongest elastic character. PBS and BSC media actually contribute to strengthen the gel, as suggested by the increase of the storage modulus, probably due to the presence of multivalent ions acting as crosslinking agents of the κ-CAR component. In particular, for BSC, and especially for the systems with lower polymer concentrations (0.5 wt% and 1 wt%), we observe an increase of G' and a slight reduction of tgδ upon prolonged permanence of the films in the medium, which suggest a rearrangement of the polymeric network.

The rheological behavior of the three samples after hydration indicated that they are almost comparable, but for a slight higher dominance of the elastic characteristics (G' and tgδ values) over the viscous ones, the AGA1_κ-CAR1.0_GRO3 formulation was selected for further experiments.

3.3 SEM investigation of the as-prepared and hydrated AGA1_κ-CAR1.0_GRO3 film

To investigate the microscopic architecture of the polymers in the AGA1_κ-CAR1.0_GRO3 films, both as prepared and after hydration, and to correlate their structure to the rheological results, scanning electron microscopy measurements have been performed. In Fig. 3 SEM images of cross-sections from the as prepared AGA1_κ-CAR1.0_GRO3 film, and from the 3 h and 48 h hydrated films in pure water, PBS and BCS are reported.

A very compact multi-lamellae organization is visible in the as-prepared film (Fig. 3A) with a compact and smooth surface (Fig. 3A''). After 3 h immersion in pure water (Fig. 3B) the structure unravels into a porous network that opens up even further after 48 hours (Fig. 3B') and, at the same time interval, the surface has lost its smoothness and large cavities are present (Fig. 3B''). The morphological analysis nicely supports the hypothesis that buffer ions act as crosslinkers of the network. Indeed, films immersed in PBS show a more compact structure, in comparison to the one swollen by pure water, and after 3 hours still present some memory of the original multi-lamellae organization even if large pores are formed between layers (Fig. 3C) and after 48-hour immersion in PBS, a large number of small pores are evident (Fig. 3C'). The layered structure is kept by both the 3-hour and 48-hour swollen films in BCS (Fig. 3D and D'), with a tendency to exfoliation upon prolonged immersion.

From examination of both cross-sections and surfaces of the films (see Fig. 3B'', C" and D" referring to systems after 48 h incubation), the lamellar morphology is found to orient parallel to the surface. This layered-oriented structure can explain the anisotropic volume expansion of films upon immersion in the various media, with significant increases in thickness but very modest variations of circumference.

3.4 Release properties of the AGA1_κ-CAR1.0_GRO3 film

Hydrogels strongly differ in pore size, network architecture, and function, and these characteristics determine their use as drug delivery systems. The network chemical structure and mesh size, that affect the hydrophilic/hydrophobic character of the material, and the dimension of the pores, spanning from nanometers to sub-centimeters, regulate the maximum amount and the fate of the incorporated molecules, the rate of their diffusion toward the

external medium and the percentage of the loaded molecules that is eventually irreversibly entrapped in the network [34]. For this reason, the design of hydrogel wound dressings, loaded with bioactive molecules/drugs, cannot disregard performing tests concerning the *in vitro* release behavior of loaded bioactive compounds.

In Fig. 4 (A and B) the release curves of Atto633 probe and *Ch*AE biophenols from the AGA1_κ-CAR1.0_GRO3 film in pure water, PBS and BCS are reported. The loading content for Atto 633 was of ca. 80 μg per gram of film, while for the biophenols present in the *Ch*AE extract was ca. 62.5 μg per gram of film.

Drug/bioactive molecules incorporated in a 3D network structure can access the external medium by diffusion through the tortuous path formed by water channels in the polymeric matrix. This process is governed by the diffusion coefficient of the drug/bioactive molecules in the penetrating dissolution medium, chemical potential gradient and osmotic pressure, and potential erosion of the polymeric structure [35, 36]. The observed profiles for both *Ch*AE biophenols and Atto633 are the typical II-phase profiles: the rapid burst due to the immediate activation of the film surface layer upon hydration and the hydrophilic nature of the *Ch*AE biophenols and Atto633, is followed by a sustained release. The burst effect is often regarded as an event to be avoided since the bioactive molecule is released in an uncontrolled pattern, but in facts it can be also a desired effect in several situations where there is the need to rapidly reach high concentrations of the therapeutic molecule in its site of action, such as in the case of emergency wound management [37].

All curves, with the only exception of the release of *Ch*AE biophenols in BCS, reach a plateau in about three hours, that is the time required for the films to reach their equilibrium swelling. The burst release, the total amount released, and the release rate are strongly affected by the nature of the swelling medium and, to a lesser extent, by the nature of the released substance. When the film is swollen by water, it retains the largest amount of the loaded compounds, but the release is faster in agreement with a higher swelling degree. When immersed in PBS and BCS, the film releases more but at a slower rate. The initial burst and the residual amount of loaded compounds entrapped in the films are controlled by the swelling medium-network interactions and the swelling medium-loaded compound interactions, that affect in turn desorption and partitioning equilibria. The release rates seem to be controlled by the swelling rates in the various media.

In order to confirm this last hypothesis, the same Weibull empirical model used to fit the swelling kinetics was applied to fit the release profiles:

$$M = M_{\infty}\left(1 - exp\left(-\left(\frac{t}{t_{c,r}}\right)^{n_r}\right)\right) \quad [4]$$

where M_{∞} is the amount of molecules released at infinite time, $t_{c,r}$ and n_r are fitting parameters. Also in this case, the agreement between experimental data and fit is good. In Fig. 4 (C and D) the correlation between the fitting parameters obtained for the release of either Atto633 or *Ch*AE biophenols and for the swelling kinetics is shown. It should be evidenced that the Weibull equation is only an empirical model, therefore its fitting parameters are devoid of any relation to diffusional constants and other physical parameters of the system [38]. Yet, it is successful in fitting all the release profiles, while Higuchi [39], Peppas [35, 40] and Case II [41] models failed, and in demonstrating the independence of the release kinetics from the nature of the released substance and their fairly good correlation with the swelling kinetics.

3.5 Evaluation of AGA1_κ-CAR1.0_GRO3 film biocompatibility

In the complex process of wound healing, fibroblasts have an important role. They come into play in the proliferative phase of the process, namely in the phase of tissue repair. They are also responsible for the production of a new extracellular matrix, necessary to provide suitable cell growth support. Once transformed in the myofibroblast phenotype, they permit the wound contraction that is essential for wound closure [42]. Accordingly, fibroblast L-929 or NIH- 3T3 cells are generally used in the ISO 10993-5 *in vitro* cytotoxicity test guideline [32].

In our study, the biocompatibility of AGA1_κ-CAR1.0_GRO3 film was verified on the NIH-3T3 fibroblasts by assessing the film effects through two different tests as suggested in the International Standard recommendations for biological evaluation of medical devices [32]: extract and direct contact tests.

The extract test showed the biocompatibility of AGA1_κ-CAR1.0_GRO3 film by determining if the material contained significant amounts of biologically harmful extractables. In this test, the volume of NIH-3T3 cell culture fresh medium was diluted 1:1 with the medium incubated 24 h in presence of AGA1_κ-CAR1.0_GRO3 film (conditioned medium). Effects of the conditioned medium on the cells were evaluated after 24 and 48 h. As shown in Fig. 5A, NIH-3T3 cell viability was comparable with cells grown without addition of conditioned medium (Control) at all incubation times investigated.

The microscopical observation demonstrated that NIH-3T3 cells did not show any change of morphology with respect to control cells (Fig. 5B).

Fig. 6A shows the viability of NIH-3T3 cells after 24 and 48 h of direct contact with AGA1_κ-CAR1.0_GRO3 film. No significant difference of the cell viability was observed for the entire observation period in comparison with the viability of cells incubated without film (Control) (Fig. 6A). Moreover, the direct contact did not cause any change of cell morphology after 24 and 48 h incubation (Fig. 6B). Data reported in Figures 5A and 6A are the mean of the results obtained by at least three independent experiments (±SE).

3.6 In vitro study on antioxidant power of unloaded- and ChAE-loaded AGA1_κ-CAR1.0-GRO3 films

Reactive oxygen species (ROS) play an important role in several phases of wound healing process. In the hemostatic and inflammatory phase, a burst of ROS is released from neutrophils to fight the wound infection. Furthermore, ROS, as secondary messengers, are involved in the recruitment of lymphoid cells to the wound site, and in the regulation of angiogenesis, cell migration, proliferation, fibrosis and remodeling. Low concentrations of ROS are required for these important functions but their excessive generation could result in prolonged inflammatory phase lastly leading to a chronic non-healing wound [43, 44]. Biophenolic compounds were demonstrated to have antioxidant capacity and activity during the wound healing process [45-47]. Therefore, the release of biophenolic compounds at wound site seems to be a potential strategy to restore the redox state of the wound [48, 49]. In a recent study [26], we demonstrated that the aqueous extract of the *Cryphaea heteromalla* (*Ch*AE) bryophyte has a high concentration of phenolic compounds. Furthermore, it contains some chemical species of jasmonates, as the 12-oxo-phytodienoic acid, that has been demonstrated suppress the oxidative stress-induced death of human neuroblastoma SH-SY5Y cells [50]. The high antioxidant activity of *Ch*AE was also experimentally demonstrated both by ORAC assay and by DCFH-DA in mouse embryonic fibroblast NIH-3T3 cells [26].

The antioxidant ability of the present formulation was tested by the ORAC assay performed with the conditioned media obtained after 24 h incubation of the ChAE-loaded and unloaded AGA1_κ-CAR1.0_GRO3 films in PBS. Results (Table 3) showed that the unloaded film already possessed already some antioxidant activity, in agreement with literature data concerning the antioxidant power of κ-carrageenan polysaccharide and its derivatives [51, 52], but the incorporation of bryophyte aqueous extract into the film caused a considerable increase (more than twice) of the antioxidant power of the whole formulation.

The antioxidant activity of the unloaded and *Ch*AE-loaded AGA1_κ-CAR1.0_GRO3 film was investigated on NIH-3T3 fibroblasts to verify if the antioxidant effect also occurred in an *in vitro* cellular model. NIH-3T3 cultured cells, in which the oxidative stress was mimicked with TBH addition, were untreated or pretreated with crude *Ch*AE at the biophenol concentration of 0.5 mg/mL, with AGA1_κ-CAR1.0_GRO3 conditioned medium or with *Ch*AE-loaded AGA1_κ-CAR1.0_GRO3 conditioned medium in which are present about 0.5 μg/mL biophenols , released from the *Ch*AE-loaded film. Results (Fig. 7) confirmed the protective effects against the oxidative stress achieved with both unloaded and *Ch*AE-loaded films and with *Ch*AE used as positive control.

Finally, we tested the biocompatibility of the *Ch*AE-loaded AGA1_κ-CAR1.0_GRO3 film by 24 h direct contact with the NIH-3T3 fibroblasts. The results (obtained as the mean of at least three independent experiments ±SE) showed neither significant difference in cell viability (Fig. 8A) nor absence of cellular morphological modification in comparison with control (Fig. 8B), demonstrating that also the *Ch*AE-loaded AGA1_κ-CAR1.0_GRO3 film did not exhibit cytotoxicity.

In general, the choice of a dressing should be appropriate to the wound characteristics and acceptable to the patient. When the progress of a wound does not regularly proceed, a chronic wound can originate. The chronic ulcers, as it occurs in the case of diabetic patients, have an annual cost of about US$ 10,000 per patient [53]. Thus, the appropriate treatment of wounds, particularly the chronic ones, has become a serious challenge. A dressing absorbing the moist formed in the wound causes a rapid re-epithelialization process and helps in the removal of the debridement surrounding the healthy skin [53].

In the last years the seaweed polysaccharides, such as carrageenan, sorghum, laminaran, and alginate, have attracted the interest of the researchers for their unique structural and functional characteristics, particularly for their swelling capability, biodegradability and non-toxic nature [54]. Furthermore, their great abundance makes possible their use in the design of low-cost medical devices [54].

The *in-vivo* curative effect of the sorghum polysaccharide hydrogel, extracted from the seeds of *Sorghum bicolor*, was demonstrated in the treatment of laser-induced burns in rat model. Results indicated a complete restoration of the healthy epidermis after eight days of treatment [55]. A laminaran-based cream was evaluated for the treatment of wounds induced in the whole skin layer of rat model. After thirteen days a complete skin repair was obtained with correct derma organization and collagen deposition [56]. A smart wound dressing film based on κ-CAR

and locust bean gum has been developed. The cranberry extract was added to the hydrogel to make it sensitive to pH of the environment and detect bacterial infection in the wound site as revealed by change of the hydrogel color. Results indicated that the formulation had suitable mechanical properties and a dose-dependent cytotoxicity against NIH-3T3 fibroblasts [57].

Calcium alginate hydrogels have been extensively used as wound dressing materials [58, 59]. Alginate hydrogels present many advantages. They can absorb wound fluid up to 20 times their weight and remain on the wound site for several days [8]. Furthermore, alginate hydrogels are able to increase homeostasis and activate platelets [60]. Their disadvantages consist in the pain caused by their removing in the case of wounds producing small amount of exudate, the request of additional dressing and their propensity to collect the exudate at the edge of the wound, causing damages to the healthy skin [61]. Thus, it can be concluded that the identification of the dressing for a particular wound type is an important element in facilitating wound healing [8].

In a previous recent work the content of the aqueous extract of *Cryphaea heteromalla* bryophyte species was elucidated [26]. Benzoic, caffeic and *p*-coumaric acids, possessing high antioxidant ability, were identified as molecules present in *Cryphaea heteromalla* aqueous extract. It was found that caffeic and *p*-coumaric acids possessed anti-inflammatory effect by inhibiting nitric oxide and inducible nitric oxide synthase. Furthermore, caffeic acid acts as anti-inflammatory agent by inhibiting NF-κB expression [62]. In NIH-3T3 cells and skin-incise mice, caffeic acid considerably stimulated the production of collagen-like polymer, making possible the acceleration of the wound healing process [63]. When a wound occurs on the skin, several types of cells, neutrophils, fibroblasts and monocytes, rush towards the injury site to start the repair process. Particularly, neutrophils produce the myeloperoxidase, an enzyme generating reactive oxygen species. This enzyme remains active up to 15 days, after which the collagen fabrication starts. It was found that caffeic acid was able to significantly reduce the enzyme activation period to 10 days, thus anticipating the collagen production [63]. It was also demonstrated *in vitro* that caffeic and *p*-coumaric acids possessed antimicrobial properties against several infectious agents, such as *E. coli*, *Staphylococcus aureus*, *Bacillus cereus*, *Listeria monocytogenes* [64]. The effect is further potentiated by environmental factors as pH, and NaCl concentration [65]. *p*-Coumaric acid was found to be suitable in decreasing the expression of inflammatory mediators, TNF-α and IL-6 cytokines, in arthritic rats. Its effect was also effective in attenuating the symptoms of the disease [66]. Peng et al. indicated that *p*-coumaric acid could be efficient in the prevention of caractogenesis by protecting human lens epithelial cells (SRA01/04) from oxidative stress and apoptosis by acting on the MAPK signaling pathway [67].

In addition to biophenols, *Cryphaea heteromalla* aqueous extract contains several types of jasmonates (JA). These oxidized lipid-derived molecules (oxilipins) are exclusively present in plants and play a fundamental role in regulating plant immunity, germination and development [68] and can be considered as the endocrine system of the plants [69]. They intervene also as a response to several plant injuries among which wounds, pathogens, insects and mechanical stress [69]. Although discovered at the end of the XIX century, only recently these molecules have attracted the interest of the researchers and, up to now, very few literature data illustrate the role of jasmonates in animal cell cultures. The *in vitro* anti-cancer activity of JA was already demonstrated [70], but these molecules possess also an anti-inflammatory action [71] obtained through modulation of the NF-κB signal [72]. Further researches demonstrated that they could perform important anti-aging properties on skin by ameliorating the epidermal mechanical properties and favoring the regeneration of the extracellular matrix [73, 74]. In 2017, Henriet and coworkers observed that JA had effects on the skin glycosaminoglycan structure [75]. Furthermore, their results demonstrated that JA enhanced cell proliferation and migration of keratinocytes, through a still unknown mechanism with the fibroblast growth factors (FGF-7), involved in the angiogenesis, wound repair and embryonic development [75].

4. Conclusions

Hydrogels designed for wound dressing are ideal materials because they provide the optimal hydration for the cutaneous wounds. Their mechanical properties, in terms of flexibility, conformability and elasticity, allow a good patient's compliance. The formulation here proposed demonstrated to be a good wound dressing candidate because of its stability, ability to absorb large amounts of water, or in general, biological fluids, and film forming properties without addition of crosslinking agents. Moreover, film transparency permits an easy observation of the wound conditions. Furthermore, the high availability of the various components of the film formulation and easy preparation procedure should result in a relatively inexpensive production. Results suggest that loading the *Ch*AE aqueous extract in the film may also prevent excess oxidative stress generation during wound healing.

Further research will be devoted to the complete assessment of the biocompatibility of the formulation, here evaluated with some preliminary tests. Hemocompatibility study will allow to determine eventual effects of the *Ch*AE-loaded AGA1_κ-CAR1.0_GRO3 film on the blood components. Keratinocyte migration and cytokine activation studies will be also performed to address the effects of the film on the rate of healing. Its ability to act as a barrier against microbial infections will be also investigated.

Acknowledgements.

The authors wish to thank Fulvio Ferrante, Roberto Megna, and Alessia Provenzano for their valuable technical contribution. The useful collaboration with Eng. Fabio D'Anca is also recognized. This work was supported by **t**he European Social Assets "Sicilia 2020 – Rafforzare l'occupabilità nel sistema R&S e la nascita di Spin Off di ricerca in Sicilia" (P.O. FSE 2014/2020 Project - CUP: G77B17000220009).

Conflict and declaration of interest

The authors declare no conflict of interest and that they do not have any financial and personal relationships with other people or organizations that could inappropriately influence their work.

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FIGURE CAPTIONS

Figure 1. Mechanical spectra of the as-prepared films. Elastic (G', solid symbols) and viscous (G'', empty symbols) moduli of the as-prepared films after achievement of a steady weight for AGA 1.0 wt% blended with 0.5 (circles), 1.0 (triangles) and 1.5 wt% κ-CAR (diamonds) and GRO 3.0 wt%.

Figure 2. Swelling behavior of the films. Swelling curves of the films in pure water (A), PBS (B) and BCS (C) for AGA 1.0 wt% blended with 0.5 (circles), 1.0 (triangles) and 1.5 wt% κ-CAR (diamonds) and GRO 3.0 wt%. Dashed lines are the best fit of the experimental data with Eq. 3. Reported data are the mean of the results obtained by three independent preparations (±SE). Each measurement was performed, at least, in duplicate.

Figure 3. SEM images of the as-prepared and hydrated AGA1_κ-CAR1_GRO3 films. Scanning electron microscopical images obtained from the as-prepared AGA1_κ-CAR1_GRO3 film, cross section (A) and surface (A''). AGA1_κ-CAR1_GRO3 film 3-hour (B, C and D, cross sections) and 48-hour (B', C' and D', cross sections; B'', C'' and D'' surfaces) hydrated films in pure water (B, B' and B''), PBS (C, C' and C'') and CBS (D, D' and D'').

Figure 4. Release profiles and Weibull fitting parameters of swelling/release. Release profiles of Atto633 (80 μg/g film, black symbols) (A) and *Ch*AE (62.5 μg/g film, white symbols) (B) from AGA1_κ-CAR1.0_GRO3 film in pure water (circles), PBS (triangles) and BCS (diamonds) media. Weibull equation fitting parameters n_r vs. n_s (C) and $t_{c,r}$ vs. $t_{c,s}$ (D) obtained from the release (n_r and $t_{c,r}$) and swelling curves for Atto633 (black symbols) and *Ch*AE (white symbols) for AGA1_κ-CAR1.0_GRO3 film in pure water (circles), PBS (triangles) and BCS (diamonds) media. For each film, three different preparations were done and the measurements were performed, at least, in duplicate.

Figure 5. Extract test results. NIH-3T3 cell viability, obtained as a percentage with respect to the untreated control (black bars) after 24 and 48 h exposure to conditioned medium (AGA1_κ-CAR1.0_GRO3 film Conditioned medium) (gray bars). Data are presented as the mean ± SE of three independent experiments (A). Microscope images of the NIH-3T3 fibroblast after 24 and 48 h without treatment (Control) and with

conditioned medium (AGA1_κ-CAR1.0_GRO3 Conditioned medium). Images are representative of three independent experiments. Original magnification 10X. Scale bar 50 μm (B).

Figure 6. Direct contact experiments. NIH-3T3 cell viability obtained as a percentage with respect to the untreated control (black bars), after 24 and 48 h direct contact of the NIH-3T3 cells with AGA1_κ-CAR1.0_GRO3 film (Film direct contact) (gray bars). Data are presented as the mean ± SD of three independent experiments (A). Microscope images of the NIH-3T3 fibroblast after 24 and 48 h without film (Control) and with AGA1_κ-CAR1.0_GRO3 film. The black arrows indicate the edge of the film. Images are representative of three independent experiments. Original magnification 10X. Scale bar 50 μm (B).

Figure 7. Antioxidant effects of the unloaded AGA1_κ-CAR1.0_GRO3 and *Ch*AE-loaded AGA1_κ-CAR1.0_GRO3 on NIH-3T3 fibroblasts. Fluorescence microscopy images, after TBH oxidative stress induction in live NIH-3T3 cells untreated (Control) or treated with *Ch*AE (*Ch*AE) at the concentration of 0.5 μg/mL, with AGA1_κ-CAR1.0_GRO3 conditioned medium (Unloaded AGA1_κ-CAR1.0_GRO3) or with *Ch*AE-loaded AGA1_κ-CAR1.0_GRO3 conditioned medium (*Ch*AE-loaded AGA1_κ-CAR1.0_GRO3) in which are present about 0.5 μg/mL biophenols , released from the *Ch*AE-loaded film. Intracellular ROS were detected by DCF fluorescence (FITC). The nuclei were stained with Hoechst 33342 fluorescent dye (DAPI). Images are representative of three independent experiments. Original magnification 20X. Scale bar 20 μm.

Figure 8. Direct contact with *Ch*AE-loaded AGA1_κ-CAR1.0_GRO3 film. Cell viability obtained as a percentage with respect to the untreated control (white bar), after 24 h direct contact of the NIH-3T3 cells with *Ch*AE-loaded AGA1_κ-CAR1.0_GRO3 film (gray bar). Data are presented as the mean ± SE of three independent experiments (A). Morphological inspection of untreated NIH-3T3 cultured fibroblasts (Control) (B). Morphological inspection of NIH-3T3 cultured fibroblasts in direct contact with *Ch*AE-loaded AGA1_κ-CAR1.0_GRO3 film. The black arrow indicates the edge of the film (C). Images are representative of three independent experiments. Original magnification 10X. Scale bar 50 μm.

Table 1. Final concentrations (wt%) of the components in the as-prepared films after the attainment of the constant weight.

Sample ID	AGA	κ-CAR	GRO	H_2O
AGA1_κ-CAR0.5_GRO3	8.9	4.5	26.8	58.8
AGA1_κ-CAR1.0_GRO3	9.3	9.3	28.0	53.4
AGA1_κ-CAR1.5_GRO3	9.1	13.5	27.2	50.2

Table 2. Average elastic modulus (G') and tgδ values after 3-hour and 48-hour immersion in pure water, PBS and BCS. Reported G' values must be multiplied by 10^4.

		AGA1_κ-CAR[X]_GRO3					
		X = 0.5		**X = 1**		**X = 1.5**	
		G' [a]	**tgδ**	**G'** [a]	**tgδ**	**G'** [a]	**tgδ**
As-prepared		6.7	0.11[b]	9.0	0.13[b]	16.0	0.15[b]
H_2O-swollen	*3 h*	0.6	0.05[c]	0.6	0.06[c]	1.3	0.07[c]
	48 h	0.6	0.05[c]	0.6	0.05[c]	1.7	0.06[c]
PBS-swollen	*3h*	1.1	0.07[c]	0.9	0.05[c]	1.7	0.07[c]
	48 h	1.0	0.06[c]	0.8	0.07[c]	2.1	0.04[c]
BCS-swollen	*3h*	1.3	0.07[c]	1.7	0.07[c]	1.3	0.07[c]
	48 h	1.7	0.06[c]	2.2	0.04[c]	1.4	0.06[c]

[a] The errors are lower than 100 Pa
[b] The errors are lower than 0.03
[c] The errors are lower than 0.003

Table 3. – Oxygen Radical Absorption Capacity (ORAC) test.

	Unloaded film	***Ch*AE-loaded film**
ORAC value (μmol TE/g film)	2.71 ± 0.10	6.35 ± 0.24