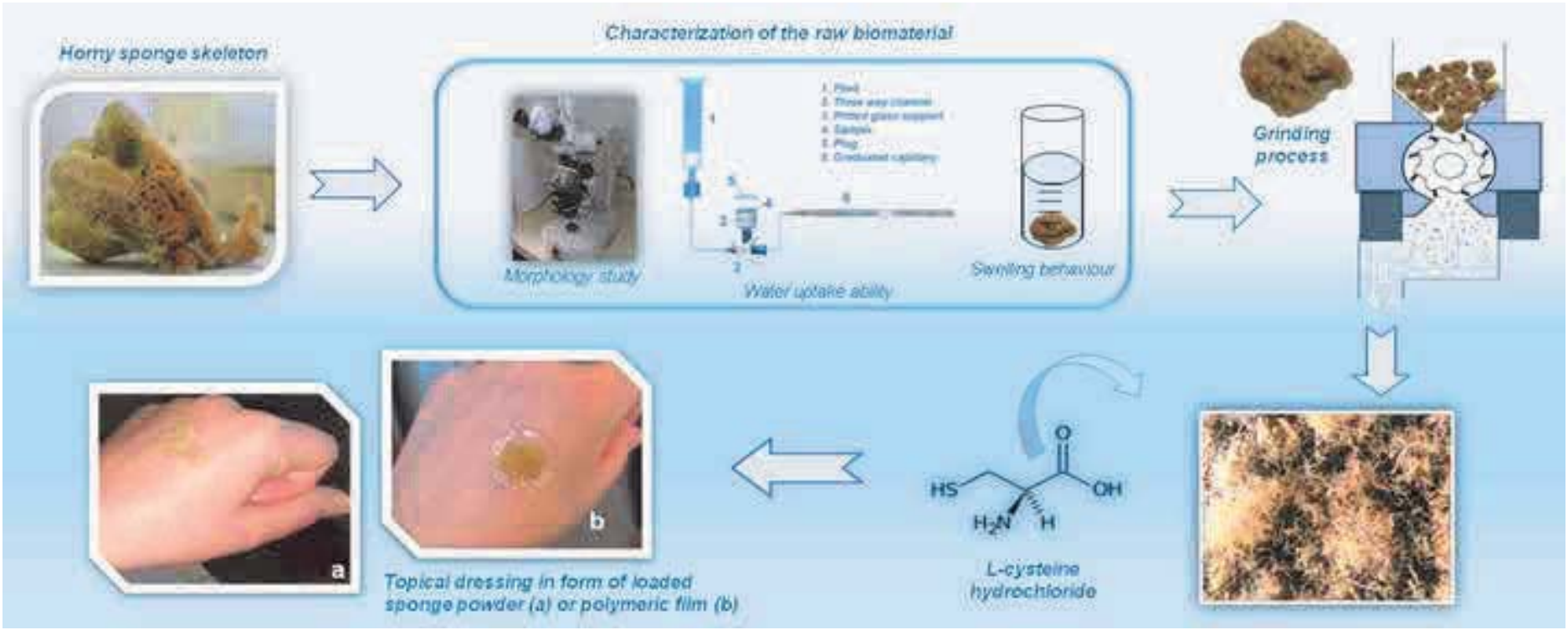
Natural collagenic skeleton of marine sponges in pharmaceutics: Innovative biomaterial for topical drug delivery

Graphical Abstract



Marine sponge skeleton is proposed as biomimetic material for pharmaceutical use

The biomaterial can be loaded with L-cystein as wound healing drug

Selected drug loaded sponge powder shows interesting morpho-functional performances

Natural material, due to its composition, can be considered as bio-functional carrier

Loaded powder was included in biocompatible film as suitable topical delivery system

Natural collagenic skeleton of marine sponges in pharmaceutics: innovative biomaterial for topical drug delivery

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Abstract

The growing interest in the use of recyclable and biodegradable natural materials has become a relevant topic in pharmaceutics. In this work, we suggest the use and valorization of natural horny skeleton of marine sponges (Porifera, Dictyoceratida) as bio-based dressing for topical drug delivery. Biomaterial characterization focusing on morpho-functional traits, swelling behavior, fluid uptake performances, glycosaminoglycans content and composition and microbiological quality assessment was carried out to investigate the collagenic skeleton properties. After grinding and sieving processes, L-cysteine hydrochloride-loaded formulations were designed in form of powder or polymeric film by testing various drug concentrations and different drying parameters. Drug content, SEM analyses and *in vitro* permeation studies were performed to test the suitability of skeleton-based formulations. To this respect, drying time and temperature are key parameters for skeleton-mediated drug crystallization. Consequently, this behavior seems to influence drug loading and permeation profiles of formulations. The high percentages of drug are found after absorption into sponge powder and *in vitro* permeation studies demonstrate that cysteine is released more slowly than the pure drug within 1h. Such a system is attractive because it combines the known healing properties of cysteine with the advantageous potentials of the collagen/proteoglycan network, which can act as biocompatible carrier able to absorb the excess of the wound exudate while releasing the drug. Furthermore, due to its glycosaminoglycans content, natural sponge skeletal scaffold might act as bioactive-biomimetic carrier regulating the wound healing processes.

Keywords

Porifera skeleton, bioactive-biomimetic material, glycosaminoglycan, alginate-based film, environmentally sustainable formulation, novel wound dressing

1. Introduction

A chronic wound is described as a break in the skin, which lasts at least 6 weeks and has a frequent recurrence. It might affect mainly epidermis and dermal inner layers. Several factors are involved in wound healing; it is indeed a complex and dynamic process of renovating cellular components and tissue layers [1]. A wide range of topical wound dressings are now available such as films, foams, hydrogels containing antiseptics, antibiotics and/or factors involved in the healing process [2-6]. The treatment of wounds will be dependent on the wound type, healing phases, condition of patient and it will include the concept of moisture[7,8]. Optimal conditions comprise a proper circulation of oxygen to support restoring cells and tissues, effective moisture around the wound and minimum microbial load [9, 10]. Despite moisture is fundamental for proper healing, excessive wetness on the wound might become problematic. A wet-wrap dressing applied to highly exudative wounds can lead to tissue maceration. Moreover, chronic wound fluid may actively interfere with the healing process, since it contains proteases that damage extracellular matrix (ECM) components, while inhibiting fibroblast proliferation. Fibroblasts produce indeed different kinds of substances important for wound repair, i.e. glycosaminoglycans (GAGs) and collagen [11]. An ideal wound dressing should remove the excess exudate without totally absorbing it, creating an excessively dried wound as a consequence. The absence of amino acids and proteins may also compromise the wound healing process [12]. In particular, effectiveness in promoting epithelial regeneration by sulfur amino acids is well known: they influence the cell proliferation and new cell formation [13]. For example, cysteine is an essential component in re-establishing wound inflammation and increasing the concentration of wound fibroblasts. Furthermore, cysteine is necessary for disulfide bonds that play an important role in the formation of the collagen triple helical structure, strengthening its components [14]. In antiquity, the collagenic skeletons of some horny sponge species (phylum Porifera, class Demospongiae, subclass Keratosa, order Dictyoceratida) now commonly known as bath sponges were recommended for wound cleaning and drying before

applying a bandage, in order to absorb fluids and promote wound healing [15, 16]. Recently the pharmaceutical interest in sponge-derived molecules/biomaterials and their sustainable production focus on a wide array of applied research[17,18]. Sponges are basal Metazoa and their *bauplan* has proved successful in evolution since Cambrian in a wide array of aquatic environments [19]. The body architecture of Demospongiaeis structured as ectosome with its skeleton as a selective and protective barrier at the outer surface, and the choanosome with its skeleton in the inner region containing flagellated chambers, choanocytes, and canals of the aquiferous system. The jelly ECM of these ancient multicellular animals resembles that of higher taxa, being composed of collagen, sulfated polysaccharides, proteoglycans (PGs), and minor amounts of structural proteins and glycosaminoglycans (GAGs) [20-26]. In the sponge ECM collagen fibrils are arranged to form thin fibrils which tend to be more densely packed to form the fibrous skeleton [23]. In horny sponges skeleton a high number of fibers are spatially arranged in a hierarchical network of primary and secondary fibers. From chemical studies on sponge collagen, glycosylated hydroxylysine together with a high content of aspartate and glutamate were found to represent the main components of fibrils [23]. In order to reproduce the interesting structure of these animals, during recent years spongy like matrices obtained by crosslinking of different polymers have been developed as new drug delivery systems targeted to wound healing [1,27, 28].

The aim of this study is to suggest a new bio-based dressing in form of powder or polymeric film for cutaneous wound healing. This biomaterial consists of marine sponge natural collagenic skeleton of a target species loaded with L-cysteine hydrochloride. The main objective of the work is to evaluate the morpho-functional performances of this bioactive-biomimetic material, easily available in the wild and/or on the market, for pharmaceutical application as natural alternative to the development of polymeric spongy-like matrices. Due to its collagenic network of fibers, it can adsorb the excess of wound exudate and modulate the release of the entrapped drug, thus contributing to a suitable environment for the re-epithelialization process. In view of that, this

biomaterial used as carrier for the drug delivery can be a new promising approach to develop environmentally sustainable formulations aimed at wounds treatment.

2. Materials and methods

2.1. Collection and sampling of sponge skeletons

The collagenic skeletons of wild marine sponges (Porifera) used in this study were collected as stranded skeletons from Sardinian beaches (Western Mediterranean Sea) and belong to four species i.e. *Spongia lamella* (Schulze, 1879), *Spongia officinalis* (Linnaeus, 1759), *Hippospongia communis* (Lamarck, 1814) (Demospongiae: Dictyoceratida: Spongiidae) and *Sarcotragus spinosulus* (Schmidt, 1862) (Demospongiae: Dictyoceratida: Irciniidae). Before to be processed each skeleton was accurately rinsed with freshwater in order to remove any contamination in form of salt, sand, shell and rock fragments entrapped in the fibrous skeletal network. Finally, they were air dried at room temperature. Samples isolated from skeletons comprised three groups selected on the base of preliminary tests: one designed for morphological characterization and swelling performances comprising all the four species; a second group for the determination of water uptake and for powder preparation (*S. lamella*, *S. officinalis*, and *S. spinosulus*); a third group for the determination of GAGs (*S. lamella*, *S. officinalis*, and *H. communis*). On the basis of the results, selected subsamples of *S. lamella* were considered as experimental target material for further studies, such as determination of viable aerobic count, sterilization, preparation and SEM characterization of both drug loaded powder and alginate-based dressing, and *in vitro* permeation assays.

2.2. Materials

L-cysteine hydrochloride (CysHCl) (>98%) Ammonium acetate (99.9%) Neocuproine (>98%)and Glycerol (99.5% Eur. Ph.)were purchased from Sigma Aldrich (Italy). Copper (III) chloride dehydrate (99% ACS) was obtained from Aldrich Chemical Company Ltd (Gillingham, England). (USA). Tryptone soya agar (oxide), Sabouraud dextrose agar (oxide), Tryptone soya broth and thioglycolate U.S.P. were purchased from Oxoid LTD (Basingstoke, Hampshire, England). Sodium

alginate (Protanal LF 120L, batch 907788) was purchased from NovaMatrix/FMC Biopolymer (Sandvika, Norway).All other chemicals and reagents were of analytical grade. Alcian Blue8GX (Cod. A9186), Papain, Hyaluronidase from *Streptomyces hyaluroniticus*, Chondroitinase-AC from *Arthrobacteraurescens*, (Cod. C8618), Chondroitinase ABC from *Proteus vulgaris* (Cod. C3667), Chondroitin sulphate A from bovine trachea (Cod. C9819), Chondroitin sulphate B from porcine intestinal mucosa (cod. C-3788), Chondroitin sulphate C from shark cartilage (cod. C-4384), Heparan sulfate from bovine kidney (Cod.H7640), were Sigma Aldrich reagents. Titan III-H cellulose acetate plates (6.0 × 7.5 cm) were from Helena BioSciences. DEAE Sephacel was from GE Healthcare (Cod. 17-0500-01)

2.3. Characterization of sponge skeletons

2.3.1. Sponge skeleton morphology

Each skeleton was observed by Light Microscopy (LM, Leitz DMRB) and Scanning Electron Microscopy (SEM Vega3 Tescan, Czech Republic)to characterize the diagnostic traits of skeletal architecture, namely texture of reticulate network, spatial arrangement of conules and of primary-secondary spongin fibers, abundance and morphometries of collagenic filaments, surface and morphometries of fibers, and presence of foreign material in fibers core. Identification of each specimen at the species level was carried out following standard techniques [29]. The taxonomic status of species was validated following Pronzato and Manconi[30].

2.3.2. Extraction, purification and characterization of glycosaminoglycans (GAGs)

GAG analyses were performed in triplicate on dry skeletons (see 2.1 section).Horny skeletons were re-hydrated for 24 h at 4°C with 0.1M sodium acetate buffer (pH 6.0) containing 5mMethylenediaminetetraacetic acid (EDTA)and 5mM cysteine (pH 6.0), and exhaustively digested with papain at 56°C for 48h (0.3U/mg of dry tissue). Free GAGs were purified by anion-exchange chromatography on (DEAE)-Sephacel, equilibrated with 50mM sodium acetate buffer (pH 6.0) and eluted with 2.0 M LiCl, followed by GAGs precipitation by incubating with 4 volumes of cold absolute ethanol at −20°C overnight. Sulfated polysaccharides were recovered by

centrifugation and dried for quali-quantitative analyses. Total hexuronic acid content was estimated by carbazole reaction, by using glucurono-lactone as a standard. Purified GAGs were submitted to discontinuous cellulose acetate electrophoresis[31]. Following Alcian Blue staining, images were acquired by means of GS-800 calibrated densitometer (Bio-Rad) and analyzed by using Quantity One v4.6.3 software (Bio-Rad). Aliquots of GAGs underwent enzymatic and chemical depolimerisation with standard methods for vertebrate GAGs characterization. The treatment with hyaluronidase (0.5 U/100 µg of hexuronic acid) was conducted in 0.15 M NaCl, 0.02 M Na acetate buffer, pH 6 for 3.5 h at 60 °C. The treatment with chondroitin-AC-or-ABC lyase (0.1 U/100 µg of hexuronic acid) was performed in 50mMTris-HCl, 50mM Na acetate buffer, pH 7.5 for 5 h at 37 °C. To establish the presence of glucosaminoglycan containing GAGs the treatment with nitrous acid was conducted as described by Cappelletti et al. [32].

2.3.3. Swelling studies

The swelling behavior of the samples, obtained by core drill from the whole skeleton (see 2.1 section) was evaluated according to Juliano et al.[33] by measuring the weight increase after contact with different media: distilled water, phosphate buffers (PB) at different pH (7.4, 6.8 and 5.5) and hydrochloric acid buffer (HAB) pH 1.2. Briefly, pre-weighed dried samples were placed in a graduated centrifuge tube containing 10 ml of each medium. At appropriate time intervals (1, 8, 24 h) they were removed and weighed after wiping excess medium with blotting paper. This operation was repeated until a constant weight was observed. Two samples per each species were examined (ectosomal and choanosomal skeleton). The degree of fluid uptake was calculated as swelling index [33]. Moreover, thickness variations of samples were measured by using a gauge and color changes were also recorded.

2.4 Powder preparation: grinding and sieving

Cross sections of the selected sponge skeletons (see 2.1 section) were ground with a mill blade (IkaWerke, Switzerland) at room temperature at4500 rpm. This process was carried out to obtain a powder from the whole skeleton, easily available for the formulative phase. After that, a particle

size analysis was carried out by certified test sieves (Giuliani, Italy) following European Pharmacopoeia 8.0 edition (Eur. Ph. 8.0) guidelines (range 140-20 mesh) [34](Supplementary material).

2.4.1. Scanning Electron Microscopy(SEM)

Samples from skeletons, their powders, and formulations were placed on double-sided tapes which had previously been secured on aluminum stubs and then analyzed after gold sputtering at 20 kV acceleration voltage, under an argon atmosphere.

2.4.2. Water uptake

The ability of samples to absorb water when placed in contact with an aqueous fluid was measured using a modified apparatus of Enslin [35]. Ten mg of powders of selected species reported in section 2.1 were lied on a disk of paper filter (d = 1 cm, A = 0.78 cm^2), saturated with different media (distilled water, PB pH 6.8 and HAB pH 1.2). The volume of solution absorbed by the samples within 60 min was taken. The results are expressed as the average of three determinations (n= 3 ±SD).

2.5. Selection and characterization of *S. lamella* skeleton

According to the results of characterization studies (see Results chapter 3.1) carried out on the different species, *S. lamella* was selected as target one with the most suitable skeleton according to the aim of the work. Selected fractions of the powder obtained from sieving process were used for the next experiments.

2.5.1. Total viable aerobic count

A microbial count test was carried out in order to assess the microbiological quality of the marine product used as carrier for the formulations. The test was carried out following Cerri et al. (2016) [36] and according to Eur. Ph. [34]:samples were prepared as reported for *"non-fatty product insoluble in water"* and the total aerobic microbial count (TAMC) and the total combined yeasts/mould count (TYMC) were determined by pour-plate method (Supplementary material).

2.5.2. Sterilization process and test of sterility

Sterilization of samples was performed by a modified UV sterilization method as described by Li et al. [37]. The sterilization was performed at room temperature for 24 h. Due to a lack of penetration of UV light samples were overturned twice during sterilization time. The sterilized material was also subject to SEM analysis, as already described, and compared with the untreated powder to assess whether the sterilization process alters the structure of the sponge powder. Moreover, test of sterility according to Eur. Ph.8.0 [34] was carried out to evaluate the success of the sterilization process. The test was carried out under aseptic conditions and inoculated media were incubated for 14 days at 37°C.

2.6. Preparation of drug loaded sponge powder

Powder of *S. lamella* skeleton loaded with CysHCl was prepared. Briefly, CysHCl was solubilized in 1.5 ml of distilled water, enough to completely wet the powder. The solution was poured on the dry sponge powder and mixed together until uniformity wetting was reached. Thus, the powder was placed in a Petri dish and dry in a forced air oven through two different methods: at 40°C for 2h and at 80°C for 1h, thereafter referred to as SCA40 (Sponge Cystein Aqua) and SCA80, respectively. Characterization of the drug loaded powder was performed on both formulations with 15% (w/w) of CysHCl content.

2.7. Characterization of drug loaded sponge powder

2.7.1. Determination of CysHCl content

Quantity of CysHCl loaded into SCA40 and SCA80 was evaluated following a modified method described by Tutem and Apak [38]. The drug loaded powders (10 mg) were transferred into a 10 ml volumetric flask containing distilled water and stirred for 10 min, in order to extract the CysHCl entrapped into the sponge. Hence, 100 µl of each solution were withdrawn and added to a mixture previously prepared: 200 µl of 0.1M $CuCl_2$, 500 µl of 3mMNeocuproin in ethanol 96% (v/v), 200 µl of ammonium acetate 1M; the mixture was vortexed, centrifuged at 13000 rpm for 5 min and analyzed at 450 nm by a UV spectrophotometer (Shimadzu 1800, Italy). The analytical determination was made by using a calibration curve in distilled water previously obtained with

standard CysHCl solutions, 10-200 mg/L (R^2 = 0.9987). The interference of the substrate was checked. Each determination was carried out in triplicate. Loading efficiency (LE%) was calculated from the ratio between the real drug content and the theoretical one, and expressed as percentage ±SD.

2.7.2. In vitro *permeation studies*

Evaluation of permeation across synthetic membranes (cellulose acetate, 0.45 μm pores diameter) was performed on formulations SCA40 and SCA80 by using three in-line flow-through diffusion cells as already described by Gavini et al. [39]. Each cell consisted of a donor and a receptor compartment. Distilled water (50 ml) was used as receptor medium due to the interferences of buffer salts with the colorimetric method previously described. The pH of the receptor medium was measured at the beginning and at the end of the test. Experiments were carried out at 37°C [40, 41] for 1 h; a quantity of SCA40 and SCA80 (35 mg) were homogeneously distributed on the upper face of the synthetic membrane. Receptor solution samples (200 μl) were withdrawn at various time intervals (5, 10, 15, 30, and 60 min) in order to evaluate the amount of drug able to pass from the donor into the acceptor chamber through the membrane. A quantity of 100 μl of each sample was employed for the colorimetric reaction previously described and analyzed. The receptor medium was refilled each time in order to keep sink conditions. Each experiment was performed in triplicate. The results are expressed as cumulative drug permeation %vs time ±SD. Results were compared with the permeation profile of the pure drug.

2.7.3. Scanning Electron Microscopy (SEM)

Shape and surface characteristics of the prepared SCA40 and SCA80 were examined using a SEM, as previously described. For comparison purpose, SEM analyses were carried out also on CysHCl plain solution and *S. lamella* powder treated at the same conditions used for formulation preparation.

2.8. Preparation and characterization of polymeric films

On the basis of the results obtained from formulation characterization, SCA40 was chosen as reference formulation to prepare a mono-layered biodegradable film by a casting-solvent evaporation technique [33]. Briefly, suitable volumes of alginate with glycerol as plasticizer were mixed under constant magnetic stirring at room temperature. The resulting solution was left to stand to remove the entrapped air bubbles, then it was poured on plastic molds (1.91 cm depth, 2.28 cm inside diameter, casting area 4.08 cm^2) equipped with a Parafilm® layer hold by a stainless-steel ring (Figure 1a) and completely dried in an oven at 37±1°C for a week. The drug loaded sponge powder SCA40 (15 mg) was added in the center of the mold after 24 h from the beginning of the drying. The Parafilm® layer was then removed (Figure 1b) and the resulting film (unloaded or loaded), translucent and flexible (Figure 1c-I,II), was carefully detached and stored at room temperature until further analyses. Film loaded with SCA40 was characterized in terms of water uptake and *in vitro* permeation behavior using a quantity of powder comparable with that used for the same tests previously described. Morphological features were studied by SEM analysis, carried out also for unloaded film for comparison purpose.

2.9. Statistical analysis

Data were analyzed using unpaired t-test and the analysis of variance (one-way ANOVA) followed by a Tukey's multiple comparison test (GraphPad Prism, version 6.02; GraphPad Software Incorporated).

3. Results

3.1. Characterization of collected sponge skeletons

3.1.1. Sponge skeleton morphology

On the basis of morphological analyses skeletons are ascribed to the families Spongiidae (*H. communis*, *S. lamella*, and *S. officinalis*) and Irciniidae (*S. spinosulus*). The *H. communis* skeleton architecture shows numerous, wide lacunae in both the choanosomal and ectosomal fibrous network. After cleaning and drying, the skeleton becomes coarsely hirsute. The primary fibers are few and exclusively present towards the surface. The skeletons of *S. lamella* and *S. officinalis* with

finely conulose surface both show elastic, resilient and very compressible consistency. The network of primary fibers together with highly developed secondary fibers, confers to these skeletons a typical flexibility and water retentive properties. *S. spinosulus* shows a soft touch skeleton but extremely tough and often difficult to cut or tear because of the high content of collagenic filaments. Its structure has cored primary fibers with notably abundant foreign debris and simple, uncored secondary fibers.

3.1.2. Glycosaminoglycans (GAGs) content and composition

Total GAG content, expressed as μg hexuronate/mg dry weight, shows some variability among the tested species, being 0.171±0.021, 0.367±0.028 and 0.460±0.081 for *H. communis*, *S. officinalis*, and *S. lamella*, respectively. The electrophoretic mobility of the sponge sulfated polysaccharides, as well as their susceptibility to specific depolymerization procedures, differ from those of standard GAGs, except for small amounts of low moving CS isomers. This suggests a lower charge density and/or a different disaccharide units composition/clusterization. Overall, data suggest that these sponge GAGs are structurally divergent from vertebrate GAGs (Figure 2). *S. spinosulus* was not processed for the high amount of filaments.

3.1.3. Swelling studies

The swelling behavior of the ectosomal (Ect) and choanosomal (Ch) skeleton of each sponge varies in different media depending on the section exposed to the medium(Figure 3). The highest buffer uptake is observed with the ectosomal skeleton of *S. spinosulus* that reaches its maximum swelling index in PB pH 7.4. Most samples show the lowest absorption in distilled water and reach their maximum swelling indexes after 8 hours, thus keeping constant these values, probably due to saturation phenomena. *S. officinalis* exhibits the lowest swelling properties, even if it is quite similar to *S. lamella* profile. On the other hand, *H. communis* shows intermediate but unregularly increasing values. As far as morphological changes and thickness increase are concerned (Table1), the most interesting changes are noticed in PB pH 6.8 and HAB pH 1.2. Specifically, *H. communis* can be considered the worst skeleton in this case because of its cleavage in the first medium that

damages its soft, fragile structure. In the same medium, *S. spinolsulus* shows a remarkable thickness increase both for ectosomal and choanosomal regions ($P<0.05$ *S. spinosulus* vs *S.lamella*vs*S. officinalis*). At extreme conditions (pH 1.2) it is observed a brightening of the native colors for *H. communis* and *S. lamella,* associated to a thickness increase for both of them. Also *S. officinalis* shows a thickness increase especially for ectosomal region, whereas no interesting changes are observed in PB pH 6.8 in terms of thickness and color variations, except a slight browning of the ectosomal region. However, a significant thickness increase ($P<0.05$) occurs at pH 7.4 for *S. lamella* compared to *S. spinosulus.*

Table 1. Morphological changes and thickness increase of sponge skeleton samples in different buffers; Ect= ectosomal skeleton; Ch= choanosomal skeleton. PB=phosphate buffer, HAB= hydrochloride acid buffer. NC= no changes. $P < 0.05$:*****_H. communis vs S. lamella_ at pH 6.8, _H. communis vs. S. officinalis_, #_S. lamella_ and _S. officinalis vs S. spinosulus_ at pH 6.8; §_S. lamella vs S. spinosulus_ at pH 7.4

	MORPHOLOGICAL CHANGES-PB pH 7.4			***MORPHOLOGICAL CHANGES-PB pH 6.8***		
	THICKNESS INCREASE (cm)		**COLOR CHANGES**	**THICKNESS INCREASE (cm)**		**COLOR CHANGES**
	DRY	**AFTER24h**		**DRY**	**AFTER24h**	
H. communis(Ect)	0.2	0.4	NC	0.3	1*	Cleavage
H. communis(Ch)	0.2	0.4	NC	0.4	1.3*	Cleavage
S. lamella (Ect)	0.7	1.1§	NC	0.4	0.7*#	NC
S. lamella (Ch)	0.3	0.9§	NC	0.4	0.7*#	NC
S. officinalis(Ect)	0.7	0.8	NC	0.5	0.7*#	Slight browning
S. officinalis(Ch)	0.3	0.6	NC	0.6	0.6*#	NC
S. spinosulus (Ect)	0.6	0.8§	NC	0.7	1.7#	NC
S. spinosulus(Ch)	0.4	0.5§	NC	0.3	1.3#	NC

	MORPHOLOGICAL CHANGES-PB pH 5.5			***MORPHOLOGICAL CHANGES-HAB pH 1.2***		
	THICKNESS INCREASE (cm)		**COLOR CHANGES**	**THICKNESS INCREASE (cm)**		**COLOR CHANGES**
	DRY	**AFTER 24h**		**DRY**	**AFTER 24h**	
H. communis(Ect)	0.3	1	Cleavage	0.3	1	Slight brightening
H. communis(Ch)	0.4	0.5	NC	0.1	0.2	NC
S. lamella (Ect)	0.3	0.7	NC	0.4	0.6	Brightening
S. lamella (Ch)	0.4	0.4	NC	0.6	1.2	NC
S. officinalis(Ect)	0.6	0.7	NC	0.6	1.2	NC
S. officinalis(Ch)	0.6	0.7	NC	0.3	0.5	NC
S. spinosulus (Ect)	0.4	0.9	NC	0.3	0.6	NC
S. spinosulus(Ch)	0.6	0.9	NC	0.3	0.7	NC

3.2 Powder preparation: grinding, sieving and SEM analyses

Powders obtained after the grinding process from each selected sponge skeleton show quite similar properties in terms of structure, related to the fibrous nature of their collagenic skeleton. Hence, they keep their native framework even in form of powder and tend to aggregate. Data from SEM images confirm this assumption, especially for *S. lamella* and *S. officinalis* powders (Figure 4a,b) because fibers appear to be cut along their longitudinal axe by shear forces. However, it can be seen that in the case of *S. spinosulus* obtained powder is heterogeneous and separate in two fractions (Figure 4c). Moreover, after the sieving process, it does not give an optimum size distribution, showing both a purely fibrous component of a high average diameter (the most abundant) and finer fractions of different sizes that include an undefined amount of sand, which cannot be easily separated from the rest of the powder(Figure S1). On the contrary, *S. lamella* and *S. officinalis* show a similar, homogenous dimensional distribution. The most abundant fractions have an average diameter ranging from 300 to 500 μm, with a higher amount recovered for *S. lamella* (Figure S1).

3.2.1. Water uptake

The water uptake along the time of skeleton powders is presented in Figure 5. All powders have a similar profile, since they quickly absorb the medium in the first 5 min ($P>0.05$). Then, regularly increasing values are registered for all species until the end of the test. As it can be seen, all powders absorb less water in comparison with buffers. The greatest uptake capability is shown by *S. lamella*, followed by *S. spinosulus* that reaches the maximum absorption in PB pH 6.8. *S. officinalis* exhibits the lowest values of absorption among the other species. At the end of the test, all powders do not show swelling properties, even if it was observed an obvious weight increase. Although the water uptake test is commonly used for powders [42], experiments were performed also on the whole skeletons (ectosomal and choanosomal regions) for comparison purpose. They have a limited capillary water absorption ability compared to powders. The ectosomal skeleton of most species seems to better absorb the fluid at physiological pH compared to the choanosomal one, probably due to the different microstructure of fibers in the two regions.

3.3. Selection and characterization of *S. lamella* skeleton

Based on the results obtained by characterization studies on both entire and powdered skeletons, *S. lamella* was selected as the most suitable material. In particular, it shows a good swelling behavior and a persistent solid structure without significant color changes in most of the media used, especially at pH close to neutrality. These color changes might be related to different factors that require a deeper analysis, so it was decided to take into account preferably samples that have not undergone important morphological variations. Furthermore, its fluid uptake ability as powder appears to be the best among the different species, especially when physiological-like conditions were used (PB pH 6.8). Moreover, *S. lamella* also shows optimal and homogenous size distributions, with the highest amount of powder recovered in the selected size range. On the other hand, *H. communis* was firstly discarded after water uptake (about the whole skeleton) and swelling test, without performing grinding and sieving processes, because of its too soft structure, not useful for the purpose of the study. Although *S. spinosulus* shows good properties in terms of swelling behavior and water uptake ability, it is not easily grinded, giving a heterogeneous dimensional distribution. Finally, *S. officinalis* does not exhibit particularly interesting properties regarding the different characterization tests.

3.3.1. Total viable aerobic count

Acceptance criteria for non-sterile pharmaceutical products are based upon the total aerobic microbial count (TAMC) and the total combined yeasts/moulds count (TYMC). *S. lamella* powder complies these acceptance criteria with TAMC and TYMC <10 UFC/g, hence below the limits (respectively, $\leq 10^2$ and $\leq 10^1$ UFC /g) established for non-sterile dosage forms for cutaneous use [33]. In this case, the relevance of the test is closely related to the safety of the product. The presence of certain microorganisms in non-sterile preparations has the potential to negatively affect the health of the patient, regardless the route of administration. This assumption becomes more important when natural-occurring products, such as sponge skeletons, are concerned.

3.3.2. Sterilization process and test of sterility

UV sterilization was selected as the most suitable method for sponge powder, because it appears to be the less invasive way for the biomaterial structure, compared to heat or chemical sterilization methods. SEM analyses show that no relevant changes occur between sterilized and not sterilized sample as concerning the structure of fibers, confirming the safety of the method used (Figure8d). The sterility test demonstrates the efficacy of the performed process in the adopted conditions [34].

3.4. Preparation and characterization of drug loaded sponge powder

Formulations were obtained as *S. lamella* powder loaded with CysHCl. Two of them (SCA40 and SCA80) with the most appropriate drug content (15% w/w) were selected and successively characterized.

3.4.1. Determination of CysHCl content

Drug content results demonstrate a slight difference between SCA40 and SCA80 that is not statistically significant (unpaired t test, $P>0.05$). In the first case, almost all the CysHCl employed during the preparation phase is entrapped in the final powder (LE%: 99.06 ± 0.23).In the second case, less than 90% of CysHCl is recovered after dispersion of SCA80 in water (LE%: 75.40 ± 0.22) probably due to a partial thermal degradation of CysHCl at this drying temperature.

3.4.2. In vitro *permeation studies*

Figure 6 reports the permeation profiles of CysHCl after releasing from SCA40 and SCA80 in comparison with the pure drug. Both formulations reach good percentages of permeated drug at the end of the test, as the final drug amount recorded is between 86-88% ($P>0.05$). SCA40 profile has a more linear trend compared to SCA80, which shows a higher value of CysHCl permeated already after 5 min ($P<0.05$). Then, it goes towards a regular increase until a “plateau” between 30 and 60 minis reached. On the other hand, the pure drug is able to pass through membranes completely after 5 min, with a significant difference from both formulations ($P<0.05$), keeping almost constant these values until 30 min. Then, it starts a decrease probably due to the scarce stability of CysHCl in neutral or alkaline aqueous solutions. It is indeed readily oxidized to yield the disulfide, cystine, which have different physical-chemical properties than cysteine [43].

3.4.3. Scanning Electron Microscopy (SEM)

SEM photomicrographs of SCA80 and SCA40 are reported in Figure 7a,b and 7c,d, respectively. The two systems show a marked difference about CysHCl crystallization. In the case of SCA80, CysHCl is distributed along the entire fibers, forming stick-like structures (Figure 7a,b). On the other hand, SCA40 shows a CysHCl distribution particularly concentrated in some tracts of fibers, assuming a characteristic sub-spherical shape (Figure 7c,d). CysHCl plain solution subjected to the same preparation conditions of SCA80 and SCA40 does not show any peculiar crystallization form, suggesting that drug crystallization morphology strongly depends on the substrate (Figure 7e,f). Furthermore, the drying treatments (40°C/2h and 80°C/1h) do not alter the fibers morphology(Figure 8).The casting-solvent evaporation technique used is a suitable method to prepare polymeric films. SCA40 is used for loaded film preparation. Compared to SCA80, it shows higher drug loading, controlled release profile and because of low drying temperatures, high drug stability might be guaranteed. Surface of the alginate-based films showed a smooth and regular aspect. *S. lamella* fibers appear to be well arranged within the loaded film matrix (pictures not reported).By comparing the water uptake of *S. lamella* powder and *S. lamella* loaded film (Figure 9) it is evident that film uptake ability is higher than the powder alone: values obtained statistically differ from *S. lamella* powder since 5min until the end of the test ($P<0.01$).Indeed, the film presents gradually increasing values, while the powder reaches a stabilization, since registered values are very similar from 40 min until the end of the test. *In vitro* permeation studies of SCA40-loaded film give a good result, with 70% of drug released after 30 min, followed by a slight decrease until 60 min and then constant values until 90 min. The comparison with SCA40 profile demonstrates a different capability of the two systems in terms of amount and kinetic of permeated drug (Figure 10).

4. Discussion

The development of a new bio-based system for topical drug delivery was the proposal of the work. The idea took into account the concept of the wound healing as potential therapeutic target, since a

sulfur amino acid involved in such process was chosen as reference drug. However, the suitability of the selected natural material chosen as bio-functional carrier was especially considered. There is indeed growing interest in the use of recyclable-biodegradable natural materials for both biomedical devices manufacturing and drug delivery systems [44], especially from bioprospecting marine and fresh water environments [45-47]. Safety, efficacy, ease of handling and cost-effectiveness were the highlights of this work, in order to propose novel delivery systems able to compete not only with traditional systems but also with others recently developed, such as nano-fibrous scaffolds or bio-functionalized materials [48-50]. Results on GAG content indicate that in sponge skeletons different sulfated polysaccharides are associated to collagenic network, according to Villanova et al.[51, 52]. Overall, morphology studies together with results on GAG content suggest that collagen fibers of sponge skeletons could be cross-linked by proteoglycans connections, as already reported for other Keratosa[51-53]. Our preliminary biochemical analyses confirm that the sulfated glycans from marine sponges deeply differ among different species, as well as from those of vertebrate ECM glycosaminoglycans[51-54]. Indeed, it is well known that sponge GAGs are species-specific and this promote self- and non-self-recognition processes[55]. GAGs and PGs, although minor ECM components, are known to be involved in different phases regulating the wound healing processes, i.e. cell adhesion, migration, proliferation and differentiation, matrix supramolecular organization and collagen fibrillogenesis [56-59]. Since the nature of GAGs/PGs may modulate drug retention, crystallisation, and delivery, glycosaminoglycans fine chemical structure, i.e. the sequence of disaccharide units, as well as their sulfation and/or iduronation patterns and degrees, deserves further investigations. Moreover, characterization studies highlight the different behavior of the investigated species, related to the typical organization of their fibrous network as well as to the various media used for the experiments. In particular, the water uptake of powdered skeletons showed that all samples are able to better absorb buffers than water. As concerning swelling studies, the ectosomal skeletons show the highest buffer uptake values. Particularly, the absorption was more evident for all samples in PB at pH close to the neutrality. It is interesting to note that the

same behavior was found also in water uptake results. This might be related to the microstructure of ectosomal fibers. The *S. lamella* skeleton was selected among the others because of its proper consistency, suitable swelling behavior and great fluid uptake ability. All these parameters were considered important for the formulative phase, especially because the skeleton morpho-functional performances are persistent in obtained powder. *S. lamella* powder loaded with CysHCl was developed by a simple and not expensive adsorption technique. Two formulations were chosen as references. Relatively low temperatures for the dry process prevented structure modifications as possible, as confirmed from SEM analyses (Figure 8a-c). However, it was found recently that these fibrous skeletons are mechanically robust and stable up to 160°C [60]. Anyway, it can be observed a different CysHCl crystallization for the two formulations, as demonstrated in Figure 7. We can assume that the crystals type significantly depends on the specific substrate, time and temperatures used for the drying process, as also confirmed by the comparative SEM analysis of the CysHCl plain solution. In addition, it can be seen that the distribution of CysHCl is not uniform along all fibers of the powder, as above mentioned. This fact, together with the different crystallization of CysHCl into the two formulations, could explain the differences in terms of *in vitro* permeation between SCA40 and SCA80. From results shown in Figure 6, SCA40 demonstrated a more slow and linear profile than SCA80, which can probably ascribed to the spatial arrangement of crystals (see also Figure 7). The type of crystal, indeed, might influence the ability of the drug to pass through a membrane and reach an aqueous medium [61]. Based on these results, SCA40 was chosen for the preparation of the polymeric film, suggested as biocompatible support and suitable system for topical application[62]. Alginate-based film demonstrated a great ability to absorb water, probably due to the good affinity with this medium. About this, Norajit et al. [63] reported that the ability of alginate to swell is facilitated by the carboxyl groups, which strongly associate with water molecules. For the same reason, we can explain the *in vitro* permeation behavior of the alginate film that dissolves during the test, thus contributing to a quicker CysHCl permeation within 30 min.

5. Conclusions

New bio-based topical formulations were developed by using a natural marine sponge skeleton consisting of a collagen/proteoglycan network as carrier for L-cysteine hydrochloride, a sulfur amino acid known for its wound healing properties. According to literature data and characterization studies performed in this work, such biomaterial can be suggested as attractive and eco-sustainable (recyclable and biodegradable) drug delivery system, able to actively contribute to the achievement of the therapeutic target. In particular, SCA40 was selected as the leader drug loaded sponge powder because of its interesting morpho-functional performances, especially when drug crystallization and optimal *in vitro* permeation profile are concerned. The presented results suggest that this formulation might be proposed as innovative and promising topical dressing in form of powder or alginate-based film for wound treatment.

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Declaration of interest

The authors declare that they have nothing to disclose regarding funding or conflicts of interest relating to this manuscript.

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Figure captions

Fig. 1 a-c. Film preparation. Plastic mold with removable parafilm layer (a); mold with a dry film after parafilm removal (b); alginate-based film, blank (c-I) and SCA40 loaded (c-II).

Fig. 2. Electrophoretic profiles. Representative profiles of standard GAGs (lane 1) and GAGs purified from the fibrous skeleton of *Spongia lamella* (lane 2), *S. officinalis* (lane 3), and *H. communis* (lane 4). DS: dermatan sulfate; HS: heparansulfate; CS: chondroitin sulfate.

Fig. 3. Swelling behaviour. Swelling indexes of fibrous sponge skeletons (four species) in different media (**A**: PB pH 7.4; **B**: PB pH 6.8; **C**: PB pH 5.5;**D**: HAB pH 1.2).

Fig. 4 a-c. Morphological study of untreated powders. SEM images of powdered collagenic skeletons of sponges belonging to *S. lamella* (a), *S. officinalis* (b), and *S. spinosulus* (c).

Fig. 5. Water uptake profiles of powdered fibrous skeletons of sponges in different media.

Fig. 6. *In vitro* permeation study. Permeation profiles of CysHCl from formulations in comparison with the pure drug in distilled water (n = 3 ± SD). P< 0.05: *CysHCl *vs* SCA80 and SCA40 at 5 min, SCA80 *vs* SCA40; §CysHCl *vs* SCA40 at 30 min.

Fig. 7 a-f. Morphological study of formulations. SEM images of SCA80 (a,b), SCA40 (c,d), CysHCl plain solution at 80 °C/1 h (e), and at 40 °C/2 h (f).

Fig. 8 a-d. Morphological study of treated powders. SEM images of *S. lamella* not treated powder (a), *S. lamella* powder at 40 °C/2 h (b), *S. lamella* powder at 80 °C/2 h (c), and *S. lamella* subjected to UV sterilization (d).

Fig. 9. Water uptake profiles of *S. lamella* powder and *S. lamella*+alginate film.P < 0.01: *S. lamella* powder *vs S. lamella*+alginate film at 5min and at 60min.

Fig. 10. Comparison of *in vitro* permeation profiles of SCA40 and SCA40-alginate film.

Figure 1
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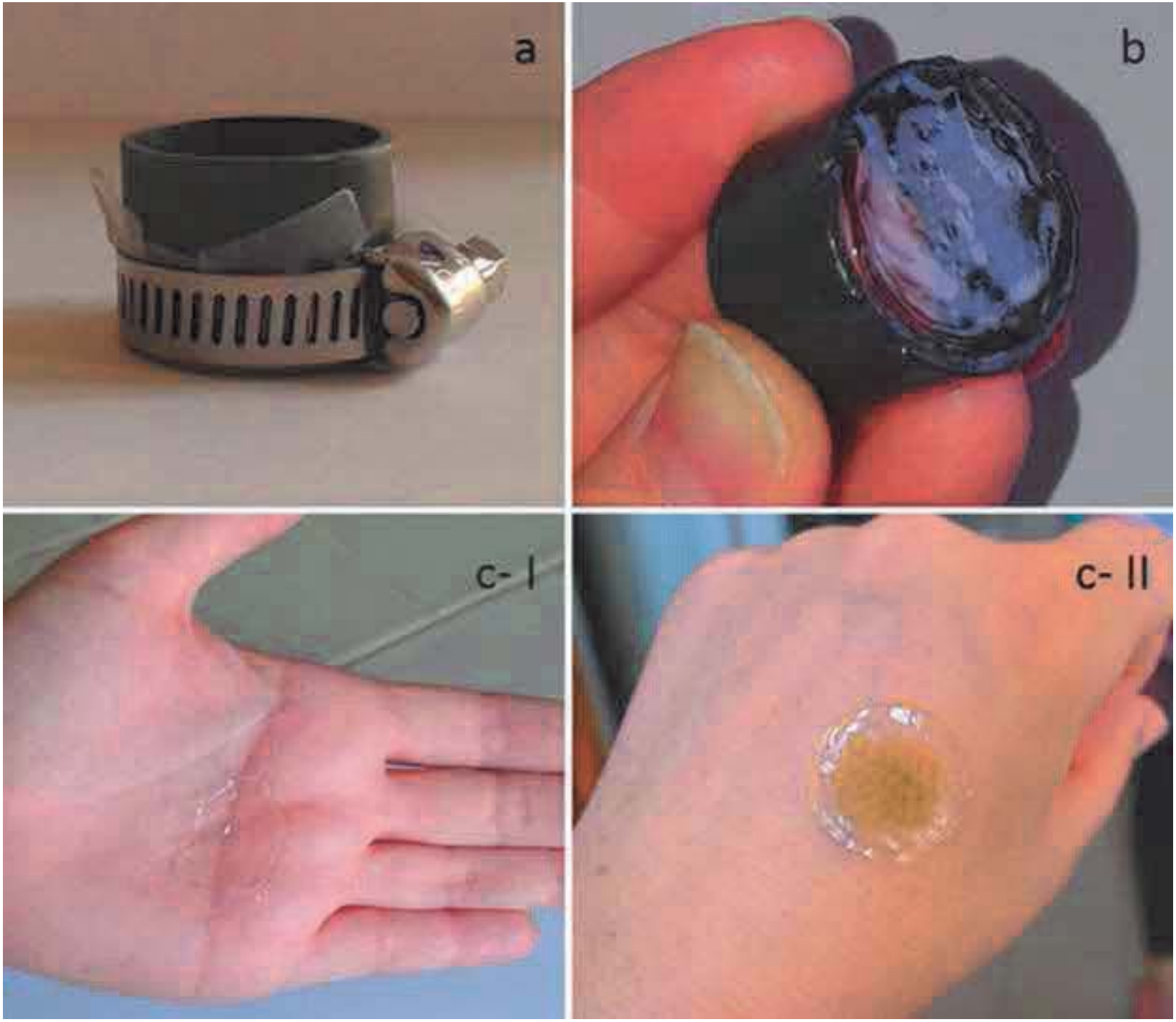

Figure 2
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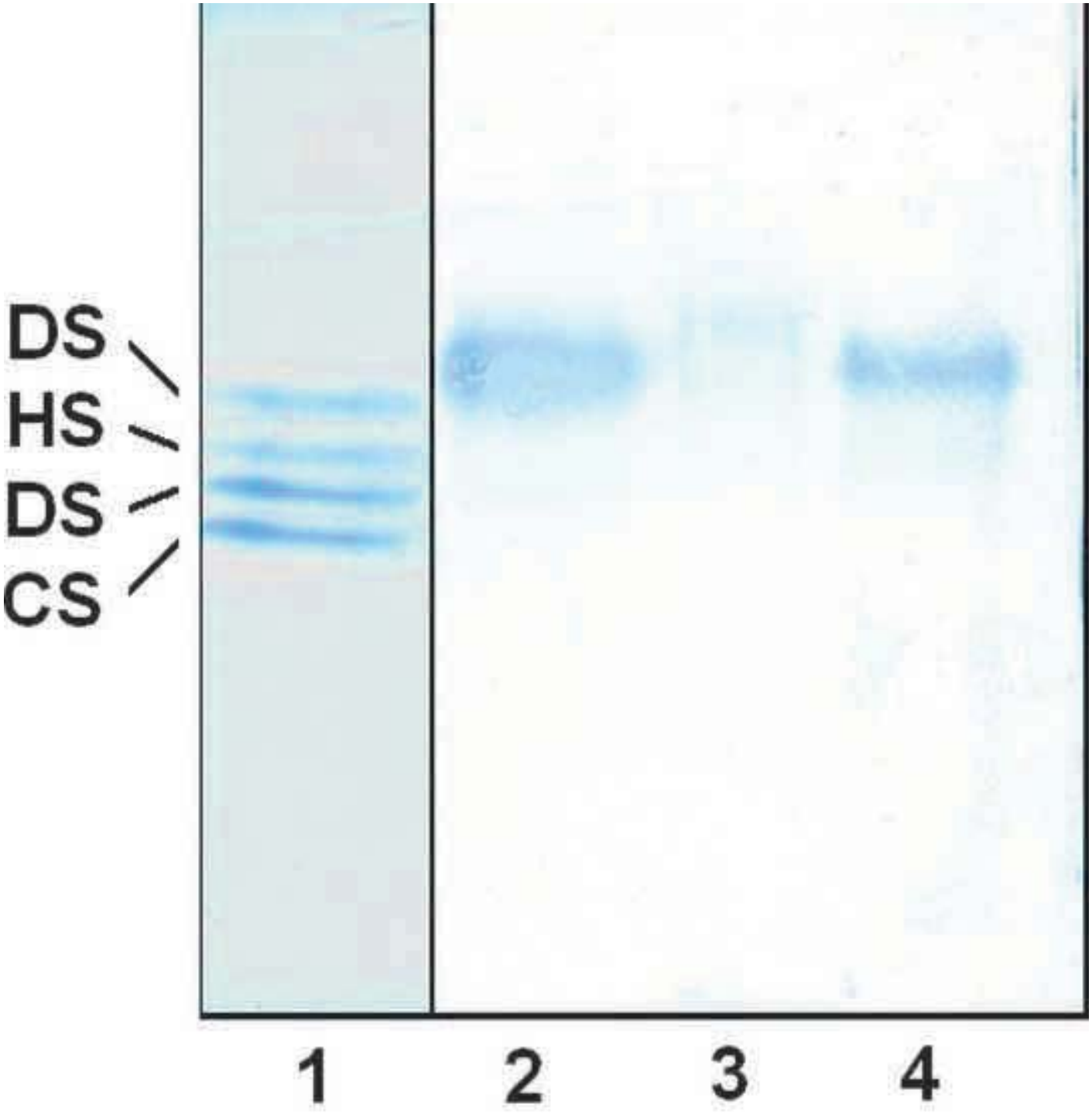

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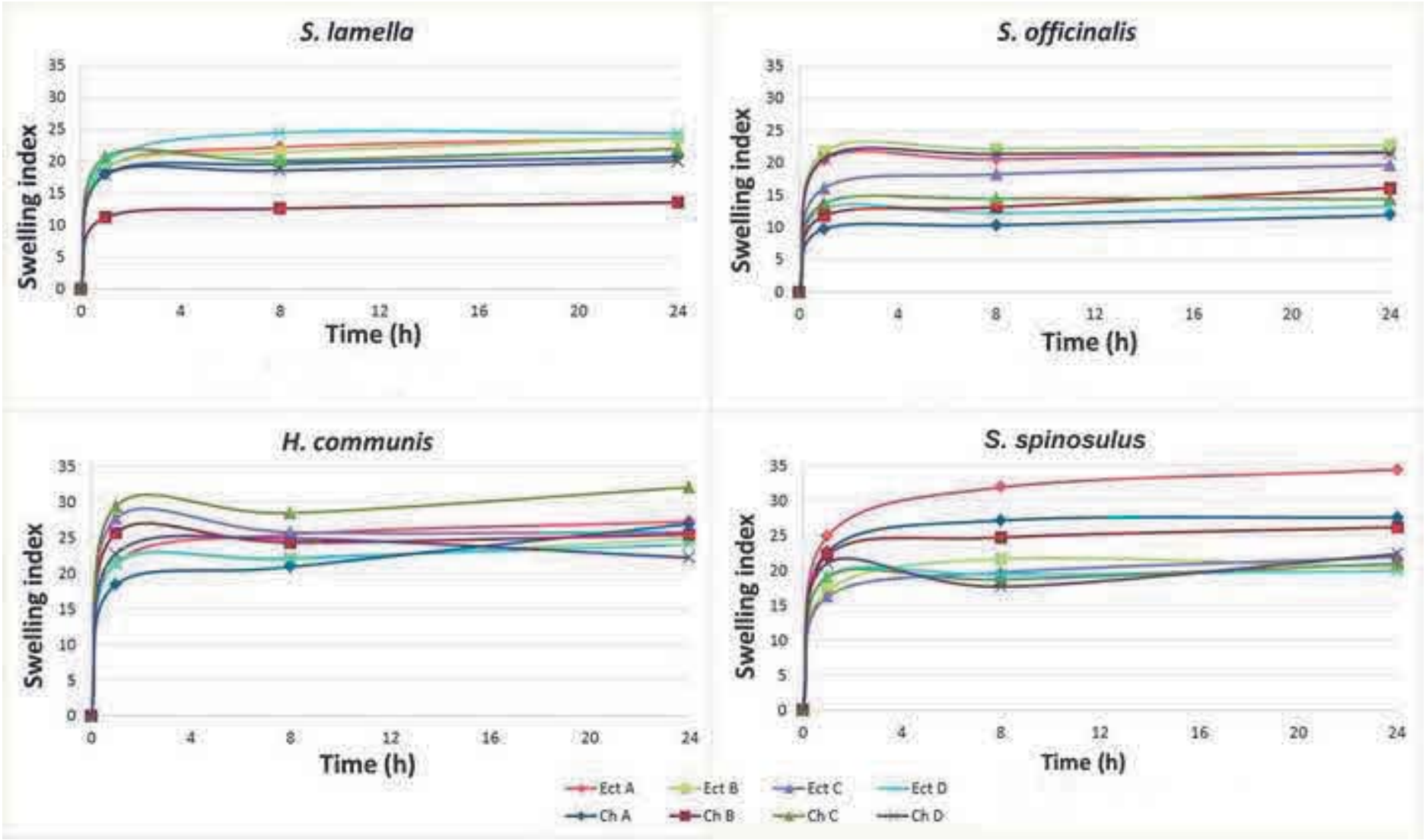

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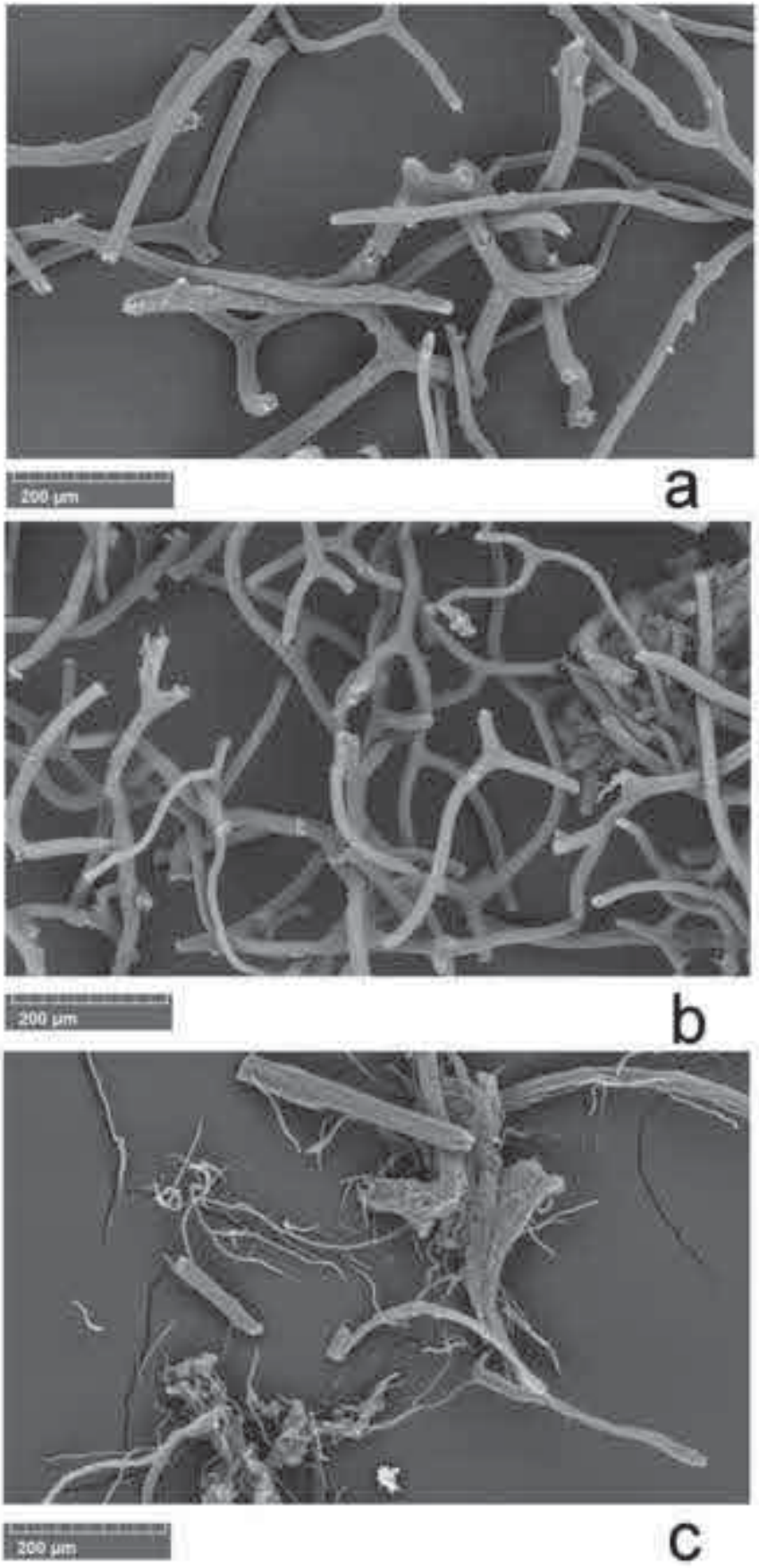

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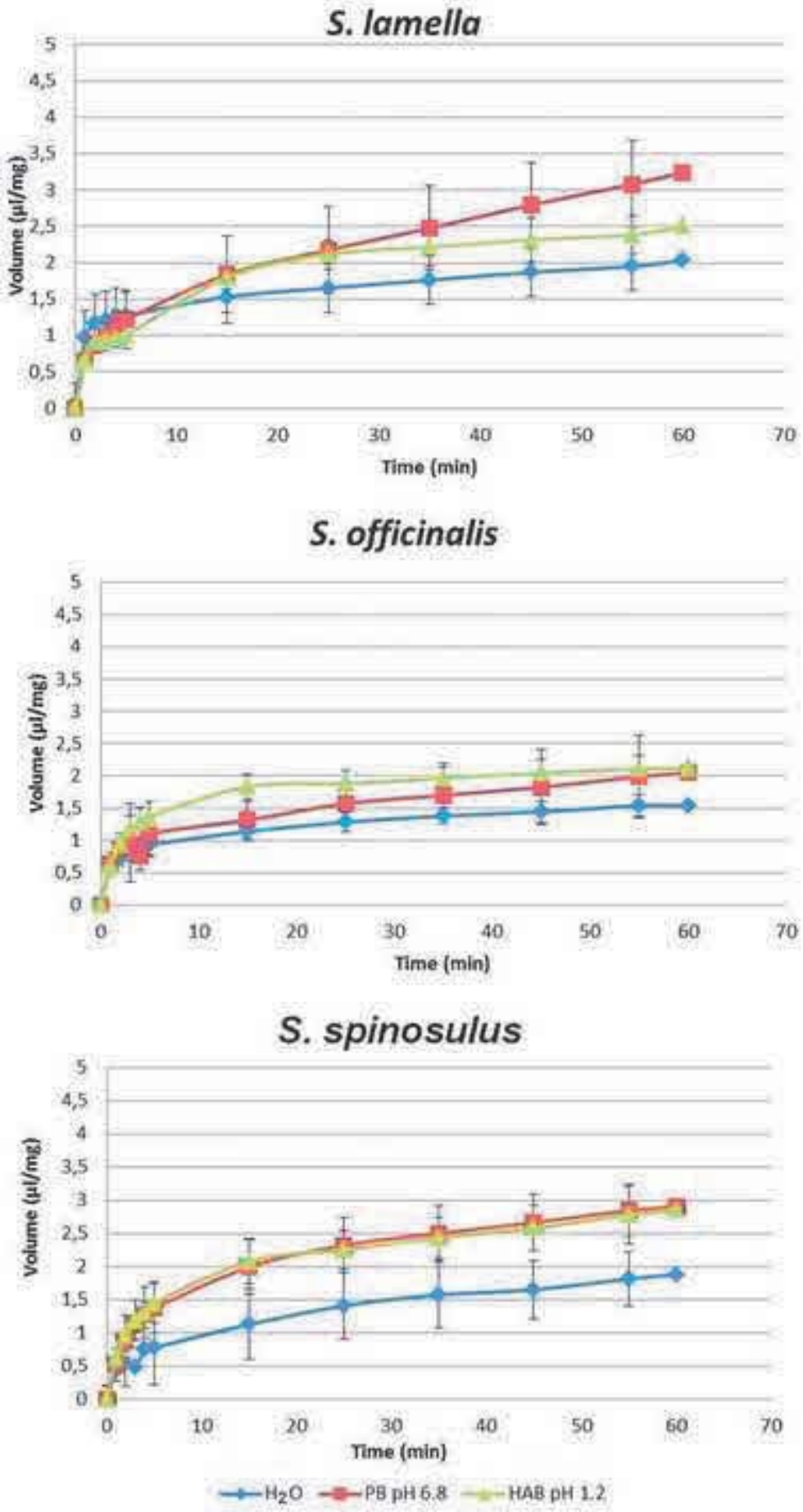

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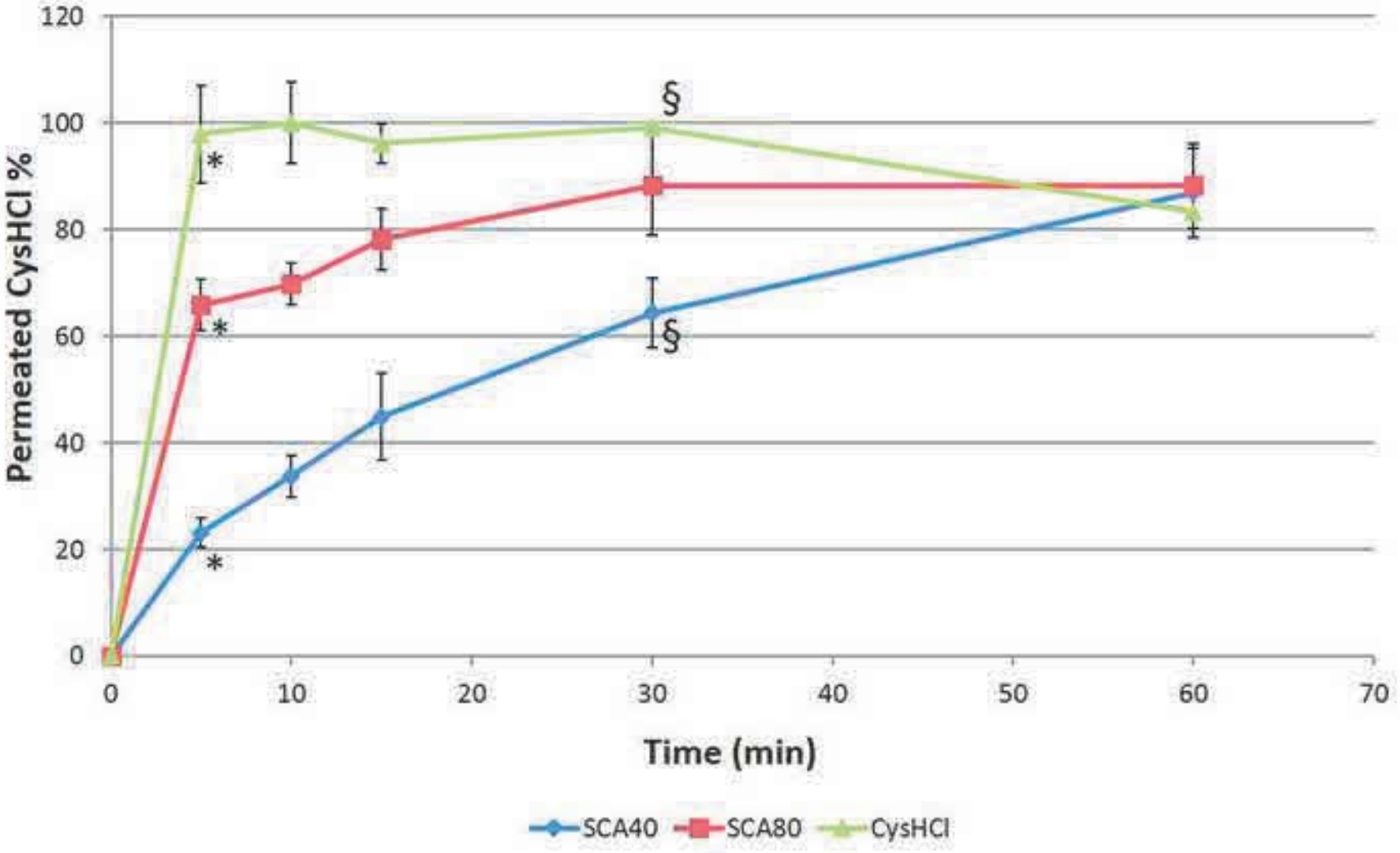

Figure 7
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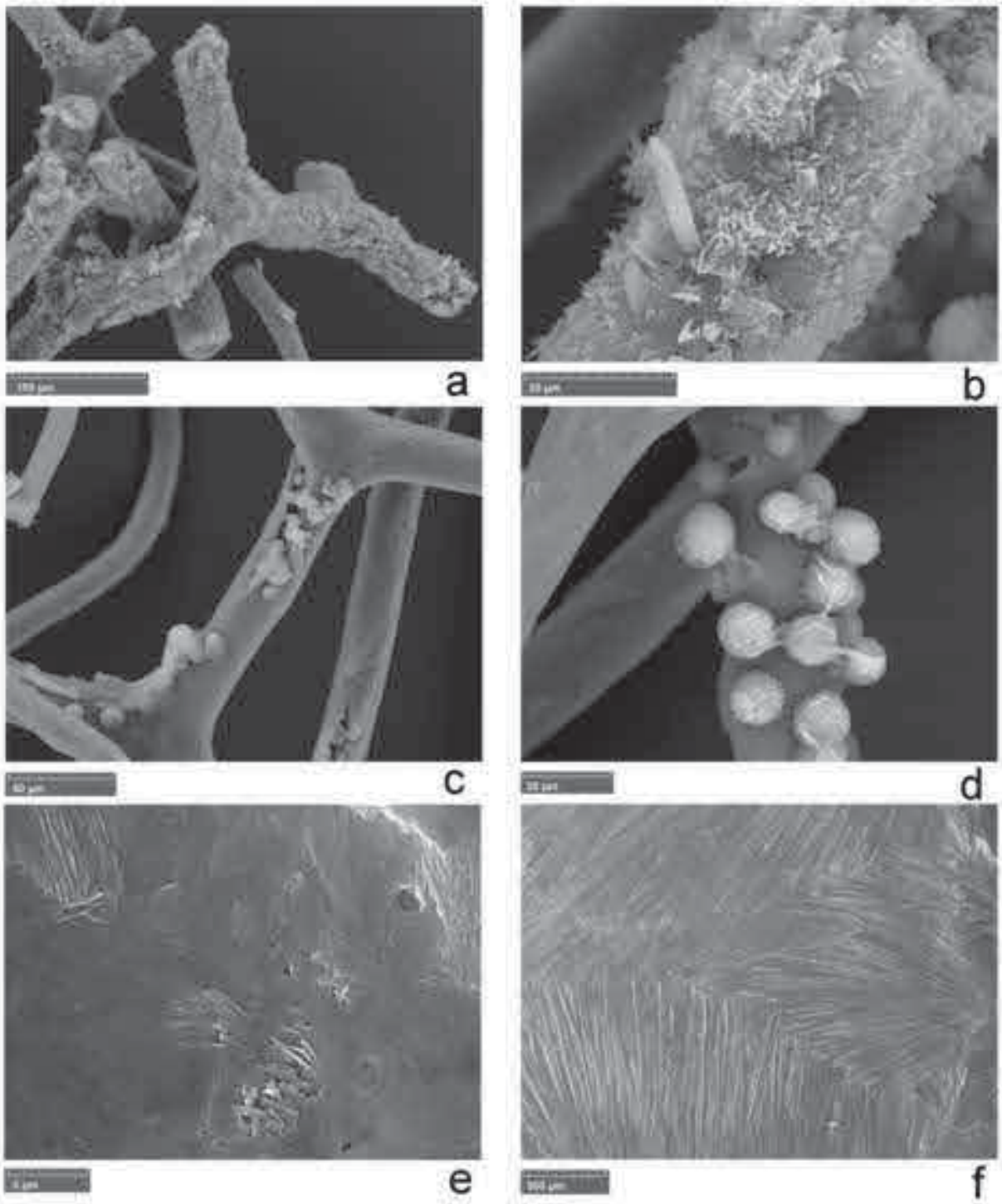

Figure 8
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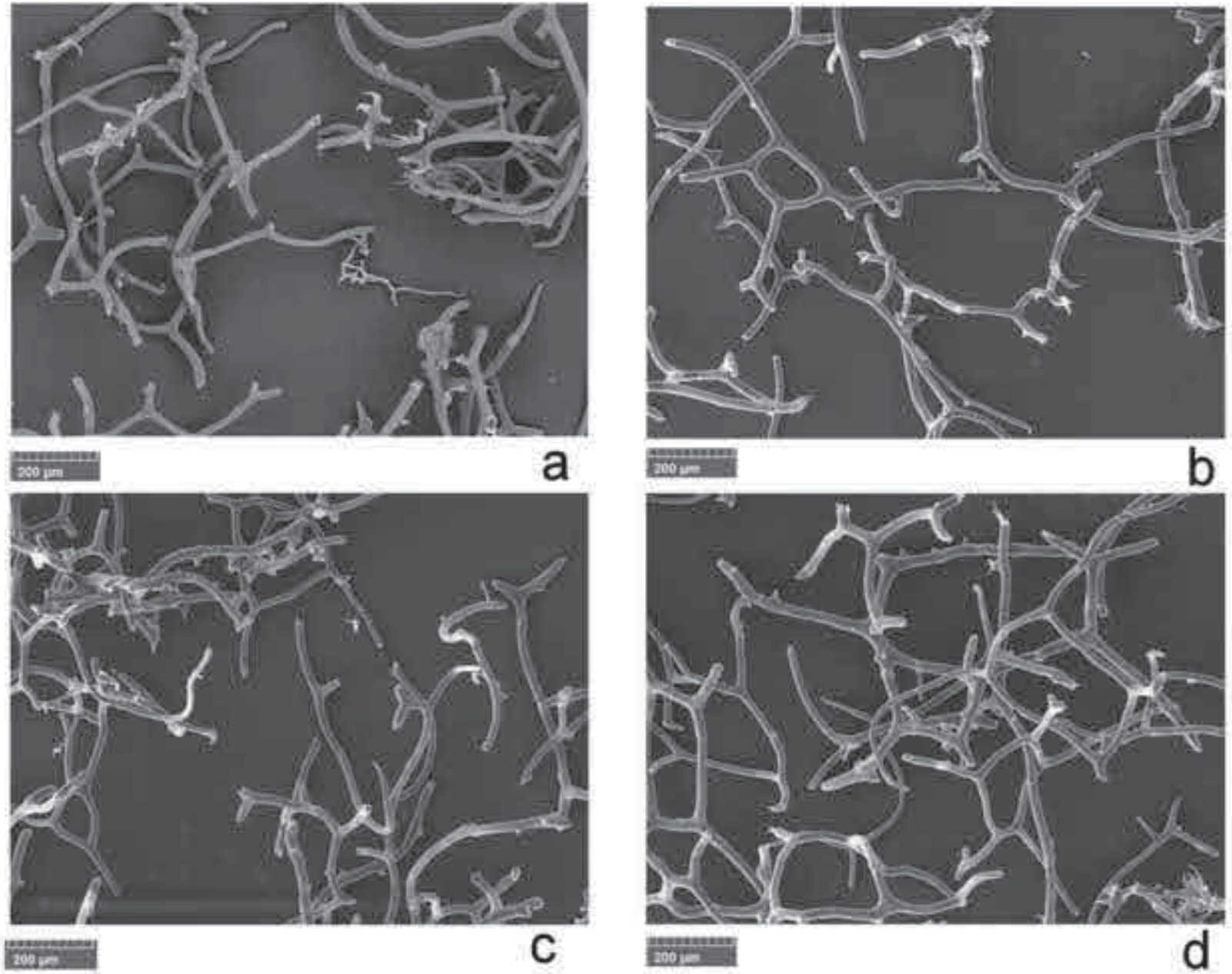

Figure 10
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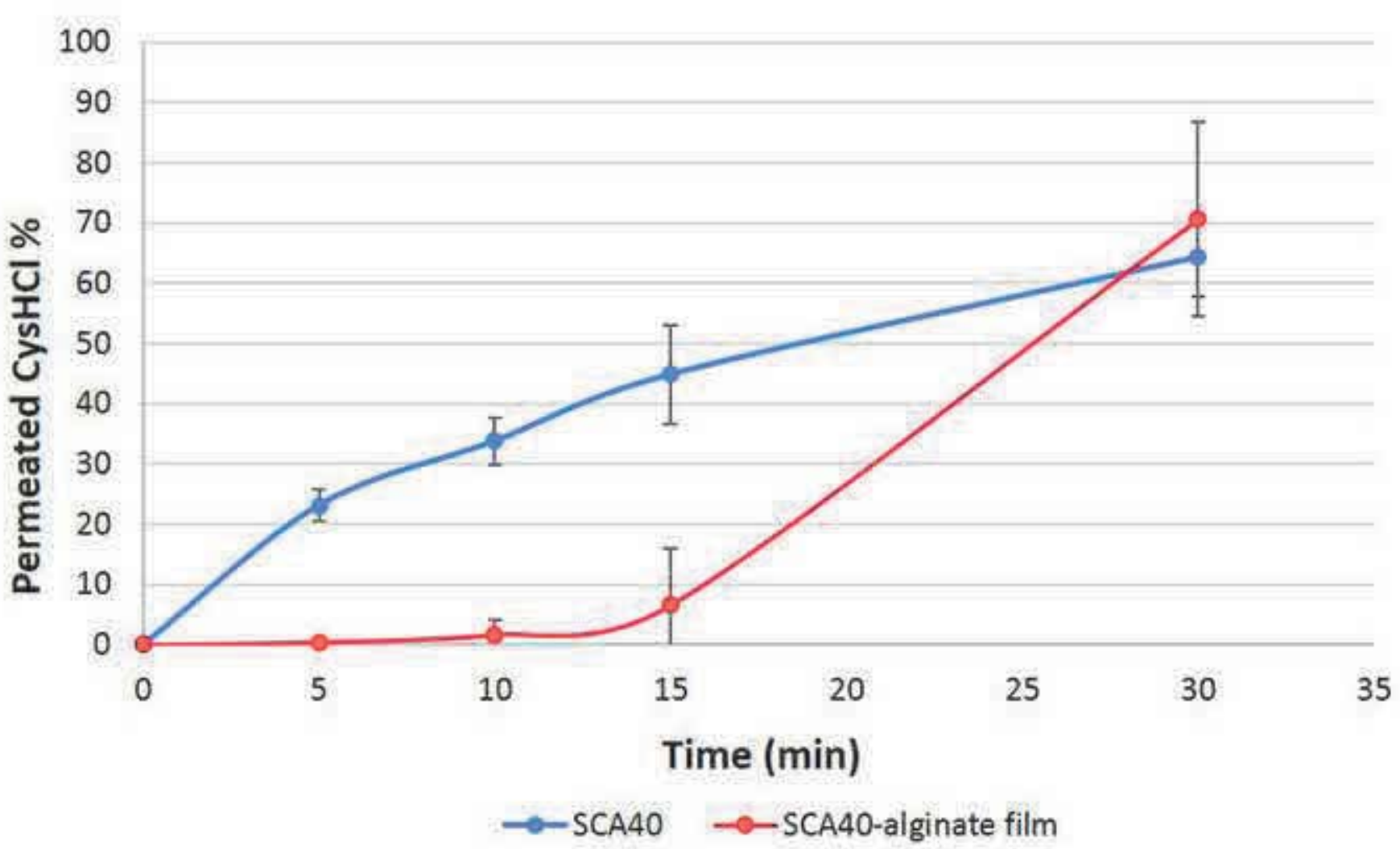

Figure 9
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