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In situ forming antibacterial dextran blend hydrogel for wound dressing: SAA technology vs. spray drying

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ABSTRACT

This study focuses on designing microparticulate carriers based on high-mannuronic alginate and amidated pectin blend loaded with gentamicin sulphate able to move rapidly from dry to soft hydrogel. Supercritical assisted atomization was used to produce microparticles in form of dry powder and characteristics were compared with those obtained by spray-drying. Particles with very high encapsulation efficiency (~100%) and small diameter (less than 2 μm) showed good flowability and high fluid uptake enabling wound site filling and limiting bacterial proliferation. Moisture transmission of the in situ formed hydrogel was about 95 g/m^{2}h, ideal to avoid wound dehydration or occlusion phenomena. All formulations presented a burst effect, suitable to prevent infection spreading at the beginning of the therapy, followed by prolonged release (4–10 days) related to drug/polymers ratio. Antimicrobial tests showed stronger effect than pure GS over time (up-to 24 days) and the ability to degrade preformed biofilms, essential to properly treat infected wounds.

1. Introduction

Wound healing is a dynamic ordered process involving a variety of cellular and matrix components which end-up in wound closure. However, in some cases, wounds fail to close due to different reasons related to local or systemic factors (Baranoski, 2008). Non-healing chronic wounds, as ulcers, can be easily colonized by different potential pathogens (Dowsett & Scanlon, 2001). Unbalanced microflora can interfere with the healing process and, if untreated, can lead to large area of necrosis and to systemic infection. Antibacterial agents must be topically administered in order to reduce wound bioburden and to avoid infection spreading.

A number of wound dressing devices loaded with active pharmaceutical ingredients (APIs) have been manufactured with different polymeric materials. The ideal dressing material should have proper adherence to the wound site and good exudate absorbance. It should be able to maintain appropriate moisture at the wound bed, nonetheless, easy application and removal. Dextran hydrogels may combine most of these properties; moreover, they are transparent allowing the monitoring of healing process. Above all dextrans, alginate is the most used in the production of fibres for exuding wounds (Agren, 1996; White, 2011). It is well known that this hydrophilic polymer is composed by a mixture of two monomeric units, guluronic acid (G) and mannuronic acid (M). High G content alginates have been demonstrated to be less liquid absorbent and gel forming than high M content alginates, nevertheless they retain a stronger structure and are able to gel in the presence of Na^+ ions highly concentrated in wound fluids (Meaume, Vallet, Morere, & Téot, 2005). Otherwise, M residues may induce cytokine production by human monocytes, a process very useful in chronic wound healing process that would take advantage of exogenous pro-inflammatory stimuli to which macrophages are receptive (Thomas, Harding, & Moore, 2000). However, conventional alginate dressing, as well as other dextran dressing, produced in form of sheets or membranes has shown conformability problems on the wounds (Berger et al., 2004; Kickhöfen, Wokalek, Scheel, & Ruh, 1986; Singh & Singh, 2012; Thu, Zulfakar, & Ng, 2012) whereas in situ forming hydrogels could fit the irregular shape of the wound without wrinkles or fluting (Balakrishnan, Mohanty, Umashankar, & Jayakrishnan, 2005; Thu et al., 2012). Spray-on films have shown partially reduced conformability and high costs, complex manufacturing and, in some cases, bacterial spreading when used over third degree wounds (Hennink & van Nostrum, 2002; Kane, Tomkins, Yarmush, & Burke, 1996; Peppas, Bures, Leobandung, & Ichikawa, 2000).

Easy swellable powders made by dextrans microparticles, forming hydrogel in wound site, may be an interesting and cheaper

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alternative to conventional dressing devices. Such microparticulate systems loaded with antibiotics, swelling in presence of wound fluid, might strongly limit the formation of fluid-filled pockets that enable the bacterial proliferation (Quinn, Courtney, Evans, Gaylor, & Reid, 1985), promoting wound repair and regeneration.

Gentamicin sulfate (GS) is an aminoglycoside antibiotic worldwide used for the treatment of severe infections caused by both Gram-positive and, especially, Gram-negative bacteria (Forge & Schacht, 2000). Due to its pharmacokinetics and biopharmaceutical properties multiple systemic daily administrations are need to achieve good local antibiotic concentrations that might lead to various adverse effects (Ahmed, Hannigan, MacDougall, Chan, & Halmagyi, 2012; Ali, 1995). In the last few years, different GS loaded polymer delivery systems intended for local administration, obtained by different techniques, have been designed to overcome drug systemic disadvantages by maintaining high local antibiotic concentration, to reduce side effects and induced bacterial (Aviv, Berdicevsky, & Zilberman, 2007; Della Porta et al., 2010; Zalavras, Patzakis, & Holtom, 2004).

In this study, we designed an in situ hydrogel forming system based on alginate-pectin blend microparticles loaded with GS as an antibiotic model with the aim to obtain a local drug controlled release. Particularly, particle carriers were produced using high M content alginate, in order to stimulates cytokine production by human monocytes (Thomas et al., 2000), and amidated pectin to increase in situ gel forming rate.

Two different technologies have been assessed and compared in order to produce such GS loaded gelifying systems: spray drying, that is one of the most reliable technique to microencapsulate different API and supercritical assisted atomization (SAA), a SC-CO_2 based technique recently proposed for the production of size controlled microparticles loaded with antibiotics (Aquino et al., 2013) as well as other API (Reverchon, Adami, Cardea, & Porta, 2009). The effect of the two encapsulation technologies have been evaluated regarding particle micromeritics, morphology, size distribution, and solid state properties. Moreover, studies on stability in accelerated storing conditions have been conducted. Properties desirable in a wound dressing such as swelling, fluid uptake and moisture transmission, as well as drug release behaviours have been tested. Finally, the antimicrobial activity of all formulations has been evaluated against both Gram positive and Gram negative bacteria, *Staphylococcus aureus* and *Pseudomonas aeruginosa*, respectively.

2. Materials and methods

Gentamicin sulfate (GS) and pectin (pectin from citrus, amidated low methoxyl grade) have been supplied by Sigma-Aldrich (Milan, Italy). Sodium alginate from brown algae (1% viscosity 35 mPa s; mannuronic acid content 70%) was kindly donated by Dompè S.p.A (L'Aquila, Italy). Carbon dioxide (CO_2; purity 99.9%) and Nitrogen (N_2; purity 99.9%) have been purchased from SON (Naples, Italy). All other chemicals and reagents were obtained from Sigma Aldrich (Milan, Italy) and used as supplied.

2.1. Powders preparation

Microparticles obtained by both supercritical assisted atomization (GAP) and spray drying (GAPS) were prepared by processing different aqueous feeds. After a series of pilot experiments, GS and alginate/pectin ratio (1:1:1 or 1:3:1) and total concentration of the feeds (0.5% or 1.0% w/V) were set. Feeds were obtained by slowly adding a GS solution to an aqueous solution of alginate and pectin blend, previously prepared, under gentle stirring. Dextrans blend solution was obtained by dissolving both polymers in distilled water under vigorous stirring for 4 h while GS solution was obtained by dissolving a carefully weighted amount of the drug in distilled water under gentle stirring.

Feeds for pneumatic atomization were spray-dried using spray drying laboratory apparatus (mini spray dryer B-191 Buchi Laboratoriums-Tecnik, Flawil, Switzerland) with process parameters and conditions set as follow: inlet temperature 120 °C, outlet temperature 66 °C, feed rate 5 ml/min; nozzle diameter 0.5 mm, drying air flow 500 l/h, air pressure 6 atmospheres, aspirator 100%.

Feeds for supercritical assisted atomization (SAA) were produced using a laboratory apparatus consisting of two high-pressure pumps (mod. 305, Gilson, Middleton, MO, USA) delivering the liquid solution/suspension and liquid CO_2 to a heated bath (Forlab mod. TR12, Carlo Erba, Milan, Italy) and, then, to the saturator. The saturator (internal volume 25 cm^3) containing stainless steel perforated saddles which assure a large contact surface between liquid solution and CO_2. The SAA process was conducted dissolving supercritical CO_2 (SC-CO_2) in the feed solutions (mass flow ratio between CO_2 and water set at 2.5). The solution obtained in the saturator was sprayed through the injection nozzle into the precipitator (internal volume 3 dm^3) operating at atmospheric pressure. A controlled flow of N_2 was taken from a cylinder, heated in an electric heat exchanger (mod. CBEN 24G6, Watlow, St. Louis, MI, USA) till 85 °C and sent to the precipitator to facilitate liquid droplets evaporation. The saturator and the precipitator were electrically heated using thin band heaters. A stainless steel filter located at the bottom of the precipitator allowed the powder collection and the gaseous stream flow out.

Each preparation was carried out in triplicate. Powders were collected and analyzed in term of production yields expressed as weight percentage of the final product compared to the total amount of the material processed. Powders were stored at room conditions and successively solubilized in distilled water were analyzed in terms of drug content by means of HPLC method described below.

2.2. Morphology and particle size distribution

The morphology of all formulations, studied by Scanning electron microscopy (SEM), was performed using a Carl Zeiss EVO MA 10 microscope with a secondary electron detector (Carl Zeiss SMT Ltd., Cambridge, UK) equipped with a LEICA EMSCD005 metallizator producing a deposition of a 200–400 Å thick gold layer. Analysis was conducted at 20 KeV.

Powders were dispersed on a carbon tab previously stuck to an aluminium stub (Agar Scientific, Stansted, UK). Samples were coated with gold using a sputter coater (mod. 108 Å, Agar Scientific, Stansted, UK). At least 20 SEM images were taken into account for each run to verify the microparticles uniformity.

Particle size distribution was evaluated by static light scattering (LS) equipped with a microliquid module (Coulter LS 13320, Beckman Coulter, Inc., Fullerton, CA, USA). The Coulter LS 13320 software uses Mie theory to produce an optimal analysis of the light energy distribution and to obtain the size distribution of the particles. Analyses were performed after the preparation of the microspheres suspensions using 30 mg of each sample dispersed in dichloromethane by sample sonication. The effectiveness of the microspheres dispersion was verified performing the measurement after different sonication times ranging between 5 and 30 min. Moreover, measurements on each sample loaded in the instrument were repeated at different time intervals, that is, every 5 min for 30 min. Results were expressed as d_{50} and span defined below:

$$\text{Span} = \frac{d_{90} - d_{10}}{d_{50}}$$

where d_{90}, d_{50} and d_{10} indicate the volume diameters at the 90th, 50th and 10th percentiles, respectively. In all time intervals, good reproducibility of results was obtained.

Sphericity coefficient (SC) was calculated by the following equation (Aquino et al., 2012; Del Gaudio, Russo, Rosaria Lauro, Colombo, & Aquino, 2009) by means of particles perimeter (P) and projection surface area (A) obtained by image analysis (ImageJ software, Wayne Rasband, National Institute of Health, Bethesda, MD, USA) using at least one hundred microparticles

$$SC = \frac{4\pi A}{P^2}$$

Surface roughness (SR) was calculated using fractal descriptors obtained by grey level distribution analysis measured on the SEM microphotographs of the microparticles. The images were taken at the same magnification. A scanned area of the SEM image, a box of 500 nm width and 500 nm length, was analyzed by ImageJ software using the algorithm known as the "box counting method" (Auriemma, Del Gaudio, Barba, d'Amore, & Aquino, 2011; Auriemma et al., 2013). The analysis was carried out on 10 different area sections for each microphotograph considered and a mean fractal descriptor was obtained. The analysis was repeated on at least 10 images for each batch.

2.3. Powders physico-chemical properties

Infrared analysis was performed using a FTIR spectrophotometer (FT-IR Nexus, Thermo-Nicolet, West Palm Beach, FL, USA) equipped with a mercury-cadmium-telluride detector. The samples were combined with small amount of potassium bromide and pressed to 6 tonnes in a manual press (OMCN s.p.a., Bergamo, Italy). The thin compacts produced were analyzed using 256 scans with a 1 cm^{-1} resolution step. Each experiment was carried out in triplicate, and results averaged.

Water content was evaluated by both Karl Fischer titration (Titromatic KF 1S, Crison, Barcellona, Spain) and Thermo gravimetric analysis (TGA, TG50 – Mettler Toledo, Columbus, OH, USA) by using about 7 mg of each GAP or GAPS powder batch. Each experiment was conducted in triplicate. Water content values obtained by the two tests were practically overlapped.

Bulk and tap density of all powders was measured by modifying the pharmacopoeia test, as reported in literature (Gainotti et al., 2006; Sansone, Aquino, Gaudio, Colombo, & Russo, 2009). Briefly, powders were loaded into a bottom-sealed 1 ml plastic syringe (Terumo Europe, Leuven, Belgium) capped with laboratory film (Parafilm® "M", Pechiney Plastic Packaging, Chicago, IL, USA) and tapped until no change in the volume of the powder was observed. The bulk and tap densities were calculated from the difference between the net weight of the plastic syringe content divided by the volume in the syringe before and after tapping, respectively. Experiments were performed in triplicate.

Fluid uptake ability of all formulations was evaluated as the ratio between fluid content at equilibrium after gelation of a carefully weighted amount of powder and dried powder. Briefly, 15 mg of formulation was spread over a previously weighted filter paper disc. The membrane was in contact with a vertical donor compartment filled with simulated wound fluid (SWF) consisting in 50% foetal calf serum (Sigma Aldrich, Milan, Italy) and 50% maximum recovery diluent (Sigma Aldrich, Milan, Italy, composed by 0.1% (w/v) peptone, peptic digest of animal tissue, and 0.9% (w/v) sodium chloride) (Bowler et al., 2012). SWF was thermostated at 37 °C and refilled during the experiment in order to maintain constant fluid volume into the donor. At regular intervals of time, the weight of the gel was measured after removing the filter paper from donor until equilibrium was reached. All experiments were performed at least in triplicate.

Water vapour transmission rate (WVTR) was obtained as described by ASTM Standard (2010). Briefly, 25 mm hydrogel disc of GAP or GAPS formulation was mounted on the top of a plastic tube containing 20 ml of distilled water. Teflon tape was used to cover the edge of the disc in order to avoid boundary loss. The assembly was kept inside an incubator at 37 °C. Weight loss was recorded at regular time intervals and plotted against time. WVTR was calculated as the ratio between slope of the plot and area of the disc, by the following formula

$$WVTR = \frac{slope}{A}$$

where A is the test area of the sample in m^2. All experiments were performed at least in triplicate.

2.4. Drug content and encapsulation efficiency

Samples of each manufactured GAP and GAPS series were dissolved under vigorous stirring, derivatized and HPLC analyzed, as reported elsewhere (Della Porta et al., 2010) using 25 mg of powder. Calibration curves were worked out and proportionality between GS concentration and AUC checked in the range 5–450 μg/ml.

Drug content was obtained as the ratio between actual GS weight and powder weight while encapsulation efficiency (e.e.) was calculated as the ratio of actual to theoretical drug content. Each analysis was performed in triplicate and the results were expressed as mean ± standard deviation.

2.5. Drug release studies

Permeation assays were performed by means of Franz-type vertical diffusion cells (Hanson Research Corporation, CA, USA) with an exposed surface area of 0.60 cm^2. The donor compartment was filled with 20 mg of GAP or GAPS formulation. The receptor phase was simulated wound fluid (SWF). The solution was thermostated at 37 °C and magnetically stirred at 150 rpm by Teflon-coated stirring bars placed in the receptor compartment in order to prevent boundary layer effects. Nitrocellulose membrane filter with pore size 0.45 μm, (Sartorius, Goettingen, Germany) previously set with SWF was used as barrier. The receptor solution, 200 μl aliquots were sampled, derivatized and analyzed by HPLC (USP 30, "gentamicins content method") for the determination of GS permeated at regular time intervals.

2.6. Antimicrobial assay

Time-kill studies were performed as previously described (Aquino et al., 2013). Briefly, an over-night culture of *S. aureus* strain A170 was diluted 30-fold with prewarmed Mueller-Hinton Broth (MHB) and incubated at 35 °C under constant shaking. The late-log-phase of growth was normalized to 2.5×10^8 cells/ml and 200 μl were used to inoculate three sterile 96-well plates further treated with the different formulations (GAP1 = 1.99 mg/ml, GAP2 = 1.78 mg/ml, GAP3 = 3.30 mg/ml, GAP4 = 2.36 mg/ml, GAPS1 = 2.44 mg/ml, GAPS2 = 2.31 mg/ml, GAPS3 = 4.07 mg/ml, GAPS4 = 4.17 mg/ml) corresponding to 0.70 mg/ml of GS used as control. At defined time points (6, 12, and 24 days), viable counts were calculated to give CFU/ml (colony forming unit per ml) by plating serial dilutions on MHA incubated at 35 °C. Kill curves were plotted with time against the number of CFU recovered. Each antimicrobial assay was performed in triplicate in separate days.

To assess the ability of both GAP and GAPS formulations to degrade *P. aeruginosa* ATCC-27853 biofilm, bacteria were grown overnight in MHB medium. Cultures were then diluted to approximately 10^7 CFU/mL in fresh MHB medium, and 200 μL was used to inoculate flat-bottom 96-well polystyrene microlitre plates. The

Table 1
Composition, process yield, particle size and encapsulation data of both GAP and GAPS formulations at different GS/polymer blend ratio.

Sample code	GS/alginate/pectin ratio (p/p/p)	Feed (w/v) (%)	Yield (%)	d_{50} (μm) and () span	Drug content (%)	Water content (%)	e.e.[a] (%)
GAPS1	1:1:1	0.5	68.4 ± 2.3	3.88 (1.19)	28.6 ± 0.5	7.6 ± 0.2	85.7 ± 0.3
GAPS2	1:1:1	1.0	57.6 ± 2.8	4.10 (1.27)	30.3 ± 0.4	7.9 ± 0.3	90.9 ± 0.4
GAPS3	1:3:1	0.5	42.7 ± 2.1	3.70 (1.29)	17.2 ± 0.3	8.7 ± 0.4	86.1 ± 0.4
GAPS4	1:3:1	1.0	49.9 ± 2.9	4.07 (1.43)	16.8 ± 0.2	9.1 ± 0.4	84.2 ± 0.5
GAP1	1:1:1	0.5	74.2 ± 1.2	1.91 (1.07)	35.1 ± 0.4	5.1 ± 0.2	>100
GAP2	1:1:1	1.0	68.7 ± 1.1	1.82 (1.19)	39.4 ± 0.6	4.7 ± 0.3	>100
GAP3	1:3:1	0.5	70.6 ± 1.2	1.45 (1.15)	21.2 ± 0.2	5.0 ± 0.3	>100
GAP4	1:3:1	1.0	66.1 ± 1.1	1.39 (1.13)	29.6 ± 0.1	4.4 ± 0.2	>100

[a] e.e. = encapsulation efficiency.

biofilms were allowed to develop up to 24 h at 37 °C, afterwards, formulations corresponding to 0.70 mg/ml of GS used as control were added on each well. After incubation for 8 h at 37 °C without shaking, the plate wells were washed twice with phosphate-buffered saline (pH 7.2) to remove unattached cells and dried in an inverted position. Finally, the adherent cells were stained for 10 min with 200 μL of Gram's crystal violet (CV). Wells were rinsed with water and dried. Images were acquired using a Canon EOS 450d and processed with Microsoft Office 2010 (Stigliani et al., 2013). The amount of biofilm mass was obtained by destaining the wells with 200 μL of 33% acetic acid and then measuring the absorbance of the CV solution in a microplate spectrophotometer set at 595 nm (Thermo Scientific Multiskan Spectrum Model #1500, Themo Scientific, Milan, Italy).

Disc diffusion test was carried out on Mueller-Hinton agar (MHA) according to Clinical and Laboratory Standard Institute (CLSI) guidelines (Wikler, 2008), with some modifications. Briefly, a sterile cotton swabs was dipped into a bacterial suspension of *S. aureus* strain A170 normalized to 10^8 cells/ml, and then spread on MHA agar plate. Similarly, a bacterial suspension of *P. aeruginosa* normalized to 10^8 cells/ml was spread on the same plate. The plate were spotted with different GAP and GAPS formulations corresponding to 0.70 mg/ml of GS, used as control. After incubation at 35° for 24 h, the diameters of the zones of clearance produced by each powder were compared.

2.7. Storage stability test

Stability test of GAP microparticles were performed following the ICH Q1AR2 C Guideline "Stability Testing of New Drug Substances and Products" by storing the powders at 40 °C and 75% relative humidity (RH) for 3 months. Samples were monitored by HPLC, DSC, and Karl Fisher titration method after 1, 2 and 3 months.

3. Results and discussion

3.1. Preparation and characterization of the powders

In order to produce in situ hydrogel forming systems, the feasibility of two atomization techniques, spray drying and SAA, was verified. GS loaded dextran based microparticles were produced processing the same aqueous feed solutions by both techniques. Feed solutions consisted of GS/alginate/pectin in two different ratios (1:1:1 and 1:3:1) with total amount of the components set at 0.5 or 1.0% (w/v).

As shown in Table 1 both microencapsulation technologies demonstrated good production yield ranging from 42 to 68% for spray drying and from 66 to 74% for SAA. As expected, particles size distribution (PSD) was affected by processing technology. In fact, while pneumatic atomization in spray-drying produced microparticles with diameters (d_{50}) ranging between 3.70 and 4.10 μm, particles obtained by decompressive atomization involved in SAA (Reverchon et al., 2009) had narrower size distribution and smaller diameter, ranging between 1.39 and 1.91 μm. Microparticle size obtained by both techniques were affected by GS/polymers ratio, the higher the ratio the larger the particles.

Both techniques produced powders with high encapsulation efficiency (e.e.); spray drying e.e. ranged between 84.2 and 90.9% depending on the feed while encapsulation efficiency obtained by SAA was always slightly higher than 100%. This phenomenon, as

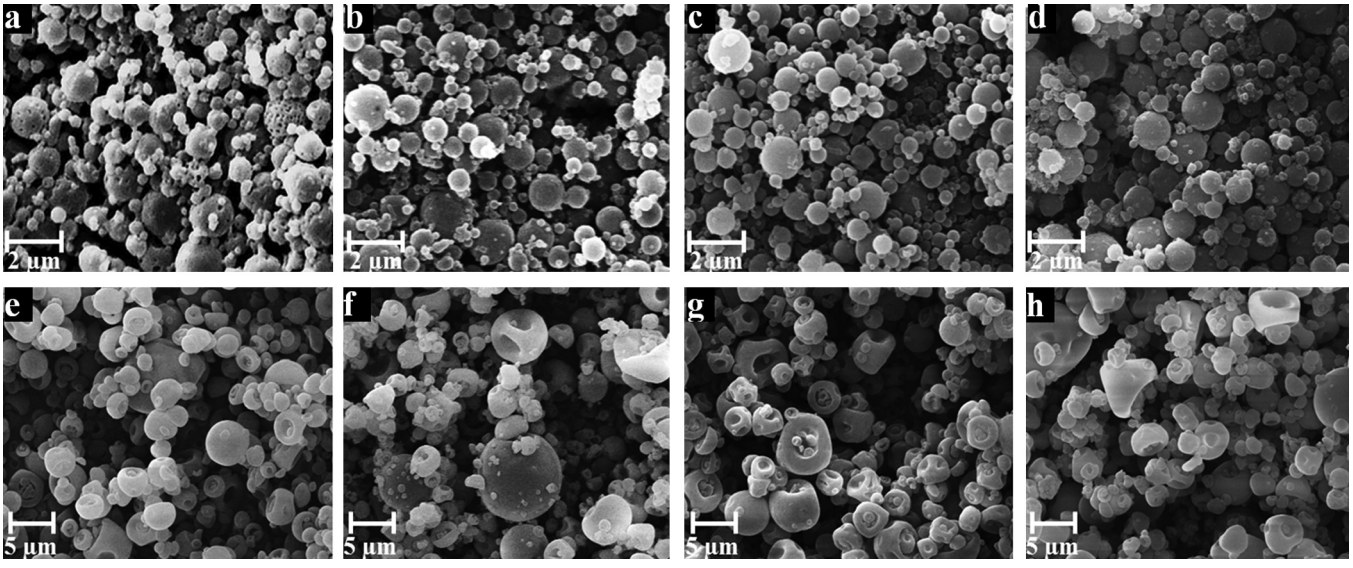

Fig. 1. SEM microphotographs of GS/alginate/pectin microparticles produced with different drug polymers ratio: GAP1 (a), GAP2 (b), GAP3 (c), GAP4 (d) obtained by SAA and GAPS1 (e), GAPS2 (f), GAPS3 (g) and GAPS4 (h) obtained by spray drying.

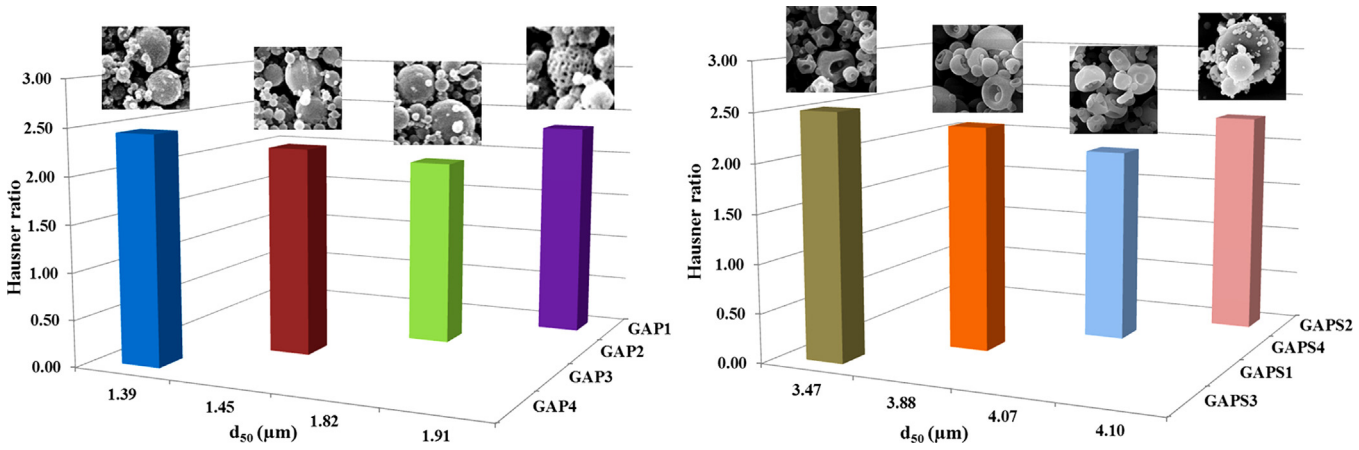

Fig. 2. Correlation between Hausner ratio and particle size of GS/alginate/pectin microparticles produced by SAA (GAP series) and spray drying (GAPS series) produced with different drug polymers ratio.

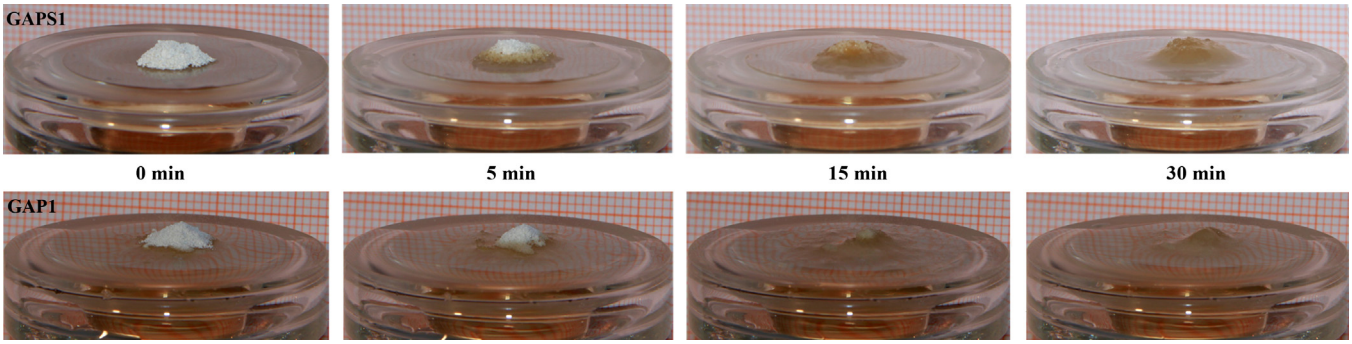

Fig. 3. Simulated wound fluid uptake of GAPS1 and GAP1 formulations moving from dry powder to gel state when deposited on cellulose membrane. Pictures are shot at different time intervals.

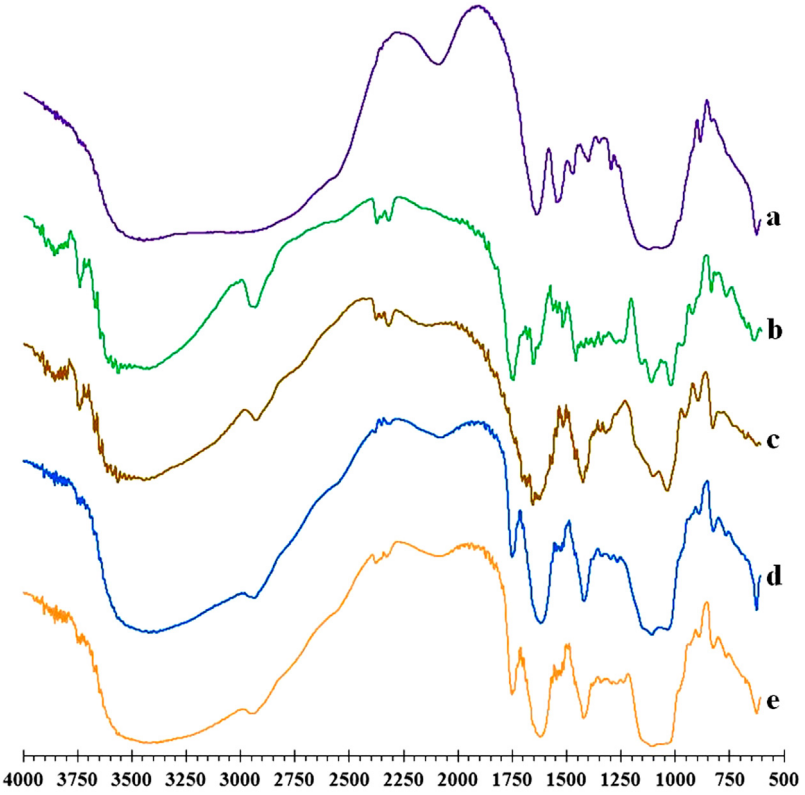

Fig. 4. FTIR spectra of GS raw material (a), pectin (b), sodium alginate (c), and GS/alginate/pectin microparticles obtained by SAA, GAP4 (d) and spray drying, GAPS4 (e).

Table 2
Morphological data, Hausner ratio, fluid uptake and transmission rate of microparticles produced by spray drying and SAA at different GS/polymer blend ratio.

Sample code	ρ_T/ρ_B	SC	SR	Fluid uptake	WVTR (g/m^{2}h)
GAPS1	2.30	0.84 ± 0.07	1.31 ± 0.06	12.43 ± 0.95	111 ± 5
GAPS2	2.28	0.85 ± 0.06	1.45 ± 0.07	11.91 ± 0.87	110 ± 7
GAPS3	2.51	0.84 ± 0.07	1.39 ± 0.09	14.81 ± 0.96	103 ± 6
GAPS4	1.97	0.82 ± 0.08	1.38 ± 0.07	14.45 ± 0.92	102 ± 8
GAP1	2.33	0.95 ± 0.02	1.61 ± 0.06	14.19 ± 0.95	98 ± 6
GAP2	2.00	0.96 ± 0.01	1.20 ± 0.05	12.96 ± 0.79	98 ± 6
GAP3	2.22	0.96 ± 0.02	1.18 ± 0.06	13.05 ± 0.85	94 ± 7
GAP4	2.44	0.97 ± 0.01	1.17 ± 0.05	13.27 ± 0.88	93 ± 8

Each value represents the mean ± S.D. ($n \geq 3$).

previously described (Aquino et al., 2013), is related to the precipitation of small quantities of polymer material into the precipitation chamber due to the so-called anti-solvent effect. Water content of spray-dried microparticles was higher than SAA's. This can suggests that during decompressive atomization a reduction in surface tension of the feed takes place, due to supercritical CO_2 mixing with feed, allowing faster and more efficient elimination of water from polymer matrix during particle formation (Del Gaudio et al., 2013; Tripp & Combes, 1998).

Stability tests were conducted in storage accelerated conditions in order to verify the stability of gentamicin entrapped in the polymeric matrix over time. Results showed that GS content was preserved even in harsh conditions and only an increase in water content around 5% was detected after 3 months for all manufactured formulations (data not shown).

GAP microparticles, produced by SAA (Fig. 1a–d), exhibited good sphericity and smooth surface with the exception of GAP1 formulation that presented porous particles whereas GAPS formulations, obtained by spray drying, were less spherical and highly wrinkled (Fig. 1e–h).

Good powder flowability, high fluid absorbing capacity as well as water permeability at the equilibrium are among the most important properties required for wound dressing preparation to maintain the optimal environment at the wound bed and to enhance healing process (Bolton, Monte, & Pirone, 2000). Therefore, flowability of both GAP and GAPS powders was evaluated as the ratio between tapped density (ρ_T) and bulk density (ρ_B), where the lower the value the higher the flowability of the powder. As reported in Table 2, values ranged between 1.97 and 2.51 or 2.00 and 2.44 for GAPS or GAP series, respectively. As well known, Hausner ratio (ρ_T/ρ_B) is strictly correlated to particles size but it is also influenced by their shape and roughness. Consequently, both sphericity coefficient (SC) and surface roughness (SR) for GAP and GAPS microparticles were evaluated.

As shown in Table 2, SC of SAA microparticles ranged from 0.97 to 0.95 (1 corresponding to a sphere) with SR around 1.20 with the exception of GAP1 formulation that exhibited the highest SR (1.61) due to the presence of particle with porous texture. Spray-dried microparticles presented lower SC (0.85–0.83) and SR ranging between 1.31 and 1.45. Hence, GAP microparticles presenting smaller diameters, had flowability very close to GAPS' homologous formulations due to the enhancement of effective contact surface area in spray-dried microparticles (Li, Rudolph, Weigl, & Earl, 2004). Moreover, on the base of SC and SR data it is possible to understand the unexpected Hausner ratio of GAP1 and GAPS2 inside the series. In fact, SEM extensive analyses showed porous texture for GAP1 and nanoparticles covered surface for GAPS2, which reduced flowability in spite of the increasing in size (see Fig. 2).

3.2. Evaluation of particles fluid uptake and permeability in simulated conditions

In order to mimic in vitro the clinical conditions and a reproducible environment in which the different formulations would be administered, a simulated wound fluid (SWF) was used to test wound exudate uptake ability of all produced powders.

Fluid uptake ability was evaluated as the weight increase of the different formulations when in contact with a SWF donor container. As shown in Table 2, GAPS formulations exhibited a ratio between gel and powders weight ranging from 11.91 and 14.81 while GAP powders presented values ranging between 12.27 and 14.19. As expected, fluid uptake was directly related to alginate content. It is interesting to point out that the lowest fluid uptake capacity of GAPS formulations is showed by GAPS2 due its lower hydrophobicity related to its high SR (Hirvi & Pakkanen, 2007) while the highest uptake in GAP formulations can be associated to porous surface of GAP1 which determines an enhancement in contact area between particles and SWF. Kinetics of the hydration process were also evaluated, resulting in about 50% of the fluid absorption in 15 min for all formulations and complete gelation within 30 min for GAPS

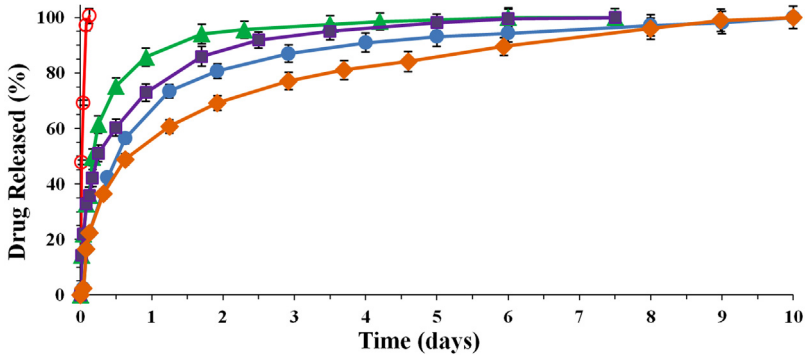

Fig. 5. Permeation profiles of GAPS and GAP microparticles manufactured by spray drying and SAA, respectively: GAPS1 (-▲-), GAPS4 (-■-), GAP1 (-●-) and GAP4 (-◆-) in comparison with pure GS (-○-). Mean ± SD; (n = 6).

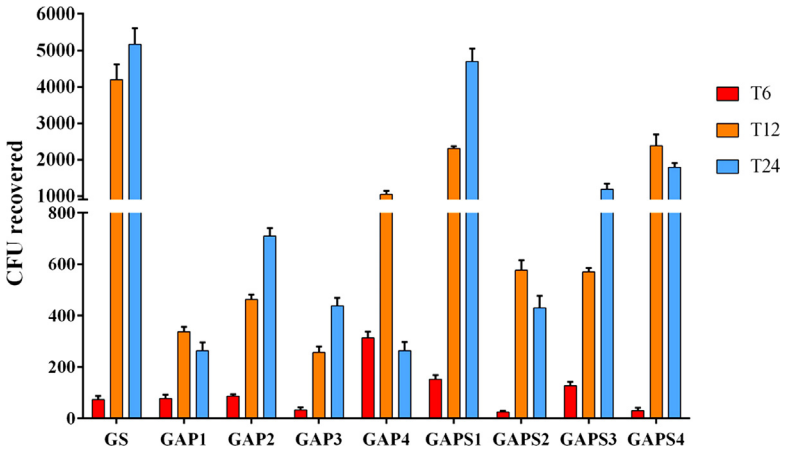

Fig. 6. Antimicrobial activity of GAP and GAPS against *S. aureus* after 6, 12 and 24 days of incubation by time-killing test. GAPS and GAP microparticles are used at concentrations corresponding to 0.7 mg/ml of gentamicin used as control; CFU recovered = number of *S. aureus* colony forming unit.

powders and 25 min for GAP's (see Fig. 3) with slightly differences in time, directly dependent on pectin content.

Fluid permeability of the different formulations was also evaluated as water loss after swelling process in order to ensure that gel layers would provide adequate level of moisture at wound bed. As reported in literature (Gwosdow, Cunningham, Lydon, Rascati, & Berglund, 1993; Queen, Gaylor, Evans, Courtney, & Reid, 1987), water vapour transmission rate (WVTR) of any wound dressing preparation should be between 80 and 105 g/m^2h in order to avoid wound dehydration or occlusion phenomena, if higher or smaller, respectively. All GAP formulations presented WVTR values between 93 and 98, then, in the appropriate range to maintain a proper fluid equilibrium. GAPS3-4 showed suitable WVTR (103 and 102) whereas GAPS1-2 WVTR was higher (111 and 110, respectively). Hence, results from these preliminary studies conducted on SWF show that gel obtained by any GAP or GAPS formulation can produce the proper environmental characteristics to enhance cellular migration and reepithelization.

3.3. FT-IR microparticles characterization

Gentamicin is a cationic drug able to interact with anionic polymer residues as alginates. Such an interaction could be useful to slow down drug release rate from microparticles prolonging residence time and drug efficacy. FT-IR studies were conducted in order to verify the presence of such interaction in powders obtained by both spray drying and SC-CO_2 assisted atomization. The characteristic peaks of GS are reported in Fig. 4 including the —NH_2 bond and —OH stretching vibration at 3250 cm^{-1} while the peaks at 1645 and 1545 cm^{-1} were related to the bending of N—H bond of the primary and secondary amines. The bands of particular interest for amidated pectin were centred at 1750 and 1630 cm^{-1} corresponding to the symmetrical stretching vibration of carboxyl group and to protonated carboxyl acid group, respectively. Alginate exhibited asymmetric stretching band from the carboxylate ion centred at 1650 and 1420 cm^{-1}. In this region, all GAP and GAPS formulations showed the peak at 1750 cm^{-1} related to the carboxyl group of amidated pectin, the peak at 1420 cm^{-1} related to the presence of alginate and a new peak centred at 1615 cm^{-1} associated to the C═O amide stretching due to the interaction between GS and carboxylic acid sites on alginate mannuronic blocks (Iannuccelli, Montanari, Bertelli, Pellati, & Coppi, 2011), confirming a strong interaction between gentamicin and alginate.

3.4. Drug permeation studies

In order to point out any differences in GS release behaviour from microparticles that could be associated to fluid uptake rate and manufacturing process, drug permeation profiles were monitored using vertical Franz-type diffusion cells with SWF (simulated wound fluid) in the acceptor compartment. Fig. 5 reports the permeation curves of both GAP and GAPS microsystems in comparison to pure GS. As expected for a very soluble drug, as GS, its total permeation was achieved in less than 3 h. By contrast, encapsulated drug showed a prolonged release, following a non-fickian diffusion mechanism, due to the formation of a gel barrier when microparticles went in contact with SWF.

Permeation profiles of microparticles were related to drug/polymers ratio and manufacturing process. In fact, microparticles produced by spray drying GAPS exhibited the faster permeation rate, achieving total release of the drug into the acceptor compartment between 4 and 6 days according to the increasing in polymer blend concentration, whereas GAP (formulations released the total amount of the encapsulated GS) between 8 to 10 days (in SWF). Moreover, both GAPS and GAP microparticles exhibited a drug burst effect within 7 h related to particles hydration kinetic, resulting in the release of almost 50% of the loaded drug in 6 h for GAP1 and GAPS1 (formulations) while GAPS4, that showed the slowest hydration rate, released in 6 h 35% of the loaded GS. Such intensive release of gentamicin in the first 6 h of administration, followed by a prolonged release, could be very useful to prevent infection spreading at the beginning of a local antibiotic therapy maintaining high local antibiotic concentration for an extended period of time.

3.5. Evaluation of gentamicin antimicrobial activity

With the aim to compare the effect of the manufacturing process on antimicrobial activity of formulations, preliminary time-killing assay, measuring the reduction of the number of CFU (colony forming unit) recovered at 6, 12 and 24 days, was conducted against *S. aureus*, one the most common Gram positive bacteria found in infected wounds (Fig. 6).

Reduction of CFU till 24 days demonstrated that both GAP and GAPS, with the exception of GAPS1, exert a prolonged bactericidal activity till 24 days whereas pure GS loosed its activity after a week. Moreover, during the first 6 days, all formulations, with the exception of GAP4, showed a bactericidal activity against *S. aureus* comparable to GS. Production technology had strong effect on bactericidal activity at 12 and 24 days. In fact, GAP series exhibited

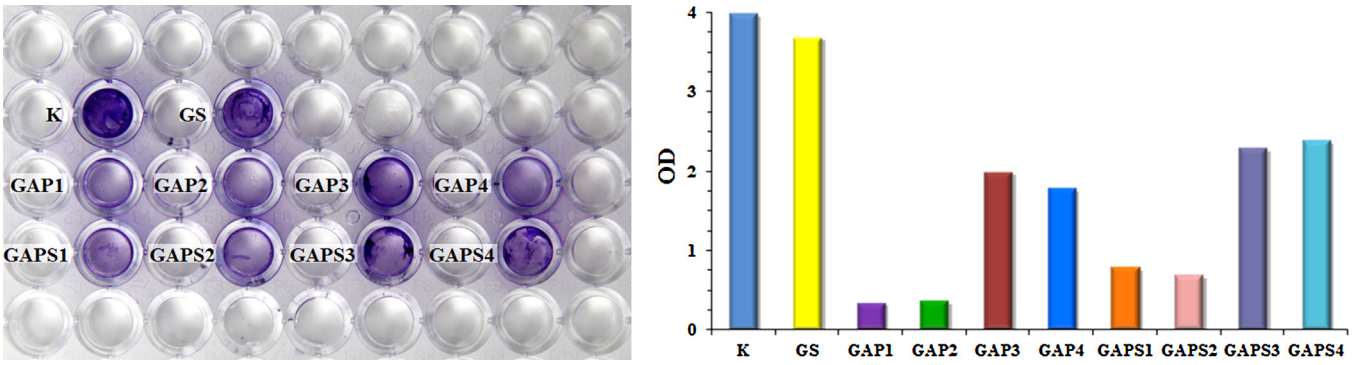

Fig. 7. *P. aeruginosa* biofilm degradation assay on GAP and GAPS dry powders and its corresponding graphic obtained by destaining the wells with acetic acid and measuring the absorbance of the CV at 595 nm (one representative of three independent experiments). GS and K (untreated sample) were used as control.

higher activity than pure GS (between 12 and 4 fold higher) at 12 days on *S. aureus* whereas GAPS formulations resulted much less active than the homologous GAP with bactericidal activity between 6 and 2 fold higher than pure GS. At the end of the assay GAPS loosed most of their activity with the exception of GAPS2 still active after 24 days. Different behaviour was found for GAP microparticle series that showed good activity even at 24 days; the best result was achieved by GAP1 and GAP4.

Moreover, given to *P. aeruginosa* attachment to surfaces and the subsequent biofilm formation are important steps in the establishment of chronic infections and persistence in host tissues, the ability of GAP and GAPS formulations to degrade preformed biofilm was investigated. Results, showed in Fig. 7, demonstrate that, while GS was able to degrade only 10% of preformed *P. aeruginosa* biofilm, GAP and GAPS formulations were able to destroy it up to 90%. In particular, biofilm degradation ability was mainly related to drug/polymers ratio. In fact, formulations with higher drug content, GAP1-2 and GAPS1-2, were able to degrade 90–80%, respectively, whereas GAP3-4 and GAPS3-4 with the lower drug/polymer ratio were able to degrade 50–40% of the preformed biofilm.

Opportunistic infections of wound are often polymicrobial. Two of the most important bacterial pathogens of humans, *P. aeruginosa and S. aureus*, are frequently coisolated from infections of catheters, endotracheal tubes, respiratory tract as well as in wound bed. For this reason an antimicrobial assay using a modified disc diffusion test was conducted on a Muller-Hinton agar plate spreaded with both *S. aereus* and *P. aeruginosa*. Fig. 8 shows the zone of clearance produced by GAP and GAPS formulations after 6 days.

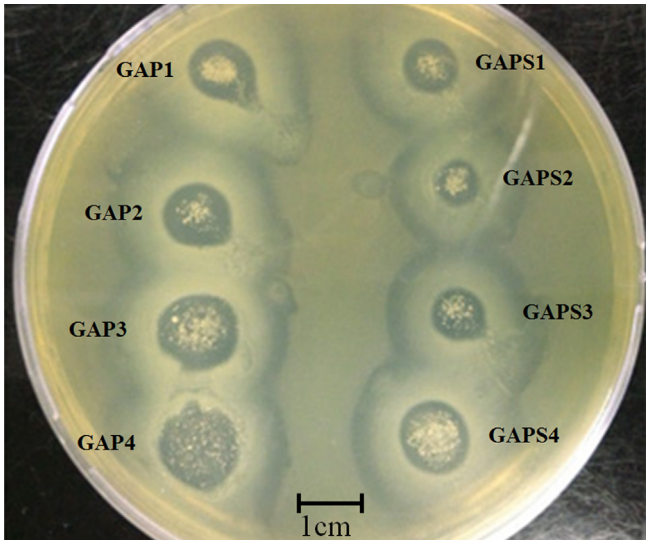

Fig. 8. Antimicrobial activity against *S. aureus* and *P. aeruginosa* at 6 days by modified diffusion assay of both GAP and GAPS microparticle formulations in concentrations equivalent to 0.7 mg of pure GS.

The inhibition zone of bacterial growth was larger around the in situ formed gel produced by GAP compared to GAPS homologous formulations. In particular, an inhibition zone diameter ranging from 12.5 to 10.5 mm and from 10.5 and 7.0 mm was recorded for GAP and GAPS series, respectively. It is interesting to point out that differently from biofilm degradation, formulations made with the lower drug/polymer ratio exhibit a larger inhibition zone for both bacterial growth at 6 days.

4. Conclusions

Spray drying as well as supercritical assisted atomization was successfully used to manufacture dextran based microsystems with high gentamicin encapsulation efficiency in form of dry powders able to gelify in wound bed. All microcarriers exhibit narrow dimensional range with mean diameter around 4 and 2 μm for spray-dried or SAA microparticles, respectively. Powders produced by both techniques also demonstrate good flowability as well as rapid fluid uptake when in contact with simulated wound fluid, moving completely into gel form in less than 45 min. Moreover, WVTR of the in situ formed gel was in the range to maintain a proper fluid equilibrium on the wound bed for most of the produced formulations, especially for GAP series that exhibit values around 95 g/m^{2}h. Release of encapsulated GS from microparticulate systems follows a non-fickian diffusion kinetics with burst effect suitable to prevent infection spreading at the beginning of a local antibiotic therapy and enabling to prolong drug release till 10 days depending on drug/polymers ratio and manufacturing technology. According to permeation profiles, a set of antimicrobial tests show that most of the microsystems are able to guarantee an appropriate initial antimicrobial effect maintained over time (till 24 days) and to degrade preformed biofilms that could be present in wound cavity. Overall, among all designed formulations, GAP1 seems to be the best choice in both inhibiting bacterial biofilm formation and to degrade a preformed one that is essential to properly treat infected wounds. The powders showed interesting fluid uptake and fluid permeability performance under simulated conditions as well as a prolonged antibacterial activity. These systems could be interesting candidates either to be incorporated in wound dressing preparations such as fibres or administered directly as self-consistent formulations on wounds.

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