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Research paper

Chitosan-alginate hydrogels for simultaneous and sustained releases of ciprofloxacin, amoxicillin and vancomycin for combination therapy

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ABSTRACT

A set of chitosan-alginic acid hydrogels were prepared for multiple encapsulation and release of antibiotics for future application in combination therapy. Vancomycin, ciprofloxacin and amoxicillin were used as models, as widely used in therapy, either individually and in combination. The hydrogels were prepared by a simple, low cost and solventfree approach based on complexation between chitosan and alginic acid followed by crosslinking with $CaCl_2$ to enhance structural properties. The antibiotics were loaded during the gelification process to maximize efficiency, and to reduce the loss of material. All the formulations were analyzed by scanning electron microscope to investigate the surface morphology, the inner structure, and the effect of the drugs loading. The swelling behavior, the cytotoxicityand the antibacterial activity, were evaluated. The investigation of the impact of simultaneous loading of the antibiotics along with the variation in the surrounding pH, on the release trend, represents a key part of work. The results revealed that the physicochemical properties of the combination of the drugs-loaded play an essential role in the morphology, structural changes and release profile.

1. Introduction

The multidrug resistance to microorganisms represents a serious issue in the infection treatment. Looking at the past, antibiotic-resistant bacteria quickly emerged after the antibiotic approval and placing on the market [1–3]. Synthetic antibacterials such as chlorhexidine, salicylate, thiazoline, and quaternary ammonium face constant threats because of the resistance acquired by microorganisms[1]. Moreover, conventional antibiotics still face issues related to poor solubility, cytotoxicity and overdose. The need for efficient and safe drug delivery systems able to reduce the onset of bacterial drug-resistance and avoid the side effects are desired [2–4].

Hydrogels are highly versatile biomaterials widely used in the biomedical field, like in drug delivery and tissue engineering as well as in the development of healthcare products such as contact lenses, and wound dressing materials [5–7]. Controlled and prolonged release, local administration, stimulate release, enhanced mechanical strength, and improved biocompatibility represent some of the hydrogels advantages compared to traditional pharmaceutical formulations. Hydrogels have been extensively studied and already successfully applied in wound dressings, catheters, contact lenses among other applications [8–10].

Generally, antibacterial hydrogels are classified in i) inorganic nanoparticle-containing hydrogel; ii) antibacterial agent-containing hydrogel; and iii) hydrogel having inherent antibacterial properties [11]. The advantages provided by hydrogels in the antibiotic therapy, compared to other systems like nano- or microparticles are; i) they could be used locally, avoiding the side effects of systemic applications; ii) provide sustainable release of the payload; iii) difficult for bacteria to develop resistance aiming at only one target; and iv) synergic effects coming from the combination of ingredients. Hydrogels based on synthetic and natural polymers containing antibiotics as ciprofloxacin [12,13], gentamicin [14,15], vancomycin [16,17], ampicillin [18,19], levofloxacin [20,21] have been largely investigated. However, a large part of the studies are focused on the development of innovative materials for hydrogels preparation; for instance, based on stimuli-responsive polymers to control the release by varying the surrounding environment, but not so much is reported about the multiple encapsulation of antibiotics in one system. One of the approaches to reduce the resistance onset is to decrease the dose or use a combination of antibiotics to achieve synergic effect. The development of hydrogels able to allocate and release more than one active compound represents an ongoing approach to avoid or limitate the development of multidrug resistance [22,23].

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The benefits of loading and release a defined combination of antibiotics from one system include; i) broadening the antibacterial spectrum; ii) activity against multiple bacterialinfections; and iii) synergistic effect.

Combination therapy is classified as; i) inhibition of the targets in different pathways, e.g. combination of isoniazid, rifampicin, ethambutol and pyrazinamide; ii) inhibition of different targets in the same pathway, e.g. combination of sulfamethoxazole and trimethoprim; and iii) inhibition of the same target through different pathways [24]. In the design of antibacterial hydrogels, several key-points have to be accounted for preserving the activity of the encapsulated agents. It includes the release time, concentration, and site.

In this work, we develop a set of hydrogels based on chitosan and alginic acid. The hydrogels experience electrostatic interactions, H-bonds, and entanglements between polysaccharides chains. As the electrostatic interactions are highly affected by the ionic strength and pH of surrounding environment, $CaCl_2$ was used as cross-linking agent to make the hydrogels more stable and suitable to hold multiple compounds.

Polysaccharides are a group of water-soluble polymers that have been utilized to prepare hydrogels due to their ability to form gels under well-defined conditions [25]. Natural polysaccharides such as chitosan, alginic acid and hyaluronic acid are already adopted in various medical applications due to their biodegradability, biocompatibility and drug interaction.

Chitosan (CS) is a linear polysaccharide made of β(1–4) linked D-glucosamine and N-acetyl-D-glucosamine units in a variable content and sequence [26]. It is obtained from chitin by partial or total deacetylation. As biomaterial, chitosan has numerous peculiarities; it is amenable to enzymatic and chemical modification, acts as an adhesive due to its positive charges at physiological pH, it is biodegradable, biocompatible and it is readily processed into different shapes [27].

Alginates (ALG) are natural polymers consisting of 1,4-linked β-D-mannuronic (M) and α-L-guluronic (G) residues organized in regions of sequential G units (G-blocks), regions of sequential M units (M-blocks), and regions of atactic organized G and M units. Due to structural similarity to the extracellular matrix (ECM), alginates are promising biopolymers in health applications; moreover, they have the advantages that gelify in mild conditions. Sol–gel transition properties of alginates are based on the formation of a stiff "egg-box" structure due to the selective binding of divalent cations to G-blocks of two adjacent polymeric chains. Therefore, the structure of alginate network is related to the monomeric composition and displacement. By varying the M/G ratio and distributions, it is possible to change the viscoelastic properties of the material. Chitosan and alginic acid are interesting macromolecules in the biomedical field, and additional advantages are coming from their combination [28–31].

Herein to develop antibacterial hydrogels, the attention has been focused on three well-known and widely used antibiotics; ciprofloxacin hydrochloride, amoxicillin trihydrate, and vancomycin hydrochloride. The antibiotics were loaded individually and in combination in the hydrogels, and the differences in encapsulation efficiency, release kinetics, and antibacterial activities were evaluated.

Ciprofloxacin hydrochloride (C), belong to the fluoroquinolone antibacterial agents, has a wide antibacterial spectrum versus $Gram^+$ and $Gram^-$ bacteria. It is widely used for topical applications, in particular in the treatment of eye and skin infections [32,33]. Ciprofloxacin binds to the DNA gyrase blocking the bacterial DNA duplication. Ciprofloxacin has dosage-related toxicity and the release through hydrogels could.

Vancomycin hydrochloride (V) is a tricyclic glycosylated non-ribosomal peptide, considered as the "drug of last resort" of sepsis and infections of the lower respiratory tract, skin, and bone, caused by $Gram^+$ bacteria. It is active against *Listeria monocytogenes*, *Streptococcus pyogenes*, *Streptococcus pneumoniae* (including penicillin-resistant strains), *Streptococcus agalactiae*, *Actinomyces* species, and *Lactobacillus* species. The bactericidal action of vancomycin results from the inhibition of the cell-wall biosynthesis. The pharmacokinetics is affected by the high water solubility, causing poor absorption and quick clearance. To control the local delivery, a suitable vehicle would be ideal. It has been reported as the use of hydrogel, e. g poly (lactic-*co*-glycolic acid) (PLGA) [34]; cyclodextrin and poliglycolic acid – g –(hydroxyethyl) methacrylate (PGA-g-HEMA) [35] based hydrogel can protect and enhance the effectiveness of vancomycin.

Amoxicillin trihydrate (A) is a β-lactam antibiotic active versus a wide range of $Gram^+$ and a limited range of $Gram^-$ bacteria. It is mostly used in the treatment of infections of the upper and lower respiratory tract, the genitourinary tract, and the skin. Amoxicillin acts by binding to penicillin-binding proteins with subsequent inhibition of the transpeptidation, leading to activation of autolytic enzymes in the bacterial cell wall. It has been reported that ampicillin-loaded polyvinyl alcohol (PVA) hydrogel exhibited enhanced antibacterial property to both $Gram^+$ and $Gram^-$ bacteria and improved hemolysis [36].

A set of chitosan-alginic acid hydrogels (CA) were prepared and loaded with ciprofloxacin, vancomycin and amoxicillin individually and in the following combination; amoxicillin -ciprofloxacin; vancomycin -amoxicillin; and vancomycin -ciprofloxacin. The inner structure and the surface morphology of the hydrogels were characterized by scanning electron microscopy, and the swelling behavior and loss of weight under different pH were evaluated. The impact of the drug(s) loading on the hydrogels properties were investigated using CA (drug(s) free) as control. The release kinetics of the antibiotics from the hydrogels were evaluated at different pH either and the antibacterial property evaluated against *S. Aureus*. The antibiotics in the free form were used as control to label the advantages of using hydrogels while gentamicin as reference. The *in vitro* cytocompatibility of the carrier was assessed on primary human adult fibroblast by direct contact and by extract.

2. Materials and methods

2.1. Materials

Low molecular weight chitosan (D.D. >75%); alginic acid sodium salt from brown algae (low viscosity); amoxicillin trihydrate, ciprofloxacin hydrochloride, gentamicin hydrochloride, and vancomycin hydrochloride were purchased from Sigma-Aldrich. Sodium chloride, potassium dihydrogen phosphate, sodium carbonate, and sodium hydroxide were acquired from Penta, Prague, Czech Republic. Acetic acid and hydrochloric acid were purchased from Chromservis, Prague, Czech Republic.

2.2. Hydrogels preparation

The drug(s) were incorporated within the hydrogels matrix by in situ-loading, meaning that the hydrogels network formation and the drug (s) encapsulation occurred simultaneously. With this approach, the release of the drug(s) is determined by diffusion, swelling, drug-polymer interactions and by the structural changes in the hydrogels structure when exposed to different environment. The chitosan-alginate hydrogels (CA) were prepared as follow; at first, the polysaccharides solution was prepared; chitosan (20 mg/mL) in distilled water containing 1% v/w of acetic acid and alginic acid in distilled water at concentration of 10 mg/mL.

Then, the solutions containing the antibiotics were prepared; vancomycin hydrochloride and amoxicillin trihydrate in distilled water while ciprofloxacin hydrochloride in dilute HCl (0.1 N). All the three solutions were prepared at concentration of 5 mg/mL. The ratio between the total polysaccharides content (alginic acid + chitosan) and antibiotic was defined to 20:1 (mg); for each mg antibiotic 20 mg of polysaccharides were used. The ratio was chosen on the basis of several investigations in which, a set of component ratio were tested. The 20:1 resulted the best option in terms of encapsulation efficiency (E.E),

release trends, and overall hydrogels properties. The control hydrogel (drug free) was prepared by mixing chitosan and alginic acid solutions and left under stirring for 30 min; afterwards, 0.5 mL of $CaCl_2$ solution (1% w/w) was added dropwise under gently shaking. The final mixture was stored at 4 °C overnight. The initial amount of polysaccharides in the free drug formulation is equal to that in the single drug loading. Conversely, in the double drugs loading, the amount of polysaccharides is doubled to preserve the polysaccharides to drug(s) ratio.

In the single and double loading, the hydrogels were prepared by mixing the antibiotic(s) solution(s) and alginic acid solution prior to add to chitosan.

2.3. In vitro hydrogels swelling behavior

The swelling properties of the hydrogels were evaluated at pH 4; pH 7; and pH 7.4. Prior to the test, the hydrogels were dried in vacuum oven at 60 °C until no variation in weight was observed. The initial dry weight was noted as W_d. Afterwards, the sample was immersed in 10 mL of media at 37 °C in a shaker water bath 75 rpm. After a determined time, the samples were withdrawn, the excess media on the surface removed by tissue paper and the weight recorded (W_w).

The swelling, expressed in percentage (S%) was calculated using the Eq 1:

$$S(\%) = \frac{W_w - W_d}{W_d} x100 \quad [1]$$

2.4. Hydration ratio

Hydrogels were lyophilized and weighted (M_0); then submerged in 5 mL of the swelling solution (pH 4, pH 7 and pH 7.4) for 24 h and then the mass recorded (M_{24}). The rehydration ratio (RR) was calculated using the Eq 2:

$$RR = \frac{M_{24}}{M_0} \quad [2]$$

2.5. In vitro hydrogel stability evaluation

The *in vitro* hydrogels stability was evaluated by recording the weight loss over time [40]. The initial weight of the dried hydrogels was noted (W_0) and then immersed in 10 mL of PBS solution containing 2 μg/mL of lysozyme and kept in water bath at 37 °C under shaking (75 rpm). At scheduled time, the hydrogels were removed, wiped with filter paper and then weighted (W_f). The weight loss expressed in percentage (WL%) was calculated using Eq 3:

$$WL(\%) = \frac{W_0 - W_f}{W_0} x100 \quad [3]$$

2.6. Morphology

The microstructure of the hydrogels was examined by scanning electron microscopy (FEI Nova NanoSEM 450). The sample were kept in oven at 60 °C for 48 h and then coated with gold to improve conductivity before analysis.

2.7. Rheological evaluation and mechanical properties of the hydrogels

The rheological properties of the hydrogels were evaluated using Anton Paar MCR 502 with cone and plate geometry.

All the tests were performed at 25 °C. The steady shear tests (g = 0.001–3000 s^{-1}) and frequency sweeps tests (0.1500 Hz) at 0.5 constant strain were performed to evaluate the flow properties and the viscoelastic behavior of the hydrogels.

Compression tests were performed on the drug free and drug loaded hydrogels cut in a rectangular block having the following size: 10 mm × 10 mm x 3.5 mm (universal testing machine Instron 5967). The hydrogels were uniaxally compressed at 1 mm/min rate to 90% strain to obtain the stress-strain curves. The toughness and compression strength were calculated through stress-strain curves. The compression modulus was obtained from the slope of the linear region of the stress-strain curves. For each sample, the test were performed in parallel and in triplicate, in parallel.

2.8. In vitro drug(s) release

The *in vitro* release studies of the antibiotic from the formulations were performed following a reported protocol with minor variations [37]. Drug(s) loaded hydrogels were weighted and then immersed in 50 mL of the media (pH 4; pH 7 and pH 7.4) at 37 °C, and 100 rpm shaking. After determined time, 2 mL of media were withdrawn and replenished with the same volume of fresh media to maintain the volume constant. The amount of vancomycin, ciprofloxacin and amoxicillin released from the hydrogels was quantified by HPLC. All measurements were performed in triplicate and the results represented as the average value and plotted as % cumulative vs time.

The release mechanism was evaluated by the Peppas and Sahln equation, which is related to the release driven by swelling. The equation includes the drug diffusion and the polymer relaxation phenomena's Eq 4;

$$\frac{M_t}{M\infty} = k_1 t^m + k_2 t^{2m} \quad [4]$$

where k_1 and k_2 are the diffusion and polymer relaxation constants, respectively; and m is a constant.

2.9. HPLC method

Vancomycin, ciprofloxacin, and amoxicillin contents in the hydrogels and in the release media, were assessed by high performance liquid chromatography (HPLC, Thermo Fisher Scientific, UltiMate TM 3000 with Diode Array Detection).

For vancomycin (rt: 9.2 min) column COSMOSIL 5C18-PAQ, 4.6 × 250mm was used. The mobile phase was Acetonitrile/THF/0.2% tryetylamine (7/1/92) adjusted to pH 3.2 with H_3PO_4 at a flow rate of 1 mL/min. The column temperature was maintained at 30 °C and vancomycin detected at wavelength 280 nm.

Ciprofloxacin (rt: 7.4 min) was detected using COSMOSIL column 5C18-PAQ 4.6 × 250mm. The mobile phase was acetonitrile/2.5 mmol H_3PO_4 (13/87) adjusted to pH 3.0 with trimethylamine at a flow rate of 1 mL/min. The column temperature was maintained at 30 °C and ciprofloxacin detected at wavelength 278 nm.

Amoxicillin (rt: 5.1 min) was detected using COSMOSIL column 5C18-PAQ 4.6 × 250mm. The mobile phase was methanol/10 mmol/L CH_3COONa (5/95) adjusted to pH 4.5 with CH_3COOH at a flow rate of 1 mL/min. The column temperature was maintained at 30 °C and ciprofloxacin detected at wavenlength 230 nm.

2.10. Antibacterial activity test

The antibacterial activity of the single and double drug loaded hydrogels versus of *S. aureus* was evaluated by KirbyBauer (KB) and dilution assay. In the KB assays, *S. aureus* was applied over a CMHB-agar surface during the exponential growth phase at a concentration of 10^8 CFU/mL.

The hydrogels containing one or combination of antibiotics were placed on the bacteria plates; drugs –free hydrogel was used as negative control while disc containing 30 μg of gentamicin hydrochloride as a positive control. The samples were incubated for 24 h at 37 °C and then the inhibition zone evaluated.

The drug(s) release was evaluated by microdilution assays. The

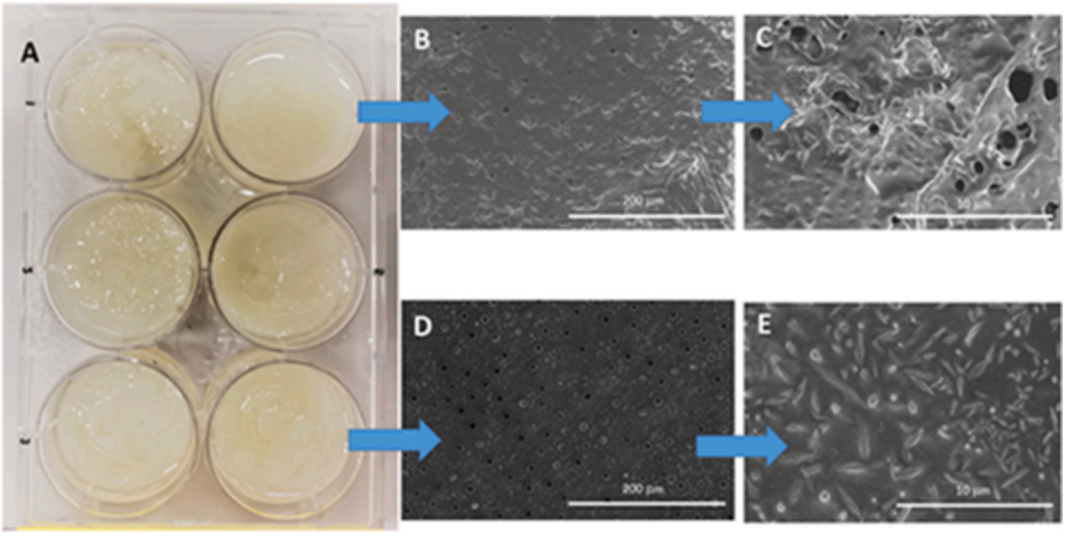

Fig. 1. A) Image of the hydrate hydrogels; B–C) SEM micrographs of the freeze-dried hydrogels; D-E) SEM micrographs of the freeze-dried hydrogels containing ciprofloxacin.

samples were placed in a 96 well plates and diluted in 2x CMHB to the final concentration of 1 x CMHB. Then, the sample was serially diluted 1:2 in 1x CMHB. Sterile 1x PBS without antibiotics was used as control. *S. aureus* in the exponential growth phase was added to the diluted sample at final concentration of 10^5 CFU/mL, along with positive control of 1x PBS without antibiotic. The negative control of 1x PBS was bacteria free. The well plates were incubated at 37 °C for 18 h under 90 rpm shaking. The optical density (OD) was measured at 600 nm. The normalized bacteria density (BD) was calculated with the Eq 5:

$$BD = \frac{OD_{600s} - OD_{600c+}}{OD_{600c+} - OD_{600c-}} \quad [5]$$

where OD_{600s} refers to the sample; OD_{600c-} and OD_{600c+} to negative and positive control, respectively.

2.11. Cytotoxicity assay

The cytotoxicity of the hydrogels was evaluated by direct contact and by extraction in Human Adult dermal fibroblasts cells line (ATCC® PCS-201-012™). The indirect cytotoxicity of the hydrogels formulations was evaluated by MTT assay. The samples were cut into circles having a diameter of 2.5 cm and placed into the cell culture media for 24 h at 37 °C. Then, different percentage of extraction media, 5%, 25%, 50%, 75% and 100% were prepared. Human derma fibroblast cells were introduced in a 24-well plate at 40,000 cells/well and incubated for 24 h at 37 °C under an atmosphere of 5% CO_2. Afterwards, the cultural media aspired and replaced by the extracts at different percentage and the plate incubated for further 24, 48 and 72 h. The cell viability was calculated at each time interval and compared to the control, untreated cells (0% extract).

In the direct contact, the hydrogels were cut in a cylinder shape with dimension to fit into the well (96 well plate), and placed on top of the cell layer attached to the well bottom. The cell viability was evaluated by MTT after 24, 48 and 72 h of contact.

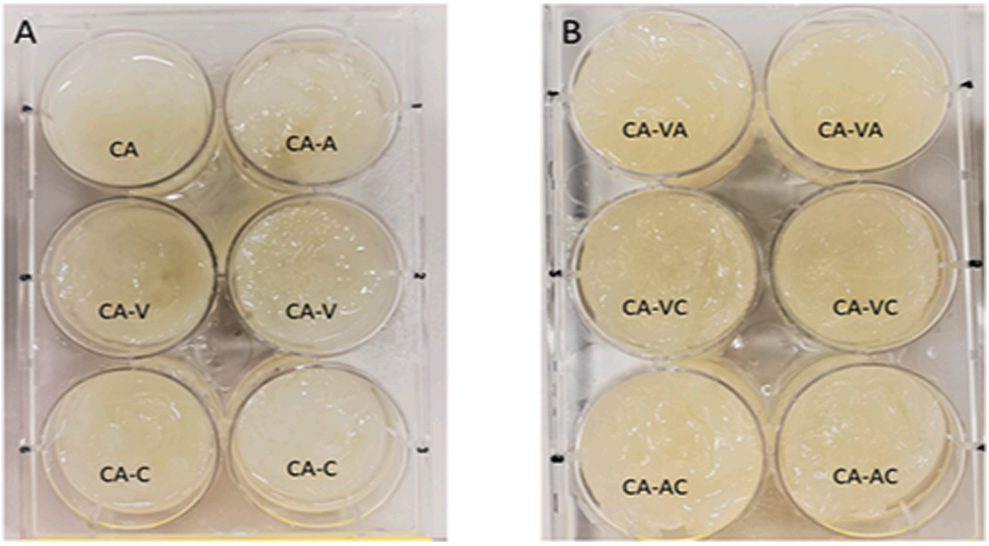

Fig. 2. Hydrogels images at the swelling equilibrium. A) Single loading; B) double loading. CA = free drug hydrogel; the others are loaded with the drug(s); single loading: CA-A = Amoxicillin (25 mg); CA-V = Vancomycin (25 mg); CA-C= Ciprofloxacin (25 mg). Double loading: CA-VA Vancomycin + Amoxicillin (25 mg + 25 mg); CA-VC = Vancomycin + Ciprofloxacin (25 mg + 25 mg); CA-AC = Amoxicillin + Ciprofloxacin (25 mg + 25 mg).

Table 1
Wt of the hydrogels after preparation. The data represents the average value and standard deviation obtained from the weight of three hydrogel samples (* without drugs; n = 3).

	Weight (g)		Weight (g)
CA*	29.7 ± 0.3		
Individual loading		*Multiple loading*	
CA-V	31.2 ± 0.5	CA-VC	37.4 ± 0.9
CA-A	29.9 ± 0.8	CA-VA	35.8 ± 0.7
CA-C	29.5 ± 0.6	CA-AC	38.4 ± 1.2

2.12. Statistical analysis

One-way ANOVA analysis was performed using the GraphPad Prism version 6.00, GraphPad Software, La Jolla California, USA, with consideration of $p < 0.05$ as statistically significant.

3. Results

3.1. Morphology and antibiotic content

The image of the hydrated hydrogels and the microstructure of the freeze-dried form are illustrated in Fig. 1The hydrogels have similar (Fig. 1A) color but differ in the surface morphology, with irregularities visible to the naked eyes. Analysis by scanning electron microscope (Fig. 1B, C,D, E) show the porous structure of the hydrogels with variations in the pores size and distribution between CA (only chitosan and alginic acid) and CA containing drugs. The formation of the pore is due to the evaporation of the water during the drying step, and herein is a random process where the size and distribution of the pore cannot be controlled. In the micrograph in Fig. 1E the antibiotic on the hydrogel surface is observable. Those molecules are responsible of the initial burst. It has to be considered that during the drying procedure the elimination of water not only produces gap, but also, it is responsible for leading a rearrangement of the polymer chains with migration of the drug(s) molecules within the structure.

The images reported in Fig. 2 show similarities among the hydrogels in term of shape and color. Moreover, they have comparable weight and the standard deviation value is acceptable for all formulations indicating the good reproducibility of the used method (Table 1). The highest weight in the hydrogels containing the combination of antibiotics is due to the higher amount of polysaccharides used to preserve the polysaccharides to drug weight ratio. Higher content in polysaccharides lead to incorporate large amount of water during the preparation. The surface morphology is not homogeneous in all samples, but it is influenced by the preparation procedure.

In Table 2 the antibiotics content, in the freeze-dried hydrogels, prior to perform release tests are expressed as percentage (of the initial amount) and in mg of the drug(s) in 100 mg of dried hydrogels.

Table 2
Encapsulation efficiency and content in mg of antibiotic(s) in 100 mg of dried hydrogel. V=Vancomycin; C= Ciprofloxacin; A = Amoxicillin. The data are reported as the average value ± SD (n = 3).

Sample	Individual loading		*Sample*	V	Multiple Loading			mg	A
	E.E. (%)	mg			E.E. (%)		V		
					C	A		C	
CA-V	92 ± 2	4.60 ± 0.10	CA-VC	87 ± 2	91 ± 2		4.35 ± 0.10	4.55 ± 0.10	
CA-A	95 ± 1	4.75 ± 0.05	CA-VA	95 ± 1		89 ± 1	4.75 ± 0.05		4.45 ± 0.05
CA-C	91 ± 1	4.55 ± 0.05	CA-AC		93 ± 1	92 ± 3		4.65 ± 0.05	4.60 ± 0.15

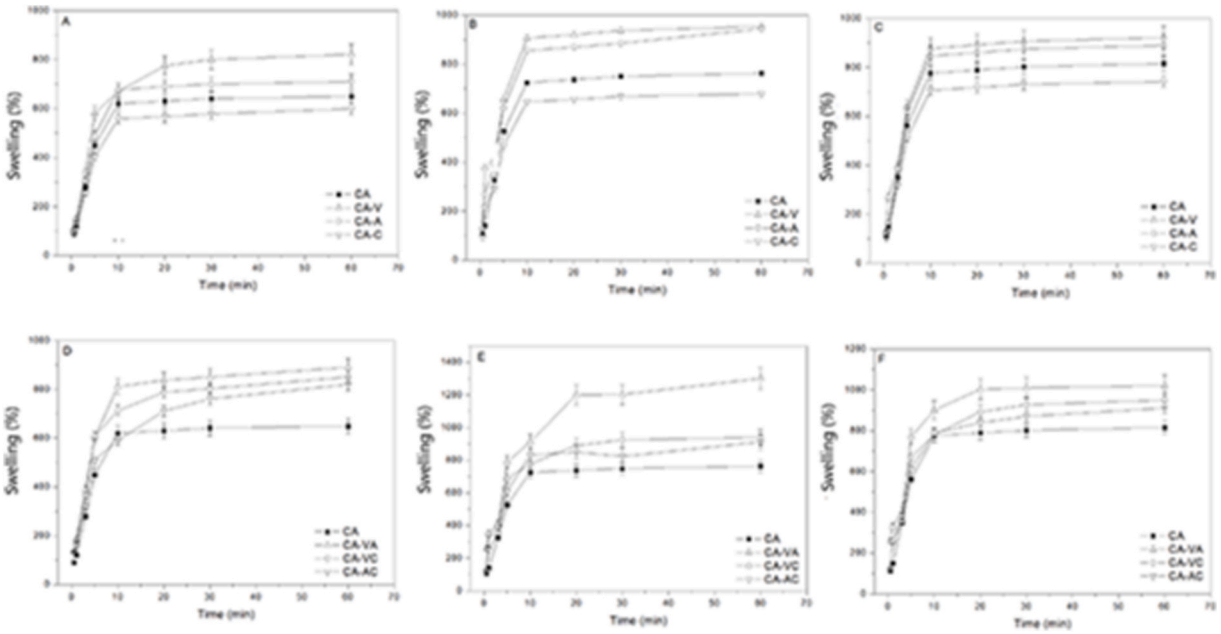

Fig. 3. Swelling kinetics at 37 °C temperature of all formulations at various pH. To evaluate the effect of the drug loading on the swelling CA (free drug hydrogel) is considered as control. Values are expressed in percentage referring to the weight of the dry sample. Graphs A,B,C refer to single drug loaded hydrogels; CA-V (Vancomycin); CA-A (Amoxicillin) and CA-C (Ciprofloxacin) while D,E,F to the double loaded formulations; CA-VA (Vancomycin + Amoxicillin); CA-VC (Vancomycin + Ciprofloxacin) and CA-AC (Amoxicillin + Ciprofloxacin). The data are reported as the average value ± SD (n = 3).

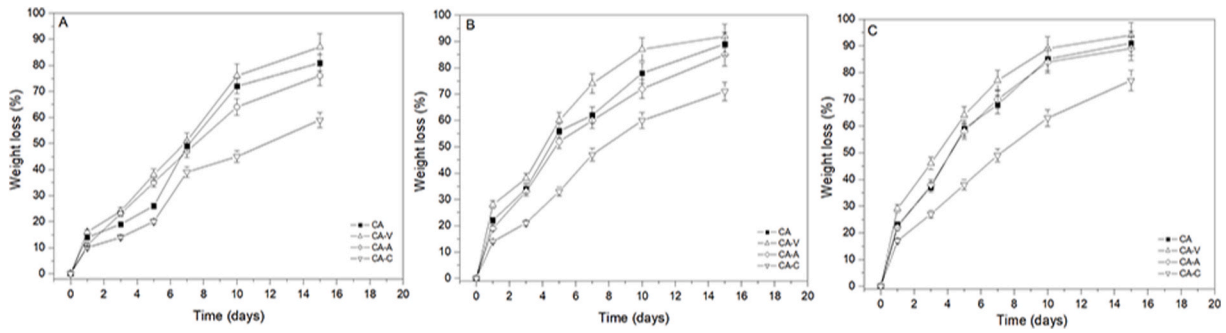

Fig. 4. Effect of pH on the hydrogels weight loss, expressed in percentage, over time. CA) free drugs hydrogel while CA-V) containing vancomycin; CA-A) containing amoxicillin and CA-C) containing ciprofloxacin. Degradation profiles at A) pH 4; B) pH 7 and C) pH 7.4 at 37 °C. The data refer to the average value ± SD (n = 3).

3.2. Swelling behavior

The swelling behavior is a key point in the characterization of the hydrogels as it directly influence the release kinetics and mechanism of the loaded compounds. In the prepared hydrogel, due to the hydrophilic nature of the polysaccharides, the swelling is driven by the phase transition from a glassy to a rubbery state. In the glassy state, the loaded molecules are immobile while in the rubbery they rapidly diffuse to the external fluid through the swollen layer of the polymers. The following graphs (Fig. 3) illustrate the swelling trend over time until equilibrium is reached.

For application in wound healing, to know the swelling behavior is highly important as the hydrogel has to absorb liquids exuded by the wound.

The trends in Fig. 3 show for all formulations, an increase in the swelling the with the incubation time and the pH of the media. The influence of the pH is related to the ionization state of the amino and carboxylic groups along the chitosan and alginate structure. When both groups are in the ionic state (pH < 6.5 the intramolecular repulsive forces and the attractive forces among the polysaccharides chains regulate the swelling. With a prevalence of attractive forces the structure lead a slower penetration of the water and the equilibrium is reached in later time. Conversely, for pH > 6.5 the amino groups are deprotonated with a prevalence of repulsive forces among the negatively charged carboxylic groups; it favours the water uptake and the maximum swelling is reached faster. Using CA as control, the presence of the drug (s) affect the swelling. The swelling rate is influenced by the hydrophilic and hydrophobic properties of the loaded compounds. The incorporation of hydrophobic drugs lead to an increase in the matrix hydrophobicity decreasing the fraction of water bound to the matrix. In the present case, all the antibiotics are hydrophilic as they are in the hydrochloride (ciprofloxacin and vancomycin) and trihydrate (amoxicillin) form. at The trends in Fig. 3 illustrate how at the same pH, vancomycin loaded hydrogel exhibit the highest swelling value followed by those containing amoxicillin while the formulations containing ciprofloxacin demonstrate the lowest swelling. In the formulations containing two drugs, the highest swelling was observed in CA-VA, while the lowest in CA-VC. Herein, all the antibiotics are hydrophilic as they

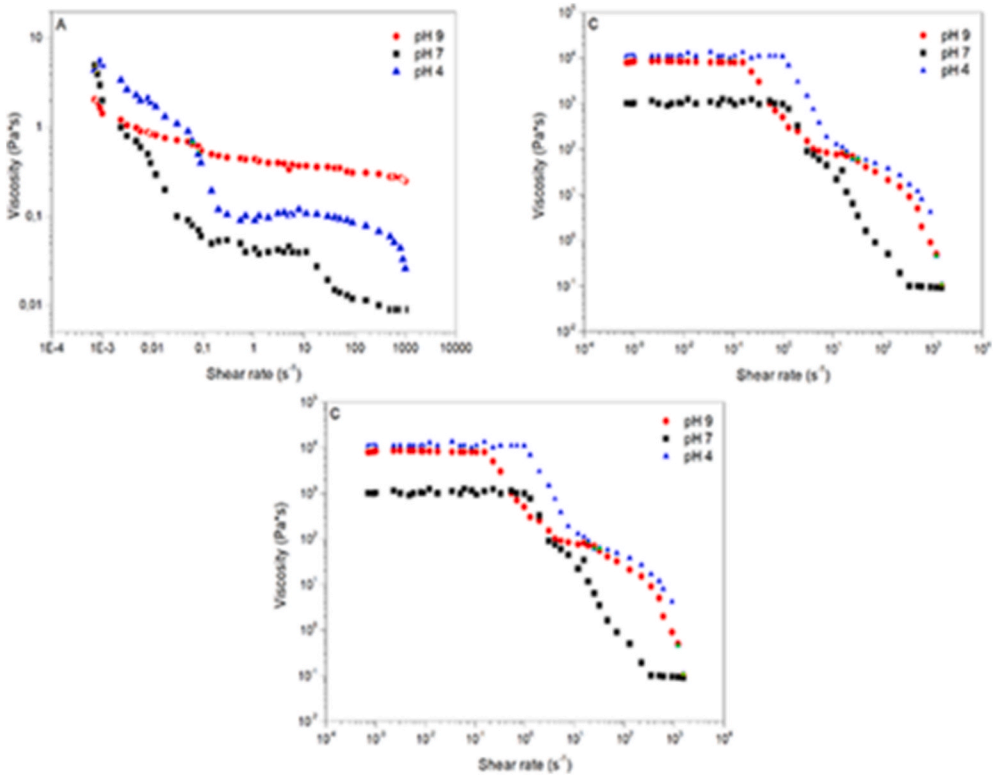

Fig. 5. Flow curves of A) alginate hydrogels, B) chitosan hydrogels and C) chitosan-alginate composite hydrogels at w/w ratio 2:1 at different pH at 25 °C.

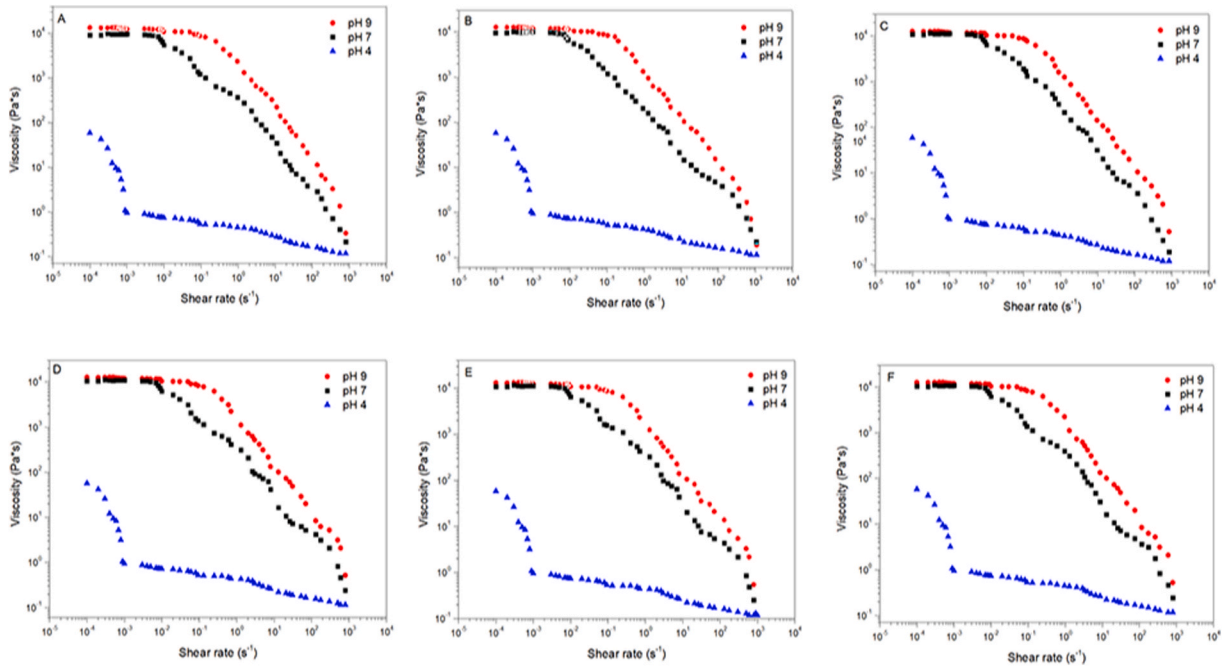

Fig. 6. Flow curves of chitosan hydrogels containing a single drug A) vancomycin; B) amoxicillin; C) ciprofloxacin; -A) or a combination, D) vancomycin + ciprofloxacin; E) vancomycin + amoxicillin and F) amoxicillin + ciprofloxacin.

are in the hydrochloride (ciprofloxacin and vancomycin) and trihydrate (amoxicillin) form, meaning that the differences in swelling in the loaded hydrogels can be related to the differences in the chemical structure of the loaded compounds which interfere with the dislocation and mobility of the polysaccharides chains.

3.3. *In vitro hydrogels weight loss*

The structural changes of the hydrogels in terms of loss of materials has been reported and evaluated as weight change versus time. As described [40] the media penetrate into the CS-ALG matrix causing swelling and dissociation of the intra and intermolecular hydrogen bonds among the polysaccharides chains, causing modification in the spatial arrangement of the chains and a subsequent loss in the structure. The pH of the media and the presence of drug(s) within the matrix influence the spatial displacement of the polymers chains. Among the samples, the fastest reduction in weight for a single drug loaded hydrogels was observed in CA-V due to the quick media intake as proved from the high swelling (Fig. 3). The two phenomena could be partially attributed to the higher hydrophilicity of the vancomycin but also to the chemical structure, which is bigger than the ciprofloxacin and amoxicillin. Amoxicillin has higher solubility in water than vancomycin but the swelling is lower and the weight reduction rate slower compared to vancomycin. It can be ascribed to the steric factor; in fact, the larger structure can affect the interactions with the chains and subsequently the compactness of the overall hydrogel macro structure.

In the drug free (CA) and the single drug loaded hydrogels CA-V; CA-A and CA-C demonstrate a pH dependent weight loss. In acidic media (pH 4) the variation in weight are 81%, 87%, 76% and 59% after 2 weeks of media exposition Fig. 4. The rate tends to increase for all formulation when the pH raise. The maximum variation is observed at pH 7.4 where CA reach the value of 96% while CA-V; CA-A and CA-C have 91%, 94% 88% and 73%. Samples containing ciprofloxacin (CA-C; CA-VC and CA-AC) demonstrate the slower rate in all the media. Such trends is comparable to those already reported [40].

3.4. *Rheological and mechanical properties*

In Fig. 5 the flow curves of chitosan, alginic acid, and the mixture chitosan-alginic acid at ration 2:1 hydrogel samples at pH 9, 7 and 4 are reported.

In chitosan 2% w/w, the behavior at neutral and alkaline pH is similar, while at pH 4 is different. In acidic condition the amino groups are protonated limiting the hydrogen bonding and the hydrophobic interactions between the chitosan chains [38]. The contribution of electrostatic and steric repulsion the viscosity decreases at moderate shear rate exhibition a Newtonian behavior. At high shear rate, slight shear thinning behavior is observable. A gel like behavior is shown by chitosan in neutral and alkaline environment due to the deprotonation of the amino groups with a reduction in the repulsive forces that allow hydrogen bond and hydrophobic interactions to dominate. In summary for the chitosan-based hydrogels, two distinct viscosity region are observed; the Newtonian and the power-law flow region. In the power-law region the viscosity decreases with the increase in the shear rate.

Alginate solution 1% w/w, shows a different trend characterized by poor viscoelasticity and the polymer melt at all tested pH, as already described [39]. The charge density along the alginate chain changes depending on the pH, due to the COO^- and COOH groups. The protonation and deprotonation of the carboxylic groups alters the hydrophilicity and the interactions among the chains. In acidic media, the carboxylic groups are protonated and an enhancement the viscosity of the solution is observed due to the suppression of the electrostatic repulsion in favor of intermolecular hydrogen bond and formation of physical entanglements [40].

While the chitosan viscosity has a direct relation with the pH, increasing pH led an increase in viscosity; the alginic acid show the opposite trend; viscosity decrease with increase in pH. Due to the opposite behavior of the two polysaccharides, the mixed hydrogels, obtained at chitosan to alginic acid weight ratio 2:1 has to be investigated.

For the composite hydrogels, the shear rate is maximum at lower pH and decreases by moving towards neutral and alkaline environment. In the mixed hydrogel, COOH and NH_2 groups belonging to alginic acid

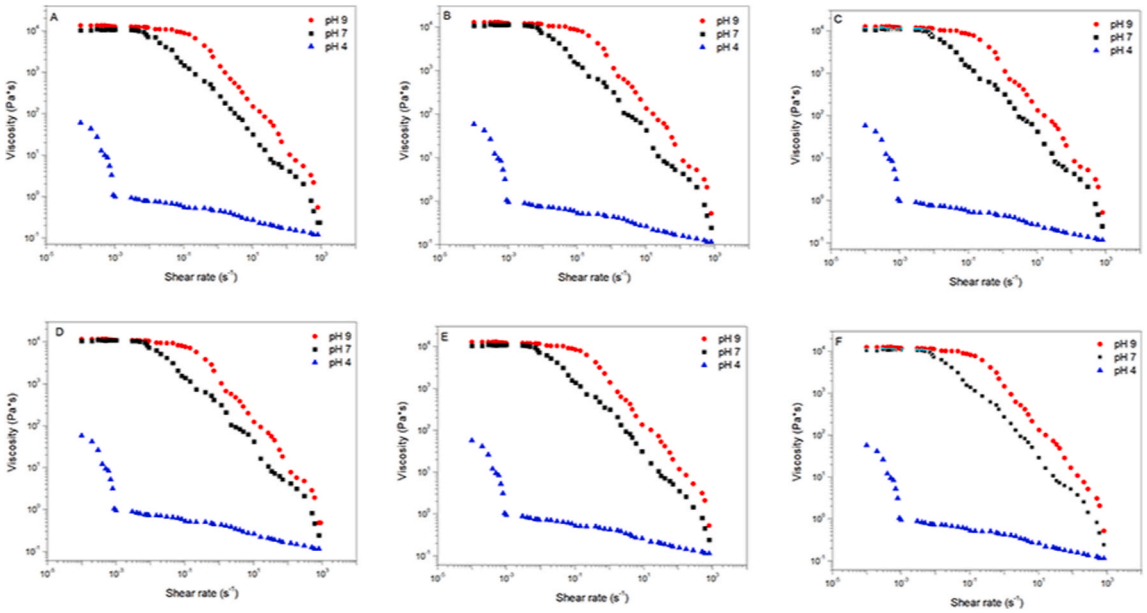

Fig. 7. Flow curves of alginate hydrogels containing a single drug A) vancomycin; B) amoxicillin; C) ciprofloxacin; -A) or a combination, D) vancomycin + ciprofloxacin; E) vancomycin + amoxicillin and F) amoxicillin + ciprofloxacin.

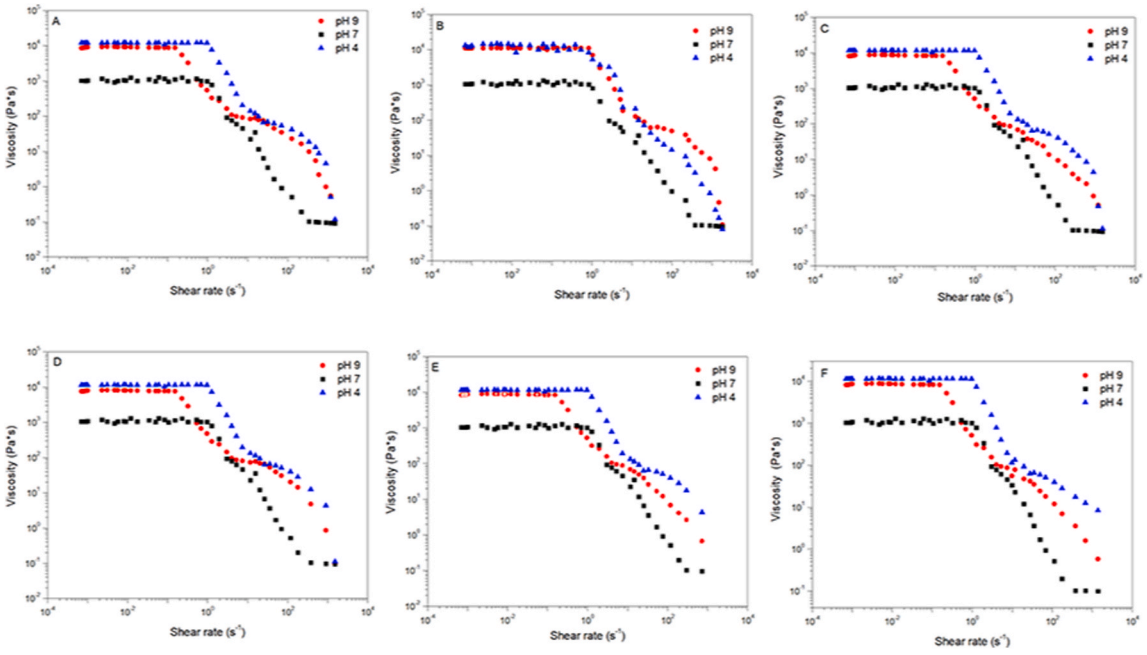

Fig. 8. Flow curves of composite hydrogels, chitosan-alginic acid at weight ratio 2:1, containing a single drug; A) vancomycin; B) amoxicillin; C) ciprofloxacin; - or in a combination, D) vancomycin + ciprofloxacin; E) vancomycin + amoxicillin and F) amoxicillin + ciprofloxacin.

and chitosan, respectively, are presented. The pK_a of the amino groups is 6.5 while for the carboxylic groups is 3.5. At pH 3 the amino groups are in their cationic state, as are protonated, while the carboxylic groups are not charged, as protonated. In the pH range between 4 and 6, either amino and carboxylic groups are in their ionic state and strong electrostatic interaction takes place making the hydrogel strong with the highest critical shear rate [40]. At neutral pH the deprotonation of the amino groups cause an unbalance in the charges with an increase in the negative one with a reduction in the electrostatic attractive forces and a decrease in the viscosity and critical shear rate. In alkaline media, pH 9, all the amino groups are in the neutral form while the carboxylic groups in the ionic form. In this condition, different forces are involved; the extent of the H-bonding between CS and ALG, which increases the viscosity, and the intramolecular repulsion between the ALG chains that decreases the viscosity. Herein, the chitosan is present in higher amount and the H-bonds are more effective with a subsequent increase in the viscosity.

Comparing the composite hydrogel to the hydrogel of pure chitosan, at the same pH the composite shows lower viscosity and critical shear rate that could be ascribed to the repulsion of the carboxylate ions in the composite hydrogel [41]. At neutral pH, the difference in viscosity is bigger, and composite shows a reduction around 100 times but shear rate raises resulting in increase in the composite elasticity [38]. The results indicates that at neutral pH the hydrogel viscosity is mainly due

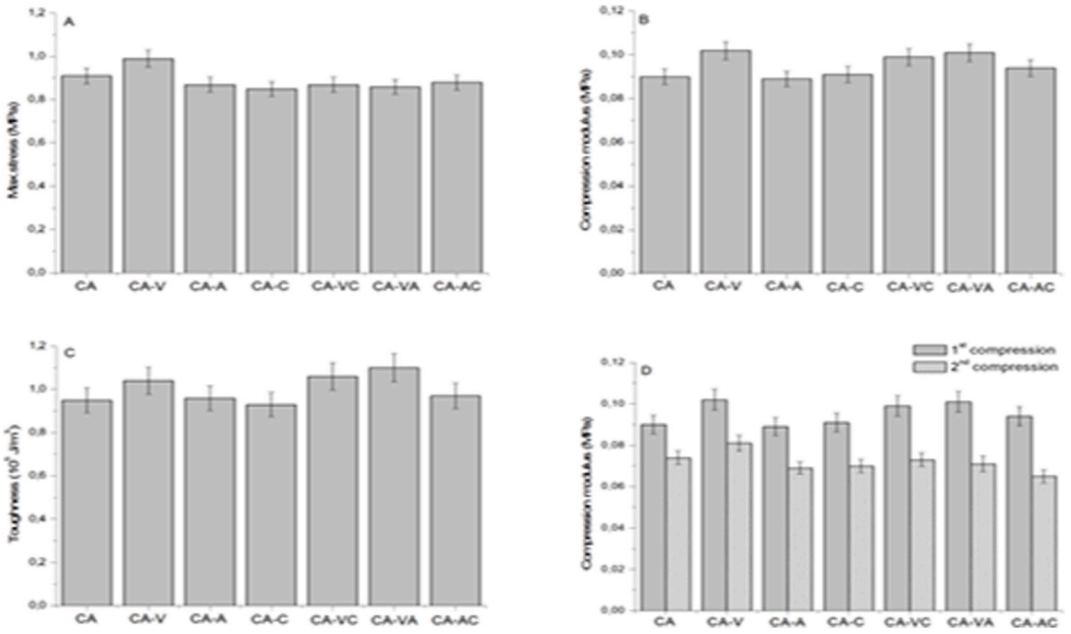

Fig. 9. Compressive properties of the chitosan-alginate hydrogels. A) maximum stress at 90% strain; B) compression modulus, C) toughness and D) compression modulus of the two compressions.

to the ionic interactions. Increase in elasticity while reduction in viscosity is an advantage for some biomedical application like in drug delivery [40].

The effect of the multiple antibiotic loading on the chitosan alginic acid and the composite was evaluated. The flow curves are reported in Figs. 6 and 7 for chitosan and alginate hydrogels, respectively. In Fig. 8, the flow curve of the composite demonstrates only a slight change compared to those of the drug free hydrogels suggesting a minor influence of the drugs on the hydrogels rheological properties at all the tested pH. Higher variations in the trend were observed for hydrogels formulations containing vancomycin, probably due to its chemical structure, which has higher molecular weight and the presence of functional groups available to be involved in H-bond with chitosan and alginate. Variation in the same percentage, around 4%, were observed for amoxicillin and ciprofloxacin loaded hydrogels.

Variations around 10% were observed in presence of simultaneous drug loading, with higher shift in the combination containing vancomycin.

The compressive properties of the composite hydrogels and the impact of the drug(s) loading were evaluated Fig. 9. The hydrogels demonstrate weak mechanical properties, probably due to the electrostatic repulsion between the excessive positive charges, coming from the excess of chitosan, which affects the electrostatic interactions between the opposite charges [42]. The hydrogels were not broken at 90% strain in the compression tests. Afterwards, the hydrogels were soaked in distilled water for 30 min and the second compression performed. After second compression, the modulus decrease indicating a loss to the resistance to fatigue.

The individual and dual loading of the drugs do not influence the compressive properties of CA hydrogels. Comparing all the formulation, those containing vancomycin demonstrate bigger variation comparing to the CA, but it does not exceed 5%.

3.5. Antibacterial activity and cytotoxicity

The antibacterial activity of the released antibiotic was evaluated by the disc diffusion assay. The control plate show a complete growth of the microorganism and absence of inhibition zone. In the plate containing the antibiotic loaded hydrogels, a clear inhibition zone is presented and it increases with the time due to the increase in the antibiotic concentration. In the double drug loaded hydrogels, the inhibition zone is slightly wider that those with a single one, confirming the antibacterial results obtained by MTT. The CA-V showed an increase in the inhibition zone from 5.3 mm after 30 min to 9.2 mm after 5 days. The inhibition zone referred to amoxicillin containing hydrogels raise from 4.7 mm after 30 min to 11.7 mm after 10 days. In ciprofloxacin loaded hydrogel

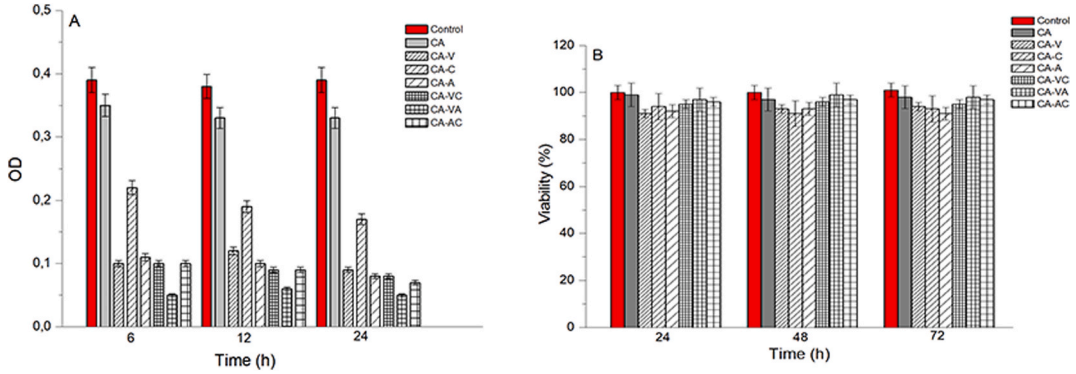

Fig. 10. A) OD and B) Cell viability referring to MTT of direct contact of the hydrogels with the cells culture. The values refer to the average value ± SD (n = 3).

Table 3
Cell viability data, by indirect evaluation up to 72 h. The data are reported as the average ± SD (n = 3). Control refers to cell culture.

Time (h)	Control	CA	CA-V	Viability (%) – 5% extract			CA-VA	CA-AC
				CA-C	CA-A	CA-VC		
24	101 ± 1	99 ± 1	96 ± 1	94 ± 2	98 ± 1	95 ± 2	98 ± 2	96 ± 3
48	100 ± 1	97 ± 2	97 ± 1	96 ± 1	96 ± 1	95 ± 1	99 ± 1	97 ± 1
72	102 ± 1	98 ± 1	97 ± 2	95 ± 1	93 ± 2	96 ± 1	97 ± 1	97 ± 1
				Viability (%) – 10% extract				
		CA	**CA-V**	**CA-C**	**CA-A**	**CA-VC**	**CA-VA**	**CA-AC**
24	101 ± 1	98 ± 1	95 ± 2	95 ± 2	93 ± 4	96 ± 2	94 ± 3	93 ± 2
48	100 ± 1	99 ± 1	96 ± 1	95 ± 1	94 ± 2	95 ± 2	92 ± 3	93 ± 3
72	102 ± 1	97 ± 1	96 ± 1	92 ± 1	91 ± 3	96 ± 1	92 ± 2	93 ± 1
				Viability (%) – 25% extract				
		CA	**CA-V**	**CA-C**	**CA-A**	**CA-VC**	**CA-VA**	**CA-AC**
24	101 ± 1	94 ± 1	93 ± 2	95 ± 1	92 ± 2	94 ± 1	92 ± 1	93 ± 1
48	100 ± 1	96 ± 1	93 ± 1	94 ± 2	92 ± 1	94 ± 1	91 ± 2	89 ± 3
72	102 ± 1	93 ± 2	92 ± 3	94 ± 2	91 ± 1	91 ± 1	91 ± 2	89 ± 3
				Viability (%) – 50% extract				
		CA	**CA-V**	**CA-C**	**CA-A**	**CA-VC**	**CA-VA**	**CA-AC**
24	101 ± 1	92 ± 1	91 ± 1	92 ± 2	88 ± 3	90 ± 1	89 ± 2	90 ± 1
48	100 ± 1	92 ± 2	90 ± 2	89 ± 2	88 ± 3	92 ± 3	88 ± 4	87 ± 2
72	102 ± 1	90 ± 2	90 ± 2	87 ± 3	86 ± 3	89 ± 1	88 ± 1	86 ± 2
				Viability (%) -75% extract				
		CA	**CA-V**	**CA-C**	**CA-A**	**CA-VC**	**CA-VA**	**CA-AC**
24	101 ± 1	90 ± 2	89 ± 2	85 ± 2	86 ± 1	85 ± 1	86 ± 1	87 ± 1
48	100 ± 1	87 ± 3	89 ± 2	85 ± 2	85 ± 2	85 ± 1	82 ± 1	83 ± 1
72	102 ± 1	88 ± 1	86 ± 2	84 ± 1	82 ± 1	83 ± 1	82 ± 1	83 ± 1
				Viability (%) -100% extract				
		CA	**CA-V**	**CA-C**	**CA-A**	**CA-VC**	**CA-VA**	**CA-AC**
24	101 ± 1	87 ± 1	85 ± 1	83 ± 2	82 ± 1	84 ± 2	83 ± 1	81 ± 2
48	100 ± 1	88 ± 1	84 ± 1	84 ± 2	83 ± 2	83 ± 1	80 ± 4	82 ± 1
72	102 ± 1	85 ± 4	84 ± 1	82 ± 2	81 ± 2	83 ± 1	80 ± 1	82 ± 2

the inhibition zone was 5.1 mm after 30 min and increase to 12.3 mm at the 15th day. The relation between the inhibition zone and the exposition time is in agreement with the release kinetics.

In the CA-VC; CA-VA and CA-AC the inhibition zone increased between 6 and 11% compared to the single loaded hydrogels. In summary, all the formulations showed antibacterial activity; in particular, those containing only vancomycin showed a short duration antibacterial activity while prolonged when in combination with amoxicillin or ciprofloxacin.

The hydrogels containing vancomycin (in combination with amoxicillin or ciprofloxacin) showed higher antibacterial effect overtime. It could be attribute to the action of vancomycin at the initial stage, as it is released quickly, followed by the contribution of the other compound (ciprofloxacin or amoxicillin) which is release with a slower rate. We assume that difference in release rate affect the duration of the antibacterial effect.

The inhibition zone of the standard, gentamicin, confirmed the antibacterial activities of the hydrogels loaded antibiotics.

The cytotoxicity of all formulations (CA; CA-V; CA-C; CA-A; CA-VA; CA-VC and CA-AC) was evaluated in human dermal fibroblast by MTT. Two approaches were used; by direct contact and by extract Fig. 10.

Data reported in Table 3 show that all formulations are cytocompatible as the viability is over 75% up to 72 h of incubation, at all the extract concentrations tested. In Fig. 10 the OD and MTT results are resumed.

3.6. Release kinetics

In Table 4 the content of antibiotics in the hydrogels prior to perform release tests are expressed in percentage (of the initial amount) and in mg per 100 mg of dried hydrogel.

The drug release kinetics are affected by several factors including composition, geometry, preparation technique of the hydrogels, and the surrounding environment during the release. For each factor, several physical and chemical phenomena are related which directly or indirectly influence the release. Among these factors, surface wettability, polymer and/or drug degradation, swelling, physical and chemical interactions between the polymers and the drugs and change in the shape and size of the hydrogel during the release process and other can be mentioned.

In the individual release patterns displayed in Fig. 11 three phases are observable; initial burst, sustained release and then a stationary phase. The only exception is observable for ciprofloxacin release at pH 4 (Fig. 11A). The intensity of the initial burst depends on the chemical properties of drug, the interaction with the hydrogel and the surrounding environment. All the used antibiotics are in the hydrochloride form; however, ciprofloxacin has lower solubility in all the tested media. The release of ciprofloxacin is slower due to the lower solubility in the media. Increasing the pH it is release faster.

In Fig. 12 the patterns referring to simultaneous delivery of antibiotics at different pH are reported. Considering each antibiotic singularly, and comparing the trends in Fig. 12 with Fig. 11 at same pH, not significant variation in the trend is observed. It probably suggests that when loaded in combination the antibiotics do not interact to each other or do not alterate the release mechanism of the other. The major factor affecting the release trend is the surrounding pH. The influence of the pH is observable in the increase in the release rate and the stationary phaseis reached faster. However, solubility of the drug is playing also a crucial role, but in the present case, considering the form of the antibiotics (hydrochloride and trihydrate) the considered pH do not alter the solubility of the drugs. The data reported in Table 5 refer to the cumulative amount, specified for each antibiotic, released at the 10th day from 100 mg of dried hydrogel.

The mg of antibiotic released after 10 days from 100 mg of dried hydrogels are resumed in Table 5. The data provide information about the effect of the pH on the individual and multiple of the antibiotics. The data, which refer to the cumulative amount of the drug released at the 10th day of exposition to the media, demonstrate that for all formulations, more than 80% is released, independently from the media pH and the type of loading (individual or multiple). Differences are in the order of microgram, which are not therapeutically significant.

Table 4
Encapsulation efficiency and content in mg of antibiotic(s) in 100 mg of dried hydrogel. V=Vancomycin; C= Ciprofloxacin; A = Amoxicillin. The data refer to the average value ± SD (n = 3).

Sample	Single loading		Sample	V	Multiple Loading			mg	A
	E.E. (%)	mg			E.E. (%)		V		
					C	A		C	
CA-V	92 ± 2	4.60 ± 0.10	CA-VC	87 ± 2	91 ± 2		4.35 ± 0.10	4.55 ± 0.10	
CA-A	95 ± 1	4.75 ± 0.05	CA-VA	95 ± 1		89 ± 1	4.75 ± 0.05		4.45 ± 0.05
CA-C	91 ± 1	4.55 ± 0.05	CA-AC		93 ± 1	92 ± 3		4.65 ± 0.05	4.60 ± 0.15

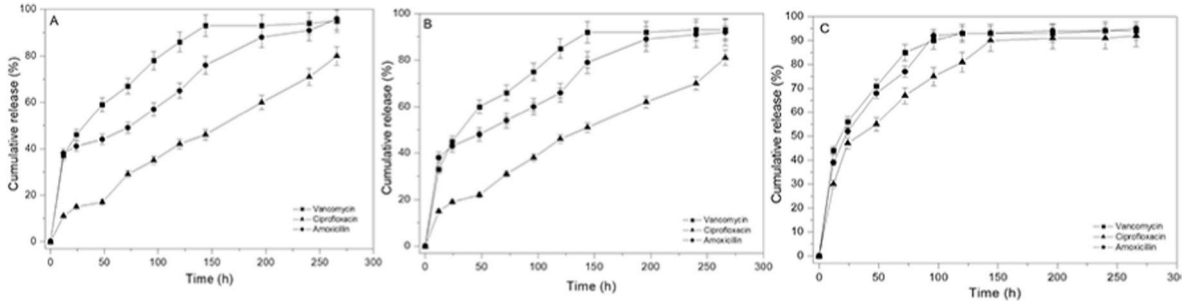

Fig. 11. Individual release patterns of antibiotic at A) pH 4; B) pH 7; C) pH 7.4. The values refer to the average value ± SD (n = 3).

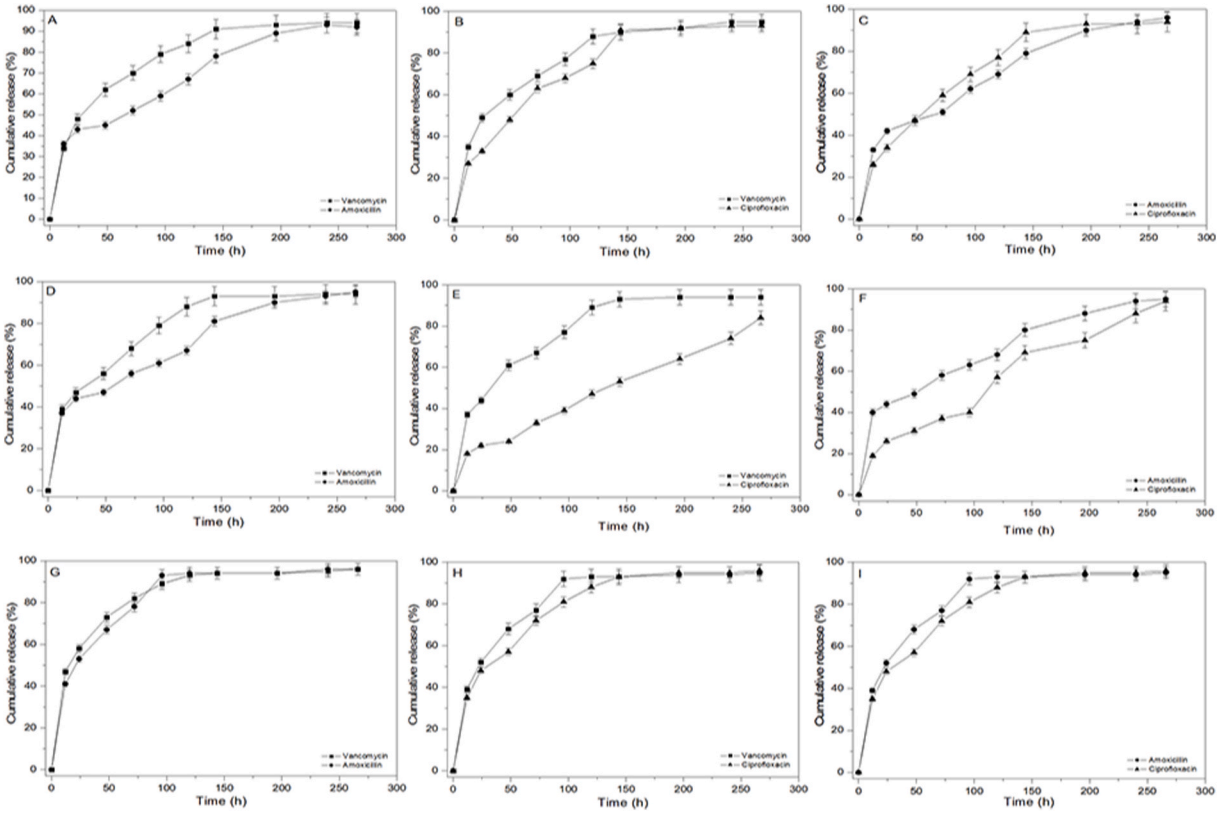

Fig. 12. Simultaneous release of the antibiotics from the hydrogels; A-C) pH 4; D-F) pH 7 and G-I) pH 7.4. The data are expressed as the average value ± SD (n = 3).

4. Conclusions

Hydrogel based on the combination of two polysaccharides, chitosan and alginic acid have been prepared by a simple, low cost and highly reproducible approach for the simultaneous delivery of different classes of antibiotics. Seven sets of hydrogels were prepared, three loaded with a single antibiotic, three with a combination of two antibiotics and one without drugs as control. All the formulations showed a porous structure with pore size and distribution not homogeneous due to the presence of the payload(s) and the drying process. The antibiotics were loaded during the preparation, and the encapsulation efficiency falls in the range 91–95% for single load and between 87 and 95% for double drug loading. Among all the formulations, a decrease in the encapsulation efficiency is observable for amoxicillin loaded in combination with

Table 5
Amount of antibiotic(s) released from the hydrogels individually and in combination. The data refer to 100 mg of dried formulations. The data are expressed as the average value ± SD (n = 3).

		mg antibiotic released per 100 mg of dried hydrogel within 10 days							
		Vancomycin		Ciprofloxacin			Amoxicillin		
pH	4.0	7.0	7.4	4.0	7.0	7.4	4.0	7.0	7.4
Individual									
CA-V	4.37 ± 0.21	4.32 ± 0.26	4.37 ± 0.12	–	–	–	–	–	–
CA-C	–	–	–	3.23 ± 0.11	4.14 ± 0.09	3.18 ± 0.16	–	–	–
CA-A	–	–	–	–	–	–	4.32 ± 0.13	4.46 ± 0.19	4.32 ± 0.09
Simultaneous									
CA-VA	4.46 ± 0.11	4.47 ± 0.17	4.51 ± 0.18				4.13 ± 0.28	4.11 ± 0.13	4.27 ± 0.25
CA-VC	4.13 ± 0.09	4.08 ± 0.17	4.12 ± 0.11	4.23 ± 0.05	3.36 ± 0.31	4.23 ± 0.16			
CA-AC				4.32 ± 0.14	4.09 ± 0.31	4.32 ± 0.27	4.32 ± 0.18	4.31 ± 0.13	4.41 ± 0.08

vancomycin and for vancomycin combined with ciprofloxacin compared to the singleloaded formulations. The prepared hydrogels demonstrate a direct correlation of the swelling ratio with the time and pH. However, the equilibrium is reached within 60 min in all the samples. The maximum swelling ratio at the equilibrium was recorded at pH 7.4 in all formulations, while the lowest at pH 4. No effect of the drugs was observed on the swelling behavior. The *in vitro* degradation studies demonstrate a pH-dependent trend. At pH 4 between a reduction in the hydrogel between 59 and 76% was observed while almost 94% at pH 7.4 in the same time interval.

Results from antibacterial tests performed on *S. Aureus* shown an increase in the bacterial growth inhibition when antibiotics were administered using the hydrogel compared to the antibiotic in the free form used as references. In the double drugs loading antibiotics, an increase between 6% and 11% in the inhibition zone was observed compared to the single-loaded due to a possible synergic effect. Among all formulations, those containing vancomycin results in higher efficacy. The relations between the time of inhibition growth and the release kinetics of the drugs are in accordance. The CA. hydrogel cytocompatibility was evaluated by direct contact with the cells culture and by extract method. In both approaches, the hydrogel resulted cytocompatible as cell viability was over 75%.

Data coming from the simultaneous and individual release of the antibiotics describe a three phase's pattern for all formulations except for Ciprofloxacin at pH 4. The three phases are characterized by an initial burst followed by a sustained release and a stationary phase. The intensity and duration of each phase are affected by the surrounding pH. All drugs are released faster when the pH increase, but in all after 10 days over 80% of the total amount loaded was released.

Comparing the individual release patterns with them in combination, no essential differences are observable, suggesting that the drugs do not interact physically or chemically with each other and do not affect the interactions between polymer matrix and surrounding environment.

CRediT authorship contribution statement

Yelena A. Khan: Writing - original draft, Investigation. **Kadir Ozaltin:** Investigation, Methodology. **Andres Bernal-Ballen:** Data curation, Visualization, Writing - review & editing. **Antonio Di Martino:** Conceptualization, Data curation, Methodology, Investigation, Supervision, Validation, Writing - review & editing, Visualization, Project administration.

Declaration of competing interest

None.

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