European Journal of Pharmaceutics and Biopharmaceutics 74 (2010) 248–254

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

journal homepage: www.elsevier.com/locate/ejpb

Research paper

Thermally sensitive gels based on chitosan derivatives for the treatment of oral mucositis

S. Rossi [a,*], M. Marciello [a], M.C. Bonferoni [a], F. Ferrari [a], G. Sandri [a], C. Dacarro [b], P. Grisoli [b], C. Caramella [a]

[a] Department of Pharmaceutical Chemistry, University of Pavia, Pavia, Italy
[b] Department of Experimental and Applied Pharmacology, University of Pavia, Pavia, Italy

ARTICLE INFO

Article history:
Received 17 June 2009
Accepted in revised form 20 October 2009
Available online 23 October 2009

Keywords:
Thermally sensitive mucoadhesive gels
Trimethyl chitosan
Methylpyrrolidinone chitosan
Benzydamine hydrochloride
Oral mucositis

ABSTRACT

The aim of the present work was the development of a thermally sensitive mucoadhesive gel based on chitosan derivatives for the treatment of oral mucositis. Trimethyl chitosan (TMC) and methylpyrrolidinone chitosan (MPC) were considered. They were mixed with glycerophosphate (GP) according to different polymer/GP molar ratios and characterized for gelation properties by means of rheological analysis in comparison with chitosan. The influence of molecular weight and substitution degree (SD) of TMC on gelation temperature and time was investigated. The mucoadhesive properties of the mixtures were also assessed using porcine buccal mucosa. The best properties were shown by TMC with high MW and low SD mixed with GP according to 1:2 molar ratio. Such mixture was loaded with benzydamine hydrochloride, an anti-inflammatory drug with antimicrobial properties and subjected to in vitro drug release and wash away test. The formulation based on TMC/GP mixture was able to prolong drug release and to withstand the removal physiological mechanisms. The antimicrobial properties of both vehicle and formulation were investigated. Also in absence of drug, TMC/GP mixture was characterized by antimicrobial properties.

1. Introduction

Recently chitosan, a high-molecular weight cationic polysaccharide derived from chitin, has been studied as thermally sensitive gel-forming agent in mixture with polyol salts. In particular, novel in situ gel systems based on chitosan combined with β-glycerophosphate (GP) or glyceryl monooleate were developed to deliver drugs via parenteral as well as oral routes [1–4].

The mechanism of thermogelation in chitosan/GP mixtures was supposed to be the result of a strengthening of hydrophobic interactions with increasing temperature [1]. Recently, some authors proposed an alternative hypothesis for the mechanism of gelation in chitosan-GP systems: heat induces transfer of protons from chitosan to glycerophosphate, thereby neutralizing chitosan and allowing attractive interchain forces to form a physical gel [5].

In the last years, many authors have studied the mucoadhesive properties of chitosan, which are markedly affected by environmental pH [6–9]. The effectiveness of chitosan as mucoadhesive agent is impaired by its insolubility at pH above 6 (for example at the physiological pH of the buccal cavity). To solve chitosan solubility problems, chitosan derivatives like methyl pyrrolidinone chitosan (MPC) – characterized by a partial substitution of the primary amino groups with methylpyrrolidinone groups, and N-trimethyl chitosan (TMC) – in which the primary amino units are partially substituted with methyl groups – were synthesized. Such polymers demonstrated to possess higher mucoadhesive properties towards buccal mucosa with respect to chitosan, due to their higher aqueous solubility at buccal pH and, in the case of TMC, to a higher charge density that corresponds to a greater interaction with the sialic acid residues of mucin [10,11].

Recently, chitosan and derivatives were also recognized as antimicrobial and wound-healing agents [12–14]. In particular, some authors prepared and characterized for their antimicrobial properties some chitosan derivatives with quaternary amine groups such as trimethyl chitosans. They found that the antibacterial activity of quaternized chitosans was stronger than that of chitosan [13].

Given these premises, the aim of the present work was the development of a thermally sensitive mucoadhesive gel based on chitosan derivatives for the treatment of oral mucositis induced by chemo-radiotherapy. The characteristic lesions of the mucositis hit the whole buccal mucosa and are often accompanied by bacterial and fungal infections. This pathology is very severe and significantly affects patient life quality. Few interventions are of proven efficacy and there are no universally accepted treatment protocols. The increasing recognition of the relevance of this affection prompts further research on this topic.

* Corresponding author. Department of Pharmaceutical Chemistry, University of Pavia, Viale Taramelli 12, 27100 Pavia, Italy. Tel.: +39 0382 987357; fax: +39 0382 422975.
E-mail address: silvia.rossi@unipv.it (S. Rossi).

0939-6411/$ - see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejpb.2009.10.003

The formulation developed should be characterized by:

- low viscosity at room temperature in order to permit an easy application onto diseased mucosa,
- gel-structure and then high viscosity at 37 °C to withstand physiological removal mechanism which washed away the formulation from the action site,
- capability to adhere to buccal mucosa i.e. mucoadhesive properties to permit a prolonged drug permanence onto mucosa lesions,
- controlled drug release properties to avoid repeated administrations and to improve patient compliance.

Two different chitosan derivatives were considered: trimethyl chitosan (TMC) and methylpyrrolidinone chitosan (MPC). They were mixed with glycerophosphate (GP) according to different polymer/GP molar ratios. In the case of TMC, polymers having different molecular weights and substitution degrees were considered.

The best polymer/GP mixture in terms of gelation and mucoadhesion properties was loaded with benzydamine hydrochloride, an anti-inflammatory drug with antimicrobial properties used in the treatment of oral mucositis. Such formulation was characterized in vitro for drug release and for its capability to withstand the removal action of saliva. The antimicrobial properties of both vehicle and formulation were investigated also in presence of albumin, to mimic the administration in presence of open wounds.

2. Experimental part

2.1. Materials

Chitosan hydrochloride having different molecular weights (CSH (MW: 580 kDa; DD: 90%) and CSL (MW: 300 kDa; DD: 92.1%)) were purchased from Sigma, Milan, I. They were used to obtain trimethyl derivates with high-molecular weight and different substitution degree (TMCH1 (MW: 580 kDa, DD: 90%, SD: 3%), TMCH2 (MW: 580 kDa; DD: 90%; SD: 78%) and trimethyl derivatives with low molecular weight and different substitution degree (TMCL1 (MW: 300 kDa; DD: 92.1%; SD: 3%), TMCL2 (MW: 300 kDa; DD: 92.1%; SD: 78%)). The trimethyl derivatives were synthesized by Prof. G. Di Colo, Department of Biorganic and Biopharmaceutic Chemistry, University of Pisa, I according to [16].

5-Methyl pyrrolidinone chitosan (MPC) (MW: 200 kDa; DD: 80%; SD: 27%) was provided by Prof. Riccardo A.A. Muzzarelli, Centre of Innovative Biomaterials, Faculty of Medicine, University of Ancona, I.

Sodium β-Glycerophosphate (GP) (Fluka, Milan, I) was used as gelation agent.

Benzydamine hydrochloride (Sigma, Milan, Italy) was used as model drug.

2.2. Preparation and characterization of TMC/GP and MPC/GP solutions

2.2.1. Preparation of TMC/GP and MPC/GP solutions

Aqueous solutions containing polymer and GP mixed in different molar ratios were prepared (Table 1). Solutions were obtained dropwising an aqueous solution of glycerophosphate to an aqueous chitosan solution, maintained at 4 °C under stirring. The samples were stored at 4 °C before testing. Different polymer concentrations were used in order to obtain solutions having viscosity values at 100 s^{-1} and 4 °C ranging between 0.1 and 0.2 Pa s. Such viscosity range was chosen since compatible with an easy vaginal administration.

Table 1
Polymer concentrations and polymer:GP molar ratios employed for the preparation of the mixtures.

Polymer	Polymer conc. (% w/w)	Polymer:GP molar ratio[a]
TMCH1 MW: 580 kDa, DD: 90%, SD: 3%	3%	1:0.5
		1:1
		1:2
TMCH2 MW: 580 kDa; DD: 90%; SD: 78%	6%	1:2
TMCL1 MW: 300 kDa, DD: 92.1%, SD: 3%	2.5%	1:2
TMCL2 MW: 300 kDa, DD: 92.1%, SD: 78%	5%	1:2
CSH MW: 580 kDa DD: 90%	2%	1:2
CSL MW: 300 kDa; DD: 92.1%	2%	1:2
MPC MW: 200 kDa, DD: 80%; SD: 27%	1.5%	1:0.5
		1:1
		1:2

[a] For the calculation of polymer:GP molar ratios, moles of deacethylated and derivatized amine groups have been considered.

2.2.2. Rheological measurements

The rheological analysis was carried out by means of a rotational rheometer (Rheostress RS600, Haake, Karlsruke, *G*), equipped with a cone plate combination (C35/1: ∅ = 35 mm; angle = 1°) as measuring system.

In particular, polymer/GP solutions were subjected to oscillation measurements, which provide to apply a constant shear stress value (chosen in the linear viscoelastic region, previously determined) and to measure the viscoelastic response of the sample expressed by the storage (G') and loss (G'') moduli. Three independent variables were considered: frequency of the stress applied, temperature and time. Oscillation measurements were performed:

- at a constant value of frequency (1 Hz) and at temperature values ranging between 5 and 50 °C (heating rate: 1 °C/min), to evaluate the gelation temperature of the samples (thermostatation time: 180 s);
- at constant values of temperature (37 °C) and frequency (1 Hz) and at increasing times, to determine the gelation rate of the sample at the physiological temperature (thermostatation time: 0 s);
- at a constant value of temperature (37 °C) and at increasing frequency values ranging between 0.1 and 10 Hz to evaluate the strength of the gel (thermostatation time: 900 s).

2.2.3. Mucoadhesion measurements

Mucoadhesive properties were determined at 37 °C by means of a tensile stress tester previously described [9]. Porcine buccal mucosa was employed as biological substrate. 100 mg of each polymer/GP mixture were layered on a filter paper disc (area = 2 cm^2) that was fixed by means of a biadhesive tape on the movable carriage of the apparatus. The mucosa was fixed, faced to the formulation, on the sample holder using a cyanoacrylate glue and hydrated with 100 μl of pH 6.4 buffer. A preload of 2500 mN was applied in order to allow the formation of the mucoadhesive joints. After a 3-min rest, the preload was removed, and the movable carriage was moved at a constant speed (4 mm/min) up to the complete separation of the two surfaces. Both displacement of the movable carriage and force of detachment data were recorded and simultaneously collected on a personal computer.

2.3. Preparation and characterization of TMCH1/GP solutions loaded with benzydamine hydrochloride

2.3.1. Preparation of TMCH1/GP solution loaded with benzydamine hydrochloride

Benzydamine hydrochloride was added at 0.15% w/w concentration to TMCH1 aqueous solution. Afterwards, GP was added

according to 1:2 polymer:GP molar ratio. Rheological behaviour of the sample was assessed as described in Section 2.2.2.

Drug concentration employed was comparable to that present in commercial products for the treatment of mouth and throat inflammation.

2.3.2. Release measurements

In vitro drug release was assessed by means of Franz diffusion cells (FDC40020FF, Crown Bio Scientific Inc., Clinton,) (Permeager, USA) [15]. The donor and receptor chambers were separated by a dialysis membrane (cut-off 12–14 kDa) in order to avoid polymer passage into receptor chamber. Before its use, the membrane was boiled in distilled water for 10 min. Two hundred and fifty milligrams of each polymer solution were spread on a filter paper disc, applied on the dialysis membrane. Phosphate buffer pH 6.4 (USP 31), thermostated at 37 °C, degassed and filtered, was used as receptor phase. At fixed time intervals, 500 μl of receptor phase were withdrawn, and drug amount was dosed by a HPLC method [17].

2.3.3. "Wash away" measurements

A modified Franz diffusion cell was used [15]. Five hundred milligrams of each formulation was layered on porcine buccal mucosa, used as model substrate. To mimic the removing action of the saliva on the formulation, 20 ml of phosphate buffer at pH 6.4 thermostated at 37 °C was continuously fluxed over the formulation by means of an HPLC pump (Gynkotek, mod. 300), at a constant rate of 0.7 ml/min (closed system). At fixed time intervals, 500 μl of the buffer was withdrawn and replaced with fresh buffer. The drug was assayed by a HPLC method [17].

2.3.4. Antimicrobial activity measurements

The antimicrobial activity of the TMCH1/GP mixture, medicated or not, was evaluated against the bacterial strains *Staphylococcus aureus* ATCC 6538, *Streptococcus pyogenes* ATCC 19615, *Streptococcus vestibularis* ATCC 49124 and against the fungal strain *Candida albicans* ATCC 10231. Minimum inhibitory concentration (MIC) and minimum bactericidal/fungal concentration (MBC/MFC) values of benzydamine hydrochloride were determined by the standard broth macrodilution method in Iso Sensitest Broth (ISB, Oxoid, Basingstoke, Hampshire, England) with an average inoculum of 10^7 CFU/ml as described by the Clinical and Laboratory Standards Institute (CLSI; formerly NCCLS) [18,19]. MIC was defined as the lowest concentration that completely inhibited bacterial growth after an incubation of 24 h at the temperature of 37 °C and fungal growth after 48 h at 25 °C. Minimum bactericidal concentration (MBC) and minimum fungicidal concentration (MFC) were evaluated by inoculating aliquots of culture medium in which the inhibition of cellular proliferation was observed. MBC/MFC was defined as the lowest concentration that kills the microbial cells.

The antimicrobial activity of TMCH1/GP 1:2 mixture was evaluated in presence and in absence of benzydamine hydrochloride. Drug concentration was, depending on the microorganism considered, about 1–4 times higher than MIC values. Bacteria were grown overnight for 18–20 h in Tryptone Soya Broth (Oxoid, Basingstoke, Hampshire, England) at 37 °C while *C. albicans* was grown in Potato Dextrose Broth (DIFCO, Detroit, USA) for 24 h at 25 °C. The bacteria cultures were centrifuged at 3000 rpm for 20 min to separate cells from broth and then suspended in phosphate buffer saline (PBS, pH 7.3). The suspension were diluted to adjust the number of cells to 1×10^7–1×10^8 CFU/ml.

Aliquots (500 μl) of the microbial suspensions were added to 500 μl of the polymer solution to obtain 1:1 solution. For each microorganism employed, a suspension was prepared in PBS without polymer solution and used as control. 1:1 solutions and controls were incubated at 37 °C. Viable microbial counts were evaluated after contact for 0, 2, 4, 6 and 24 h with the polymer solution and in control suspensions; bacterial colonies were enumerated in Tryptone Soya Agar (Oxoid, Basingstoke, Hampshire, England) after incubation at 37 °C for 24 h, *C. albicans* colonies in Sabouraud Dextrose Agar (SAB, Oxoid, Basingstoke, Hampshire, England) at 25 °C for 48 h.

The microbicidal effect (ME value) was calculated for each test microorganism and contact time according to the following equation [20]:

$$ME = \log Nc - \log Nd \quad (1)$$

where Nc is the number of CFU of the control microbial suspension and Nd is the number of CFU of the microbial suspension in presence of the polymer solution.

Tests were also performed in presence of bovine serum albumin (Sigma–Aldrich, I) to mimic "dirty conditions" like those occurred in vivo in presence of open wounds. In particular, albumin has been added at the concentrations of 0.3% and 3% w/w to microbial suspension.

3. Results and discussion

3.1. Characterization of TMC/MPC–GP solutions

3.1.1. Rheological measurements

In Fig. 1, G' vs. temperature values for TMCH1 solution in presence of different amounts of GP are reported. All the mixtures considered start to gelify at temperature close to 35 °C; such a behaviour is compatible with an in situ gelation after buccal administration. The gelation temperature is evidenced by a steep increase in G' values on increasing temperature. As reported in Fig. 2, an increase in GP amount corresponds to a decrease in loss tangent values measured at 37 °C. Since loss tangent is calculated as the ratio between G" and G', lower values of such parameter indicates the formation of a more elastic gel.

In Fig. 3, G' vs. temperature profiles observed for the solutions of GP and TMCs characterized by high MW and different substitution degree (SD) (TMCH1 e TMCH2) and for a solution of the high MW chitosan (CSH) used to synthesize trimethylated polymers are compared. A polymer/GP molar ratio equal to 1:2 was considered. CSH and TMCH2 (high MW and high SD) are not able to gelify at physiological temperature when mixed with GP according to a molar ratio equal to 1:2. Such a behaviour could be due to the influence of polymer charge density and hydrophilicity on polymer capability to undergo gelation. They have opposite effects: an

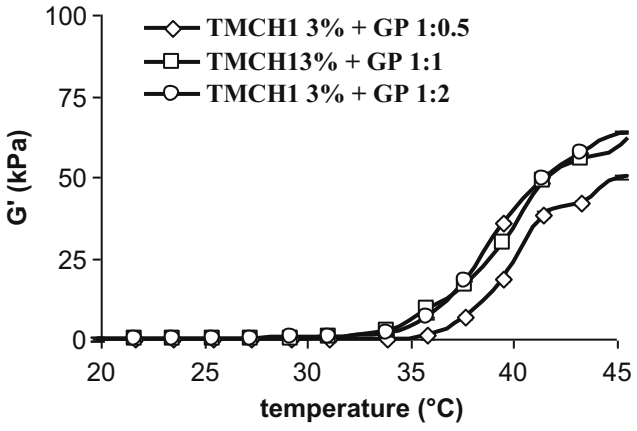

Fig. 1. G' vs. temperature profiles observed for the solutions of trimethyl chitosan with high MW and low SD (TMCH1: MW: 580 kDa, DD: 90%, SD: 3%) mixed in different molar ratios with glycerolphosphate (GP) (mean values ± se; $n = 3$).

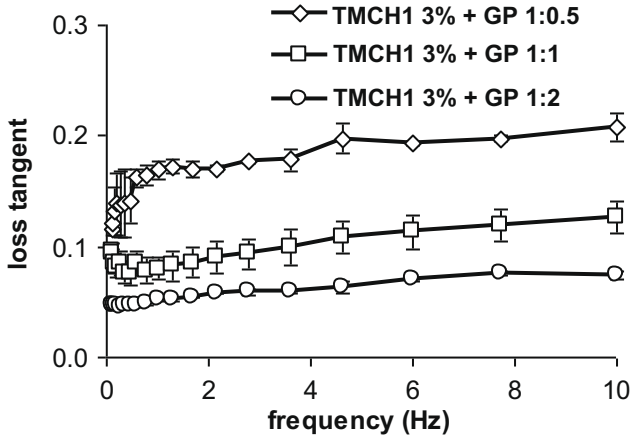

Fig. 2. Loss tangent values observed at 37 °C for the solutions of trimethyl chitosan with high MW and low SD (TMCH1: MW: 580 kDa, DD: 90%, SD: 3%) mixed in different molar ratios with glycerolphosphate (GP) (mean values ± se; $n = 3$).

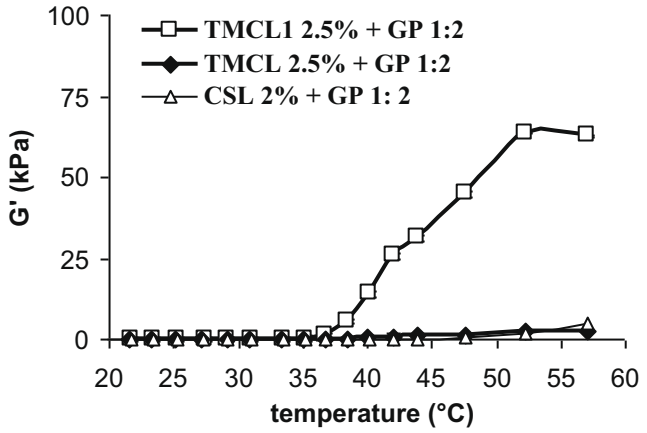

Fig. 4. G' vs. temperature profiles observed for the solutions of glycerolphosphate (GP) and trimethyl chitosans characterized by low MW and different SD (TMCL1 (SD: 3%) and TMCL2 (SD: 78%)) and for the solution of the low MW chitosan (CSL) used to synthesize trimethylated polymers (mean values $i \pm$ se; $n = 3$).

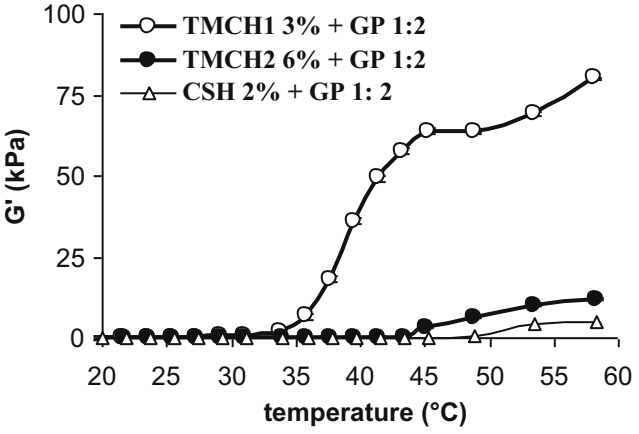

Fig. 3. G' vs. temperature profiles observed for the solutions of glycerolphosphate (GP) and trimethyl chitosans characterized by high MW (580 kDa) and different SD (TMCH1 (SD: 3%) and TMCH2 (SD: 78%)) and for the solution of the high MW chitosan (CSH) used to synthesize trimethylated polymers (mean values ± se; $n = 3$).

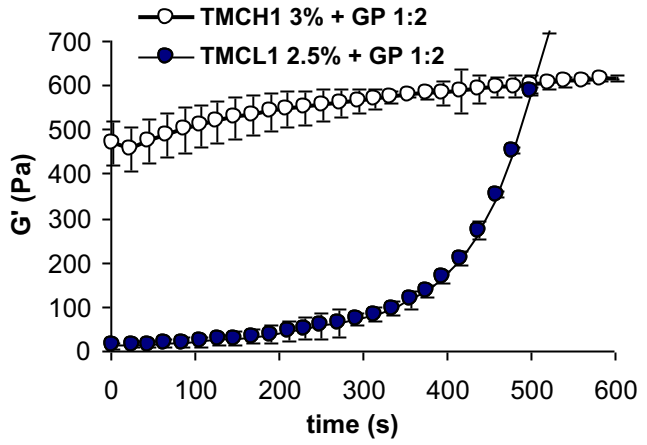

Fig. 5. G' vs. time profiles observed for the solutions of trimethyl chitosan with high MW and low SD (TMCH1: MW: 580 kDa, SD: 3%) and trimethyl chitosan with low MW and low SD (TMCL1: MW: 300 kDa, SD: 3%) mixed with glycerolphosphate (GP) (1:2 polymer:GP molar ratio) (mean values ± se; $n = 3$).

increase in charge density obtained by quaternization is likely to produce a higher interaction with the anionic GP and then favours gelation; on the other hand the increase in polymer hydrophilicity due to quaternization hinders gelation. This is the case of TMCH2, for such polymer the effect of the increased hydrophilicity prevails on that of charge density. Moreover, for such polymer interchain repulsion due to the high cationic charge can hinder polymer gelation, even if the high amount of GP should make less relevant such an effect.

In Fig. 4, G' vs. temperature profiles observed for the solutions of GP and TMCs characterized by low MW and different SD (TMCL1 and TMCL2) and for a solution of the low MW chitosan (CSL) used to synthesize trimethylated polymers are compared. Also for these polymers, the polymer/GP molar ratio employed was 1:2. Analogously to that observed for TMCH polymers, TMCL with higher SD does not gelify at physiological temperature values.

Beside the gelation temperature, another factor to be considered for buccal administration of drugs is the gelation time; it should be as short as possible to avoid a quick removal of the polymer solution by saliva.

Fig. 5 shows G' profiles as a function of time for the solutions based on TMCH1 and TMCL1 mixed with GP according to 1:2 molar ratio. It can be seen that the solution based on TMCH1 gelifies in few seconds as evidenced by the high values of G' at the beginning of the measure. On the contrary, the solution based on TMCL1 is characterized by an exponential increase in G': it means a slow gelation of the sample.

In Fig. 6, G' vs. temperature values of MPC solutions in presence of different amounts of GP are reported. In the insert, the same graph with an expanded y scale is reported to better compare the behaviour of the mixtures based on 1:0.5 and 1:1.5 polymer/GP molar ratios. An increase in the gelation temperature is observed on the decrease in GP amounts. In particular, the solution based on MPC and GP mixed according to 1:2 molar ratio start to gelify at 33 °C, while for MPC solutions based on 1:0.5 and 1:1.5 molar ratios gelation occurs at temperature values higher than the physiological one.

Fig. 7 shows G' vs. time values observed for MPC/GP mixture prepared according to 1:2 molar ratio. The mixture is characterized by a slow gelation as indicated by a slow increase in G' that is again increasing after 40 min. This behaviour is probably due to the low MW of the polymer. The same behaviour was shown by TMC characterized by low SD and low MW. Such polymer, when mixed with

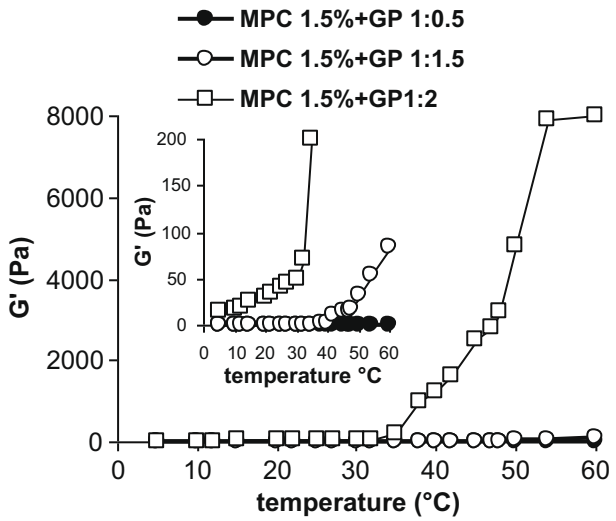

Fig. 6. G' vs. temperature profiles observed for methylpyrrolidinone chitosan (MPC) solutions (1.5% w/w), mixed with glycerolphosphate (GP) in different molar ratios (mean value ± se; $n = 3$). In the insert the same graph with an expanded y scale is reported.

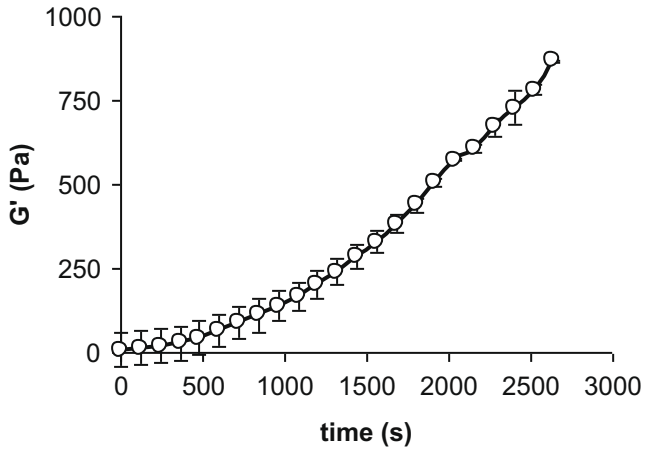

Fig. 7. G' vs. time profiles observed for methylpyrrolidinone chitosan (MPC) and glycerolphosphate (GP) (1:2 polimer/GP molar ratio) (mean value ± se; $n = 3$).

GP, is able to gelify at physiological temperature but more slowly with respect to the high MW grade.

3.1.2. Mucoadhesion measurements

In Fig. 8, the values of the mucoadhesion parameter work of adhesion are reported for all the polymer/GP mixtures considered. The addition of increasing amounts of GP to TMCH produces an increase in the mucoadhesive potential of the polymer, probably due to the higher consistency of the mixture at the physiological temperature. In the case of TMCH, the increase in the substitution degree produces a decrease in mucoadhesive properties. The mixtures based on TMCH1 and MPC show the highest work of adhesion values indicating the best mucoadhesive performance. TMCH1 mixed with GP according to 1:2 molar ratio is characterized by higher mucoadhesive potential with respect to high MW chitosan mixed with the same amount of GP. No significant differences (ANOVA one way) are observed between the work of adhesion values of the two TMCL characterized by different SD and the relating low MW chitosan.

3.2. Characterization of TMCH1/GP solution loaded with benzydamine hydrochloride

3.2.1. Rheological measurements

The presence of benzydamine at 0.15% w/w concentration does not produce any change in the rheological behaviour of TMCH1/GP mixture (data not shown).

3.2.2. Release and washability measurements of benzydamine hydrochloride

Fig. 9 shows the release profile of benzydamine loaded in TMCH1/GP 1:2 mixture. The gelation of the solution at 37 °C permits to control drug release. In fact, after 5 h a drug amount close to 70% of the drug loaded is released.

In Fig. 10, the percentage of drug "washed away" vs. time profile observed for the mixture TMCH1/GP 1:2 is reported. The results obtained from "wash away" measurements prove that the gel formed at 37 °C withstands the removing action of the saliva. In fact after 5 h %, drug amount remained on the mucosa was about 40%; this indicates a prolonged permanence of the formulation on the mucosa.

3.2.3. Antimicrobial activity measurements

In Table 2, MIC and MBC/MFC values of benzydamine hydrochloride against *S. aureus*, *S. pyogenes*, *S. vestibularis* and *C. albicans* are reported. As for inhibitory activity similar results are obtained for the different microorganisms with the exception of *S. aureus*. Such microorganism results less sensitive to the drug. Benzydamine hydrochloride shows higher bactericidal activity against the two streptococci with respect to *S. aureus*. Benzydamine hydrochloride shows also antifungal activity.

In Table 3, the microbicidal effect values of TMCH1/GP 1:2 mixture in absence and in presence of drug are reported as a function of contact time. The mixture without drug produces a valuable reduction of bacterial count after 24 h; it is characterized by higher ME values for the two streptococci with respect to *S. aureus*. The mixture is inactive against *C. albicans*: a reduction of vital microorganisms number is not observed for contact time up to 6 h.

The TMCH1/GP mixture loaded with drug shows a marked disinfectant effect against bacteria and *C. albicans*; in particular a complete inactivation of bacterial cells is observed after 2 h for *S. pyogenes* and after 4 h for *S. vestibularis* and *S. aureus*; for *C. albicans* the complete inactivation is observed after a contact of 6 h.

To simulate drug administration in presence of open wounds, antimicrobial activity of the mixture loaded with benzydamine hydrochloride was determined in the presence of albumin. In particular, two different albumin concentrations (0.3% and 3% (w/v)) were considered.

In Table 4, the microbicidal effect values obtained in presence of 0.3% and 3% (w/v) of albumin for TMCH1/GP 1:2 mixture loaded with the drug are reported.

The disinfectant action of the mixture against *S. aureus* is evident after 24 h with a ME value close to 3; on the contrary for *S. pyogenes* there is a complete inactivation of bacterial cells just after 4 h. *S. vestibularis* is less sensitive against benzydamine hydrochloride in the presence of albumin at the concentration of 0.3% w/v, also *C. albicans* is less sensible in the presence of albumin, ME value is in fact 2.65 after 24 h.

The increase in albumin concentration produces a further decrease in microbicidal activity of the mixture. *S. aureus* is viable also after 24 h contact; the complete inactivation of *S. pyogenes* is observed after 6 h contact, while a significant ME is observed for *S. vestibularis* and *C. albicans* after 24 h contact.

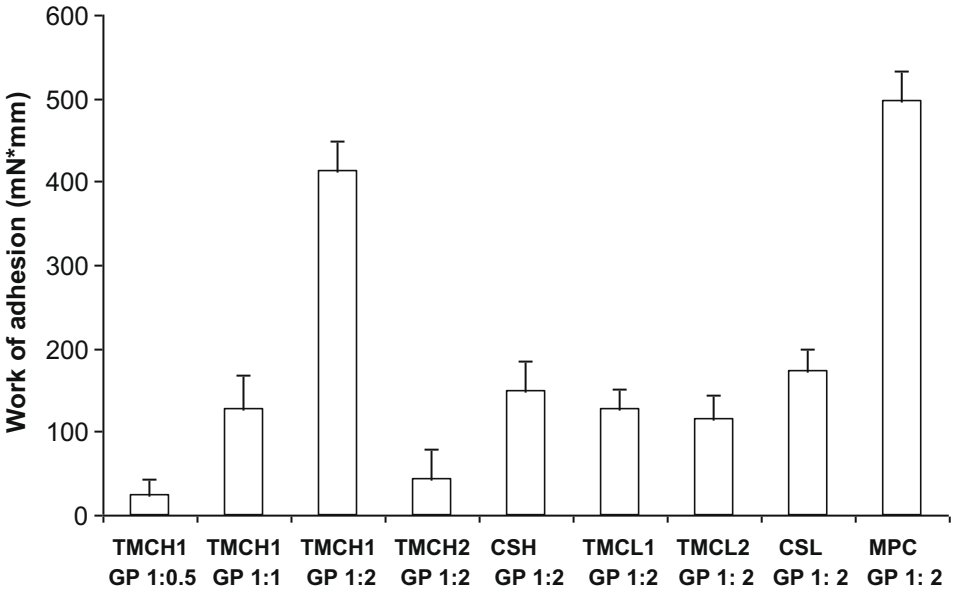

Fig. 8. Values of adhesion work observed for all the polymer/ glycerolphosphate mixtures (mean values ± se; $n = 9$).

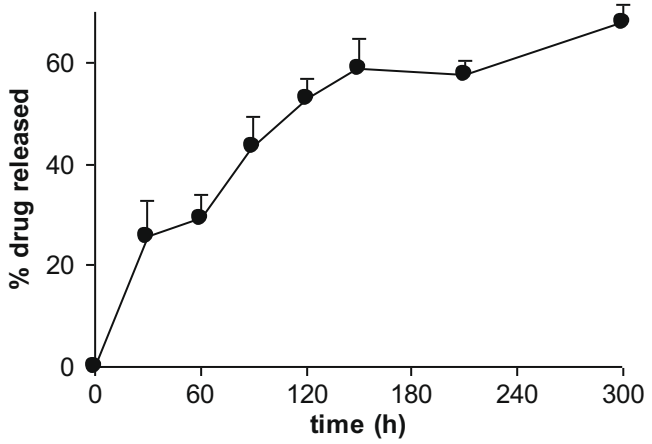

Fig. 9. % Drug released profile observed for the mixture TMCH1/ glycerolphosphate 1:2 mixture loaded with benzydamine hydrochloride (mean values ± se; $n = 6$) TMCH1: trimethyl chitosan MW: 580 kDa, DD: 90%, SD: 3%.

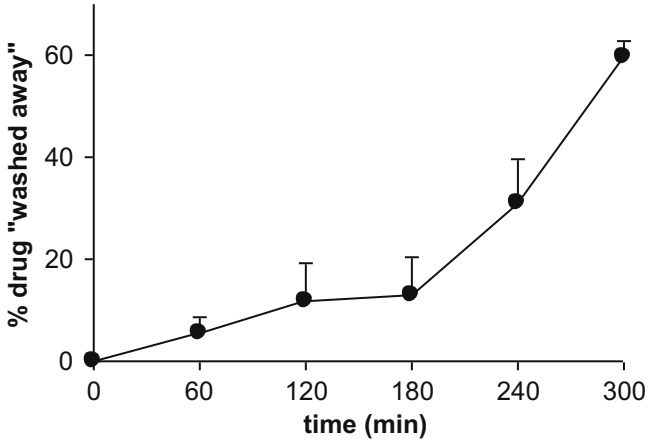

Fig. 10. % Drug "washed away" profile observed for the mixture TMCH1/ glycerolphosphate 1:2 mixture loaded with benzydamine hydrochloride (mean values ± se; $n = 6$) TMCH1: trimethyl chitosan MW: 580 kDa, DD: 90%, SD: 3%.

Table 2
Minimum inhibitory concentration (MIC) and mininimum bactericidal/fungicidal concentration (MBC/MFC) values of benzydamine hydrochloride.

Strain	MIC (μg/ml)	MBC/MFC (μg/ml)
Staphylococcus aureus	600	800
Streptococcus pyogenes	250	250
Streptococcus vestibularis	200	250
Candida albicans	250	500

4. Conclusions

Both chitosan derivatives show a higher propensity to gelify at physiological temperature when mixed with glycerophosphate (GP) with respect to chitosan. The gelation temperature of TMC/GP mixtures is affected by polymer/GP molar ratio and by the substitution degree of the polymer. At the physiological temperature, only TMCs characterized by a low substitution degree (TMCH1 and TMCL1) undergo gelation. This is probably due to the high hydrophilicity of the polymers characterized by a high substitution degree. The presence of glycerophosphate is not sufficient to produce desolvatation and then gelation of the polymer. A stronger gel is obtained when GP was mixed according to polymer:GP molar ratio equal to 1:2.

The gelation time is affected by the molecular weight of TMC: the low MW TMC (TMCL1) is characterized by a slow gelation, while the high MW TMC (TMCH1) shows an instantaneous gelation at 37 °C. Moreover, such a polymer shows, upon gelation, the best mucoadhesive properties.

Release and wash away measurements proved that the formulation based on TMCH1 is able, upon gelation, to control benzydamine release and to withstand the physiological removal mechanism.

In absence of benzydamine hydrochloride, the formulation developed shows antimicrobial properties proving the capability of TMCH1 to interact with bacterial cell also upon mixture with GP. It suggests the possibility to obtain therapeutic effect loading lower amounts of drug.

As expected, the antimicrobial activity increases in presence of benzydamine hydrochloride, it occurs after brief contact times

Table 3
Microbicidal effect (ME) of TMCH1/GP mixture in absence and in presence of benzydamine hydrochloride after different contact times with microorganisms. In the brackets are indicated the CFU/ml of the control suspension.

Contact time (h)					
Strain	0	2	4	6	24
TMCH1/GP mixture					
Staphylococcus aureus	0 (8.9×10^8)	0.93 (1.2×10^8)	1.26 (1.7×10^8)	1.31 (1.8×10^8)	4.30 (1.0×10^8)
Streptococcus pyogenes	0 (6.9×10^7)	0.78 (6.1×10^7)	1.00 (5.0×10^7)	1.60 (4.5×10^7)	>5.30[a] (2.0×10^5)
Streptococcus vestibularis	0 (9.0×10^7)	0 (8.0×10^7)	0 (8.4×10^7)	0.33 (3.2×10^7)	>7.30[a] (2.0×10^7)
Candida albicans	0 (1.3×10^6)	0 (1.6×10^6)	0 (1.7×10^6)	0 (1.3×10^6)	0.43 (1.2×10^6)
TMCH1/GP mixture loaded with benzydamine					
Staphylococcus aureus	0 (1.6×10^8)	4.20 (1.6×10^8)	>8.30[a] (2.0×10^8)	–	–
Streptococcus pyogenes	0.08 (6.9×10^7)	>7.8 (6.1×10^7)	–	–	–
Streptococcus vestibularis	0 (6.3×10^8)	4.30 (2.1×10^8)	>8.40[a] (2.4×10^8)	–	–
Candida albicans	0.27 (1.3×10^6)	1.6 (2.1×10^8)	5.33 (1.7×10^6)	>6.11[a] (1.3×10^6)	–

[a] A complete inactivation of the microorganisms occurred.

Table 4
Microbicidal effect (ME) of TMCH1/GP mixture loaded with benzydamine hydrochloride in presence of albumin (0.3% and 3% w/v) after different contact times with microorganisms.

Contact time (h)					
Strain	0	2	4	6	24
0.3% (w/v)					
Staphylococcus aureus	0 (3.0×10^8)	0 (1.9×10^8)	0 (3.0×10^8)	0 (2.2×10^8)	2.92 (1.0×10^8)
Streptococcus pyogenes	0 (4.3×10^7)	4.85 (5.0×10^7)	>5.9[a] (8.0×10^6)	–	–
Streptococcus vestibularis	0 (3.7×10^7)	0.78 (6.0×10^8)	0.66 (6.0×10^6)	1.44 (5.0×10^8)	>5.40[a] (2.5×10^5)
Candida albicans	0 (1.3×10^6)	1.30 (1.0×10^6)	2.0 (1.0×10^6)	2.61 (1.0×10^6)	2.65 (1.0×10^6)
3% (w/v)					
Staphylococcus aureus	0 (3.0×10^8)	0 (1.9×10^8)	0 (3.0×10^8)	0 (2.2×10^8)	0 (1.0×10^8)
Streptococcus pyogenes	0 (4.3×10^7)	3.10 (5.0×10^7)	4 (8.0×10^7)	>6.90[a] (9.0×10^6)	–
Streptococcus vestibularis	0 (3.5×10^8)	0.45 (8.5×10^8)	0.58 (4.0×10^8)	0.67 (7.0×10^8)	4.14 (1.0×10^7)
Candida albicans	0 (1.3×10^6)	0.40 (1.0×10^6)	0.70 (1.0×10^6)	1.56 (1.0×10^6)	2.09 (1.0×10^6)

[a] A complete inactivation of the microorganisms occurred.

with the microorganisms. A decrease in activity is observed in presence of albumin employed to simulate the administration on open mucosal wounds; this result suggests the employment of benzydamine hydrochloride only in the early stages of mucosa inflammation when no open wounds have formed.

The overall results demonstrate that TMCH1/GP mixture is a promising candidate for buccal administration of benzydamine hydrochloride in the treatment of oral mucositis.

Acknowledgements

The authors wish to thank Prof. G. Di Colo, Dr. Y. Zambito (University of Pisa) and Prof. R.A.A. Muzzarelli (University of Ancona) for the preparation of chitosan derivatives.

References

[1] A. Chenite, C. Chaput, D. Wang, C. Combes, M.D. Buschmann, .C. D Hoemann, J.C. Leroux, B.L. Atkinson, F. Binette, A. Selmani, Novel injectable neutral solutions of chitosan form biodegradable gels in situ, Biomaterials 21 (2000) 2155–2161.
[2] A. Chenite, M. Buschmann, D. Wang, C. Chaput, N. Kandani, Rheological characterization of thermogelling chitosan/glycerolphosphate solutions, Carbohydr. Polym. 46 (2001) 39–47.
[3] E. Ruel-Gariépy, M. Shive, A. Bichar, M. Berrada, D. Le Garrec, A. Chenite, J.C. Leroux, A thermosensitive chitosan-based hydrogel for the local delivery of paclitaxel, Eur. J. Pharm. Biopharm. 57 (2004) 53–63.
[4] S. Ganguly, A.K. Dash, A novel in situ gel for sustained drug delivery and targeting, Int. J. Pharm. 276 (2004) 83–92.
[5] M. Lavertu, D. Filion, M.D. Buschmann, Heat-induced transfer of protons from chitosan to glycerol phosphate produces chitosan Precipitation and gelation, Biomacromolecules 9 (2) (2008) 640–650.
[6] C.M. Lehr, J.A. Bouwstra, E.H. Schacht, H.E. Junginger, In vitro evaluation of mucoadhesive properties of chitosan and some other natural polymers, Int. J. Pharm. 78 (1992) 43–48.
[7] S. Senel, M.J. Kremer, S. Kas, P.W. Wertz, A.A. Hincal, C.A. Squier, Enhancing effect of chitosan on peptide drug delivery across buccal mucosa, Biomaterials 21 (2000) 2067–2071.
[8] S. Rossi, G. Sandri, F. Ferrari, M.C. Bonferoni, C. Caramella, Buccal delivery of acyclovir from films based on chitosan and polyacrylic acid, Pharm. Dev. Technol. 8 (2) (2003) 199–208.
[9] S. Rossi, M. Marciello, G. Sandri, M.C. Bonferoni, F. Ferrari, C. Caramella, Chitosan ascorbate: a chitosan salt with improved penetration enhancement properties, Pharm. Dev. Technol. 13 (6) (2008) 513–521.
[10] G. Sandri, S. Rossi, F. Ferrari, M.C. Bonferoni, C. Muzzarelli, C. Caramella, Assessment of chitosan derivatives as buccal and vaginal penetration enhancers, Eur. J. Pharm. Sci. 21 (2004) 35–39.
[11] G. Sandri, S. Rossi, M.C. Bonferoni, F. Ferrari, Y. Zambito, G. Di Colo, C. Caramella, Buccal penetration enhancement properties of N-trimethyl chitosan: influence of quaternization degree on absorption of a high molecular weight molecule, Int. J. Pharm. 297 (1–2) (2005) 146–155.
[12] R. A.A. Muzzarelli, Chitins and chitosans for the repair of wounded skin, nerve, cartilage and bone, Carbohydr. Polym. 76 (2009) 167–182.
[13] J. Zhishen, S. Dongfeng, X. Weiliang, Synthesis and antibacterial activities of quaternary ammonium salt of chitosan, Carbohydr. Res. 333 (2001) 1–6.
[14] S. Rossi, M. Marciello, G. Sandri, F. Ferrari, M.C. Bonferoni, A. Papetti, C. Caramella, C. Dacarro, P. Grisoli, Wound dressings based on chitosans and hyaluronic acid for the release of chlorhexidine diacetate in skin ulcer therapy, Pharm. Dev. Technol. 12 (4) (2007) 415–422.
[15] S. Rossi, M.C. Bonferoni, F. Ferrari, C. Caramella, Drug release and washability of mucoadhesive gels based on sodium carboxymethylcellulose and polyacrylic acid, Pharm. Dev. Technol. 4 (1) (1999) 55–63.
[16] G. Di Colo, S. Burgalassi, Y. Zambito, D. Monti, P. Chetoni, Effects of different N-trimethyl chitosans on in vitro/in vivo ofloxacin transcorneal permeation, J. Pharm. Sci. 93 (11) (2004) 2851–2862.
[17] H.A. Benson, J.C. McElnay, High-performance liquid chromatography assay for the measurement of benzydamine hydrochloride in topical pharmaceutical preparations, J. Chrom. 394 (2) (1987) 395–399.
[18] NCCLS, Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically, in: Approved Standard M7-A6, 6th ed., Wayne, PA, 2003.
[19] NCCLS, Methods for determining bactericidal activity on antimicrobial agents, in: Approved Guideline, Wayne, PA, vol. 19, 1999.
[20] A.P. Fraise, P.A. lambert, J.-Y. Masillard (Eds.), Russell, Hugo Ayliffe's Principles and Practice of Disinfection, Preservation & Sterilization, sixth ed., Blackwell Publishing, 2004.