5-Methyl-Pyrrolidinone Chitosan Films as Carriers for Buccal Administration of Proteins

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ABSTRACT

The purpose of this research was to investigate 5-methyl-pyrrolidinone chitosan (MPC) films as carriers for buccal delivery of protein drugs. Placebo and protein-loaded MPC films were prepared by casting and were then cross-linked with tripolyphosphate at different pH conditions. Myoglobin (MHb) was chosen as the model protein because its molecular weight is under the permeability limit of the buccal mucosa. The observed characteristics like bioadhesiveness, swelling behavior, and in vitro release of MHb from loaded films furnish information on the functional behavior of these films. The results obtained show that the modulation of MHb release was achieved only through chitosan cross-linking; the best results in release rate control were obtained by cross-linking performed at pH 6.5. Good bioadhesion properties were maintained even with high cross-linking degrees; the swelling index of MHb-loaded films at different cross-linking degrees evaluated at pH 7.4 and pH 6.4 were comparable to those of placebo films. By setting suitable tripolyphosphate cross-linking conditions for MPC films, one can control protein release without affecting bioadhesion.

KEYWORDS: 5-methyl-pyrrolidinone chitosan, tripolyphosphate cross-linked films, protein delivery, buccal cavity.

INTRODUCTION

In recent years, great attention has been devoted to the use of chitosan for pharmaceutical and medical applications[1] since it exhibits several desirable biological properties, such as nontoxicity, good biocompatibility, and biodegradability, as well as being widely available in nature, being low cost, and having high flexibility in use.

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5-Methyl-pyrrolidinone chitosan (MPC) is a chitosan derivative in which the amino groups of glucosamine units are partially replaced with 5-methyl-pyrrolidinone. Beyond its good biocompatibility and biodegradability, MPC has several properties that make it an interesting compound for use in the pharmaceutical field. It is a highly hydrophilic substance; it is soluble in water, saline, and water-alcohol mixtures; and it is able to yield viscous solutions or/and gels at physiological pH values.[2]

Mucoadhesive and penetration enhancement properties have been assessed for MPC solutions via buccal and vaginal mucosae.[3] Several studies have shown that MPC promotes ordered tissue reconstruction and vascularization. Moreover, MPC is a promising candidate for the production of wound dressings because it couples excellent wound healing promotion and antimicrobial properties,[4] although this polymer has not been evaluated yet for the manufacture of drug delivery systems such as films.

Buccal drug delivery is a novel application of chitosans involving bioadhesion and transmucosal drug transport.[5,6] Buccal drug delivery systems for local and systemic treatments should be formulated to prolong the drug's retention time in the oral cavity, fulfilling the needs of adhesion to the moist surface of mucosa and resistance to the flushing action of saliva.[7] Moreover, the buccal mucosa is a potentially important route for the delivery of peptide or protein drugs. Chitosan solutions have been evaluated for buccal delivery of proteins[8]; but in order to prolong drug release, films or hydrogels should be more suitable.[6]

Chitosan films should degrade slowly under physiological conditions, and for this reason they need to be cross-linked.[9] The cross-linking process, performed with the aim of achieving control of the drug release rate, can also affect one of the main properties of the systems, such as mucoadhesion.[10] Tripolyphosphate (TPP) has been used as a safe ionic cross-linking agent for the preparation of cross-linked chitosan films.[11] Ionic cross-linking requires multivalent counterions as cross-linkers to form bridges between polymeric chains: in particular, as chitosan is a polycation, TPP allows one to obtain ionic cross-linking in simple and mild conditions without the need for auxiliary molecules.

For the modulation of the TPP cross-linking process, one must attend to the global charge density, which depends on pK_a values and on solution pH during the reaction. The global charge of chitosan and the cross-linker must be sufficiently high to allow interactions and formation of a network.[12,13] The ionic reaction of the chitosan-TPP network is significantly influenced by the pH value of the TPP solution,[14] and the ionic cross-linking density could be improved by changing the pH of the curing agent.

In this study, MPC films were prepared and evaluated as protein delivery systems for application in the oral cavity. Myoglobin (MHb) was chosen as the model protein because its molecular weight (MW) is under the permeability limit of buccal mucosa and it is easily detectable by a spectrophotometric method without using specific markers and/or reagents for proteins.[8] MPC films loaded with MHb were prepared by casting and were cross-linked with different TPP:MPC molar ratios at 2 different pH values. The films were characterized in terms of bioadhesiveness and swelling behavior. For the evaluation of the buccal bioadhesive properties of the films, we used a method newly assessed by our research group and based on use of buccal cell suspensions.[10]

The swelling properties of MPC films cross-linked with different TPP:MPC molar ratios were analyzed for both placebo and MHb-loaded films.

The in vitro release tests were performed on MHb-loaded films by a spectrophotometric method exploiting the model protein chromophore.

Material and Methods

Materials

MPC with MW 180 kDa, degree of substitution 27%, and degree of acetylation 10% was obtained from squid chitosan supplied by France Chitine (Marseille, France)[2]; MHb with MW 18.8 kDa and TPP with MW 367.9 Da were supplied by Sigma Chemical Co (Milan, Italy); and polylactic acid (PLA50) with MW 31.5 kDa was supplied free of charge by Phusis (Saint Ismer, France). All other reagents were of analytical grade.

Young, healthy volunteers at the University of Pavia provided the human buccal cells. The experiments on buccal cells were conducted in accordance with Standard Institutional Guidelines. Informed consent from the healthy volunteers was obtained before performing cell withdrawal.

Methods

Preparation of Films

MPC films of fixed area (4.5 cm^2) were prepared by the casting method.[10] Placebo films were prepared from 3.5 g of 2% wt/wt chitosan aqueous solution containing 1% wt/wt glycerol as plasticizer. The solution was spread onto a glass plate and kept at 37°C for at least 2 days to allow complete solvent evaporation. MHb-loaded films were prepared by mixing 350 µg of protein with 3.5 g of 2% wt/wt MPC solution to obtain a final protein concentration of 0.01% wt/wt.

PLA films, chosen as nonbioadhesive reference films, were prepared from 3.5 g of 2% wt/wt PLA50 dichloromethane solution. The PLA50 solution was spread onto a glass plate thermostated at 2°C, and then the solvent evaporation was performed at room temperature.

Cross-Linking of Chitosan Films

Placebo and MHb-loaded films were cross-linked by immersion in 2 mL of TPP solution and subsequent drying at 37°C overnight (18 hours). Different TPP concentrations in the cross-linking solutions were used to get TPP:MPC molar ratios (moles TPP/100 moles MPC) of 50 and 100. TPP solutions were used at pH 8.9 or at pH 6.5 adjusted by 0.1N HCl, to modulate the cross-linking density. Table 1 reports the compositions and cross-linking degrees of the films prepared.

Morphological Characterization of Films

Shape surface texture and film thickness were evaluated by scanning electron microscopy (SEM) using the electron microscope Jeol JX 840-A (Jeol LTD, Tokyo, Japan). Film samples were gold-sputtered under vacuum and examined at 80 kV.

Films were also evaluated by visual inspection. Dry and hydrated films were photographed with a Digital Mavica Camera MVC-FD73 (Sony, Tokyo, Japan).

In Vitro MHb Release Test

The in vitro release profiles of MHb from plain films and films cross-linked by TPP solution at pH 8.9 or 6.5 were determined as follows: films were placed in individual test

Table 1. Composition and Cross-Linking Degree of MPC Films*

	TPP:MPC Molar Ratio (moles TPP/100 moles MPC)			TPP
Type of Film	0	50	100	Solution pH
Placebo	P_0	P_{50}	P_{100}	8.9
		$^{\Delta}P_{50}$	$^{\Delta}P_{100}$	6.5
MHb loaded	MP_0	MP_{50}	MP_{100}	8.9
		$^{\Delta}MP_{50}$	$^{\Delta}MP_{100}$	6.5

*The labels P_0 and M_0 refer to plain films, either placebo or MHb-loaded, respectively. The numbers 50 and 100 indicate the TPP:MPC molar ratios. The presence of $^{\Delta}$ means that TPP cross-linking solutions at pH 6.5 were used. MPC indicates 5-methyl-pyrrolidinone chitosan; TPP, tripolyphosphate; MHb, myoglobin.

tubes containing 15 mL of 10mM TRIS(hydroxymethyl)methylamine, 150mM NaCl, and 0.02% Na azide tris buffered saline (TBS) at pH 7.4 and continuously stirred at 37°C; at appropriate intervals, 10 mL of the release medium was collected and 10 mL of TBS was replaced in the test tube. Samples were assayed for MHb at 409 nm using a spectrophotometer (Beckman DU 7500, Fullerton, CA).

Placebo films were tested with the same protocol, as blanks.

Sodium Dodecylsulfate Polyacrylamide Gel Electrophoresis

The structural integrity of MHb released in the in vitro test was detected by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE), and it was compared with native MHb and reference markers. The protein released from plain and cross-linked MPC films after 1 hour of the in vitro release test was incubated with buffer solution containing 2% SDS, 0.5% dithiothreitol, 10% glycerol, and 0.01% bromophenol blue in 125mM TRIS(hydroxymethyl)aminomethane sodium chloride. The samples were heated at 99°C for 5 minutes, they were centrifuged, and 40 µL of supernatant was subjected to electrophoresis in vertical slab gels. Protein was visualized by Coomassie picric acid containing 1 volume of 0.2% Coomassie brilliant blue R250 (Bio-Rad, Milan, Italy) in 45% methanol and 10% acetic acid plus 4 volumes of 0.1M picric acid.

In Vitro Bioadhesion Test

The in vitro bioadhesion test was performed as reported in our previous work[10] and is explained below.

Donors (24-50 years old) provided the human buccal cells by gently scraping the inner cheeks of the oral cavity. The cells were suspended in phosphate-buffered saline (PBS) at pH 7.4, then centrifuged, washed with PBS, and finally resuspended in 2 mL of TBS at pH 7.4 at room temperature.[15] Cell concentration was determined by counting the cells in a hemocytometer. The cell suspension was diluted with TBS to a final concentration of 10^5 cells/mL, a value that represents the amount of buccal cells to be placed in contact with each film sample.

A piece (25 mm^2) of each type of film was suspended in a vial containing 1 mL of 10^5 cell suspension at pH 7.4. The vials were incubated for 30 minutes at room temperature under gentle shaking. Then the cell suspension was withdrawn, it was washed once, and the cells were counted in a hemocytometer.

The bioadhesive properties of the films, expressed in terms of percentage of buccal cells that adhered to the films, were calculated as follows:

$$\% \text{ Adhesion} = \frac{(X - Y)}{X} \times 100, \qquad (1)$$

where $X = 10^5$ buccal cells and Y = amount of cells that did not adhere to the films.

The results are the average of 6 determinations (± SD) for each film.

Swelling Study

Swelling studies were performed on placebo and MHb-loaded MPC films in the same experimental conditions (pH and volume of swelling medium, temperature, and time of contact between medium and buccal cells) used in the bioadhesion test. The media used were TBS at pH 7.4 or 6.4 and 150mM NaCl aqueous solution adjusted to pH 5.5.

Each film sample (surface area 25 mm^2) was weighed (W_0) and placed in a vial with 1 mL of swelling media. After 30 minutes of soaking, the films were blotted to remove excess fluid and then weighed (W_t).

The swelling degree is calculated as the weight increase and expressed as the Swelling Index (SI), as follows:

$$SI = \frac{(W_t - W_0)}{W_0}, \qquad (2)$$

where W_t = weight of hydrated film after 30 minutes of immersion and W_0 = weight of dry film at time zero. SI represents the swelling degree per weight unit. Six replicates were performed for each sample.

Statistical Analysis

Data are expressed as mean ± SD. Comparison of mean values was performed using 1-way analysis of variance. A difference is considered to be statistically significant when $P < .05$.

Results and Discussion

Morphological Characterization of Films

Placebo and MHb-loaded films were flexible, smooth, and easy to handle. The brownish color of MHb-loaded films is ascribed to the red color of the protein; placebo films were transparent (Figure 1).

SEM evaluations of shape and surface texture revealed no significant difference in surface morphology between placebo and MHb films. In both cases, film thickness was ~300 µm (285 ± 33 µm) for all films tested; the values were independent of the cross-linking degrees considered.

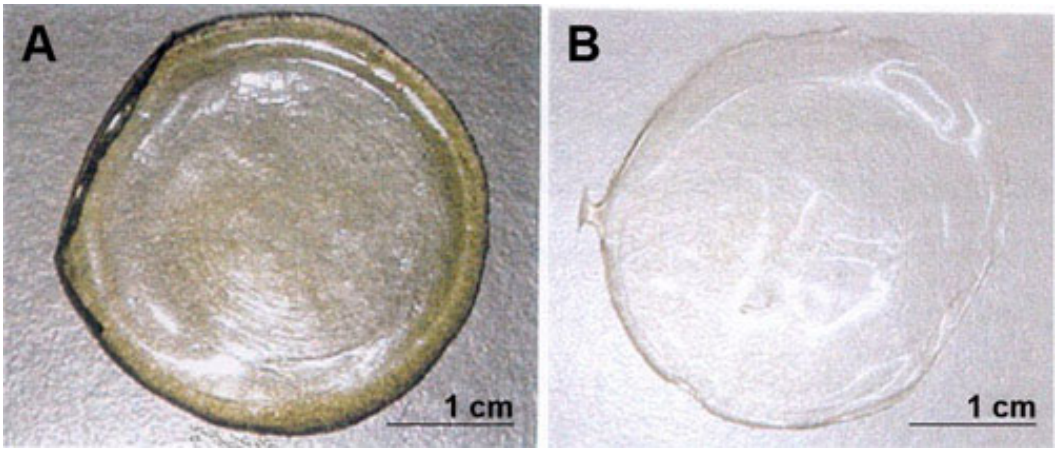

Figure 1. Pictures of myoglobin-loaded film (A) and placebo film (B).

In Vitro MHb Release Test

The in vitro release studies were performed for 6 hours at pH 7.4 to establish a possible control of drug release in the physiological environment.

As a preliminary step proving that the preparation and the cross-linking process of the films did not alter the protein structure, MHb solutions were added to the same TPP amounts used in the preparation of the cross-linked films. The spectrophotometric scanning of these mixed solutions revealed no difference in the MHb spectrophotometric profile with respect to the solution of the native protein (data not reported). Moreover, Figure 2 shows the results of SDS-PAGE performed on MHb released from the MPC films after 1 hour of the in vitro release test. These data show that neither the casting preparation process of the films nor the TPP cross-linking condition at different pHs modified the MW of protein with respect to the standard.

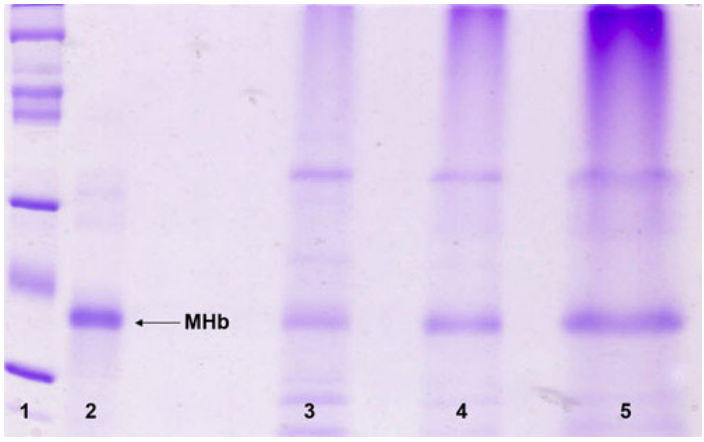

Figure 2. Sodium dodecylsulfate polyacrylamide gel electrophoresis MHb bands released from 5-methyl-pyrrolidinone chitosan films: low-molecular-weight reference markers (lane 1), MHb standard (lane 2), and MHb released from MP_0 (lane 3), MP_{50} (lane 4), and MP_{100} (lane 5) after 1 hour of the release test. MHb indicates myoglobin.

The MHb release from MPC films was determined by performing the in vitro release test on 4 pieces cut from each film. The release profiles from the 4 samples of each film were always superimposable, indicating that there was a good uniformity of MHb distribution in the MPC films and, in the case of cross-linked films, of cross-linking density.

Figure 3 shows the release profiles of MHb from MPC films with different cross-linking degrees in 6 hours. The release of MHb from the plain films was very fast: ~50% (48.29 ± 5.15%) of the protein was released after 30 minutes of incubation, and in 4 hours this percentage became ~80% (78.20 ± 10.02%).

The use of TPP in the cross-linking process and the pH of the cross-linking agent solution greatly modified the MHb release rate.

After 6 hours of the release test, MP_0 and MP_{50} evidenced the highest percentage of MHb released (84.91 ± 9.41%), while for the other samples these percentages were lower than 50%. This significant reduction in MHb release rate can be explained by the high cross-linking density when the process is performed at pH 8.9 with a 100 TPP:MPC

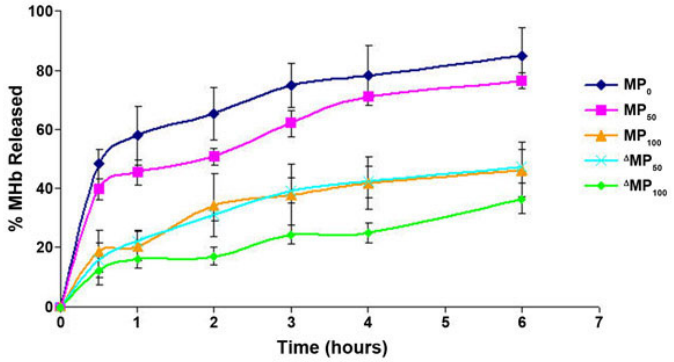

Figure 3. Percentages of MHb released from plain and cross-linked 5-methyl-pyrrolidinone chitosan films. MHb indicates myoglobin.

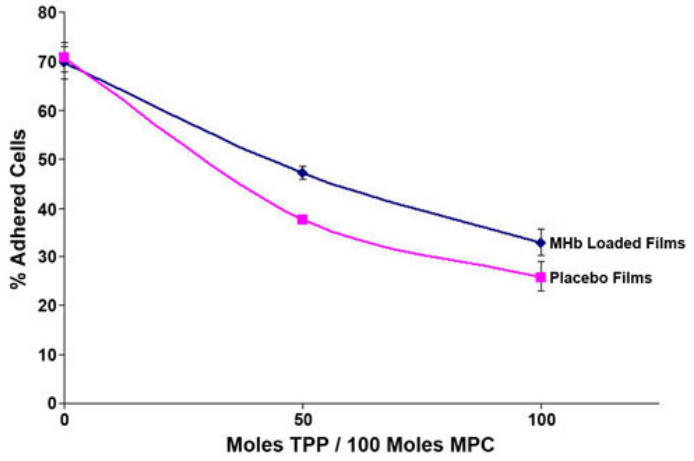

Figure 4. Bioadhesion behavior of MHb-loaded and placebo MPC films expressed as a percentage of adhered buccal cells versus moles TPP/100 moles MPC (cross-linking reaction at pH 8.9). The reference value of nonspecific adhesion is 28.7 ± 1.2% (mean ± SD; n = 6). MHb indicates myoglobin; TPP, tripolyphosphate; MPC, 5-methyl-pyrrolidinone chitosan.

molar ratio, or by improved ionic interaction between TPP and MPC for the cross-linking performed at pH 6.5 ($P < .05$). In this latter case, chitosan chains present a stronger positive charge repulsion between the $-NH_3^+$ groups and could form a complex with TPP with a higher binding ratio.

The MHb release profile from MP_{100} films is superimposable on the release profile from $^{\Delta}MP_{50}$. Moreover, SDs are lower for films cross-linked by TPP solution at pH 6.5. This can be explained by the higher cross-linking density, which makes the matrix more homogeneous.

This higher cross-linking density leads to slow release of MHb from these films: 17.08 ± 2.97% of protein released from $^{\Delta}MP_{100}$ in the first 2 hours, and 24.91 ± 3.50% after 4 hours.

In Vitro Bioadhesion Test

In a previous work,[10] the bioadhesion tests were performed on placebo plain and cross-linked films to evaluate the different cellular morphology with the hemocytometer inspection and, in this way, to confirm the biocompatibility of MPC films even after cross-linking. Moreover, MPC placebo films cross-linked with different TPP:MPC molar ratios (from 20 to 100) were also tested. A good compromise between bioadhesion and biocompatibility was observed for the TPP:MPC molar ratios of 50 and 100. TPP cross-linking did not alter cell morphology, even though it was possible to find cell agglomerates for the highest TPP:MPC molar ratio (100).

The results of the in vitro bioadhesion test (Figure 4) permit the comparison of the different behavior of placebo and MHb-loaded films: with the percentages of adhering cells versus TPP:MPC molar ratio plotted, chitosan films showed a linear decrease of adhesiveness properties when the TPP:MPC molar ratio was increased up to 100. MPC films cross-linked at pH 6.5 do not show any significant difference in bioadhesive behavior with respect to films cross-linked at pH 8.9 (data not reported). Cell adhesion to PLA films (28.70 ± 1.21%) is the reference value for nonspecific adhesion.

As expected, plain films were more adhesive than the cross-linked ones. MP_0 and P_0 were observed to be the most adhesive films, showing superimposable percentages of adhered buccal cells with, respectively, 69.66 ± 3.38% and 70.73 ± 2.97%.

The adhesion percentage began to decrease with a 50 TPP:MPC molar ratio, and there was a significant difference between MP_{50} and P_{50} (respectively, 46.26 ± 1.32% and 37.66 ± 1.02%).

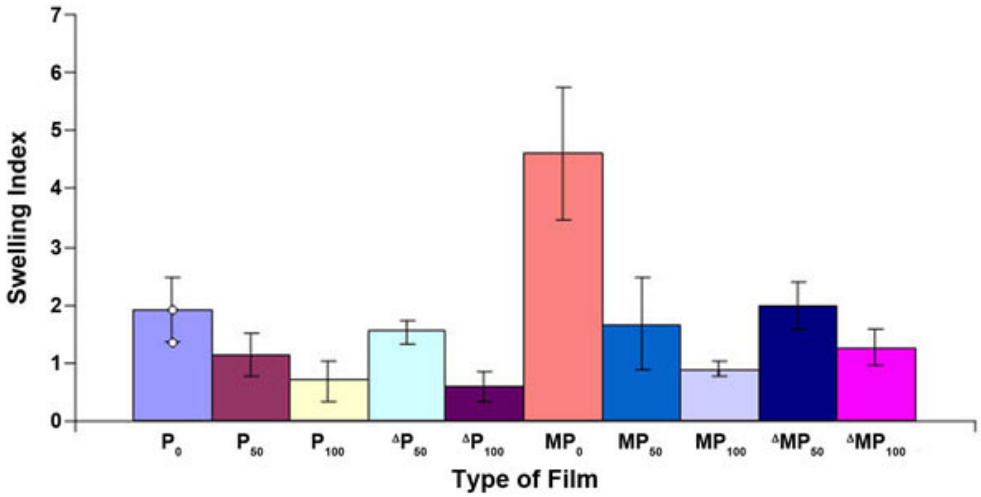

Figure 5. Swelling properties of myoglobin-loaded and placebo 5-methyl-pyrrolidinone chitosan films at pH 7.4.

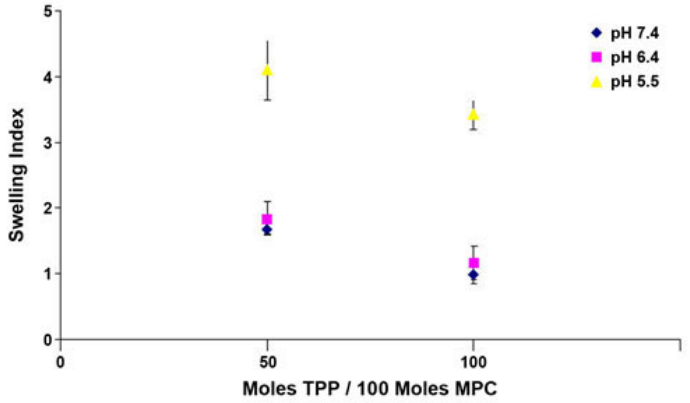

Figure 6. Swelling properties of myoglobin-loaded MPC films at different pHs (pH of TPP cross-linking solutions = 8.9; results are mean ± SD; n = 6). SI indicates swelling index; TPP, tripolyphosphate; MPC, 5-methyl-pyrrolidinone chitosan.

Both types of films showed good bioadhesion properties, even with a high cross-linking degree. MP_{100} obtained 32.93 ± 2.72% of adhered cells. This value was even higher than that of PLA films (28.70 ± 1.21%).

Swelling Properties

The SI of placebo and MHb-loaded films was determined at pH 7.4 to establish the mechanical properties of films in the physiological environment. Figure 5 reports SI values for placebo and MHb-loaded films cross-linked at both pH 8.9 and pH 6.5.

It is interesting to note that P_0 and MP_0 showed different swelling behavior, 1.92 ± 0.56 and 4.62 ± 1.15 ($P < 0.05$), respectively. Moreover, MHb films presented higher SDs.

When MHb-loaded films were cross-linked, their SI values were superimposable on the SI values of corresponding cross-linked placebo films, for each TPP:MPC molar ratio. There was no significant difference in swelling behavior between MHb-loaded films cross-linked at 2 different pHs (8.9 and 6.5). $^{\Delta}MP_{50}$ and $^{\Delta}MP_{100}$ presented SI values of 2.00 ± 0.41 and 1.26 ± 0.31, respectively; these values were superimposable on MP_{50} and MP_{100} SI values. In the presence of MHb, the swelling properties of films were reduced 2.3 and 3.5 times, respectively, for TPP:MPC molar ratios of 50 and 100 with respect to plain films. In this way, placebo and MHb-loaded films had the same SI values after cross-linking. This experimental evidence confirms the effectiveness of cross-linking. The hydrophilic nature of MHb is likely to cause the higher swelling performances of MHb-loaded plain films with respect to placebo films.

The swellability of ionically cross-linked hydrogels not only in neutral conditions but also in acidic conditions could extend the potential applications of these films, if they maintain their network integrity and thus the potential of sustaining drug release.[9] SI evaluation was performed on MHb-loaded films also in swelling media with different pHs (7.4, 6.4, and 5.5) to simulate the buccal environment: 7.4 and 6.4 represent extreme pH values of the buccal physiological pH range, while pH 5.5 is a value related to oral cavity disorders (candidiasis, gingivitis, dental caries, etc).

SI values at pH 5.5 were significantly different from SI values at pHs 7.4 and 6.4; moreover, the TPP:MPC molar ratio did not influence swelling. SI values were between 0.98 ± 0.13 and 1.83 ± 0.25 at pH > 6.4 and between 4.11 ± 3.42 and 3.42 ± 0.22 at pH 5.5 (Figure 6).

Conclusion

MPC proved to be a promising polymer for the manufacture of bioadhesive films. After ionic cross-linking, these films enabled the modulation of the release of the model protein MHb.

MHb-loaded MPC films kept their bioadhesive properties even after cross-linking up to a TPP:MPC molar ratio of 100. The modulation of protein release up to 6 hours was achieved when cross-linking of the polymeric matrix was performed at pH 6.5.

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