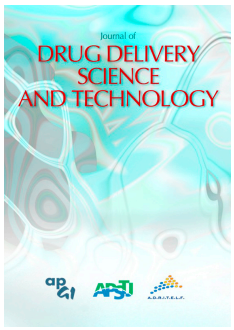

Bilayer chitosan-based patches for steroidal drug delivery on the oral mucosa

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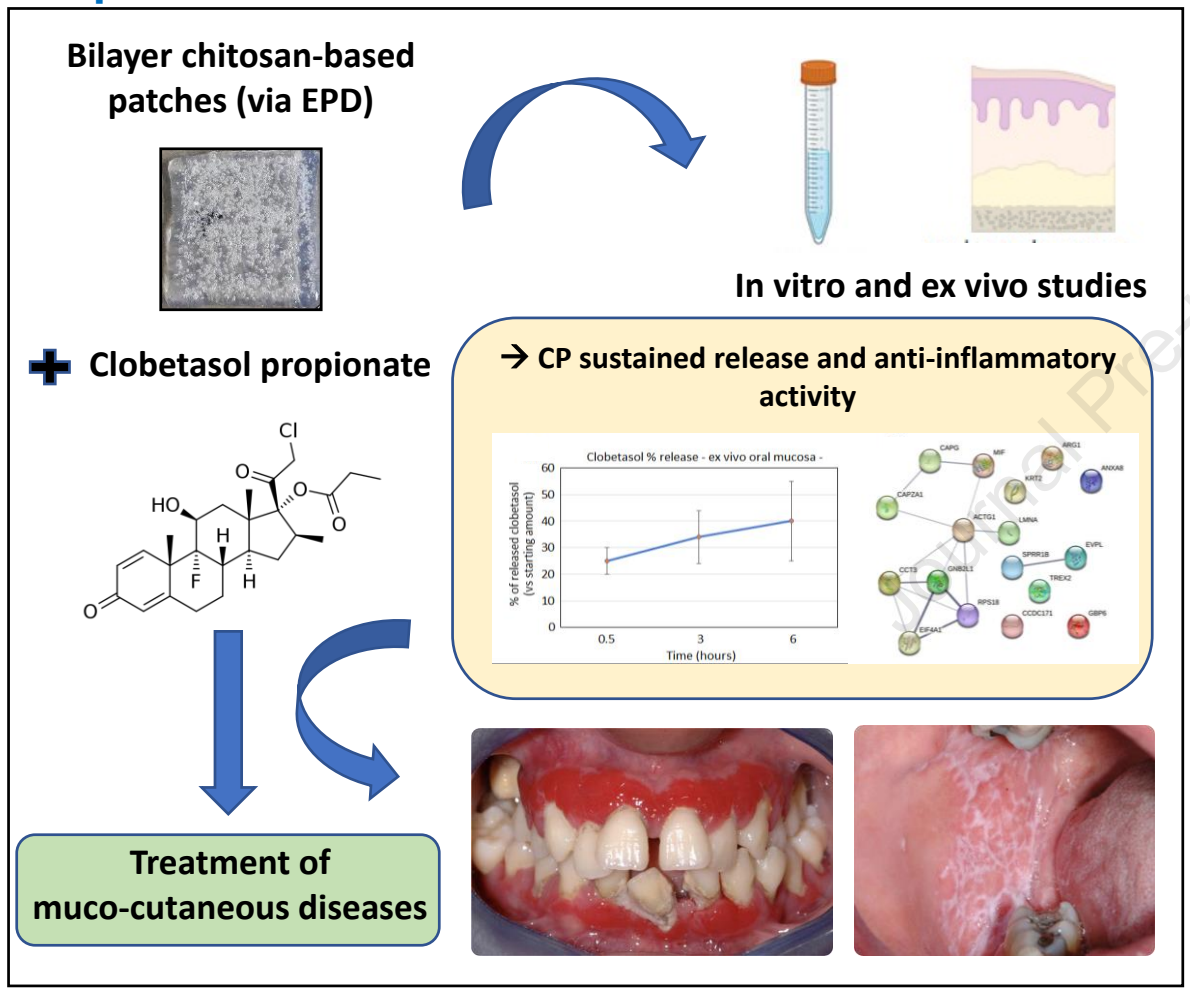
Graphical Abstract



Research Highlights

- Highly lipophilic clobetasol proprionate (CP) is successfully loaded on chitosan-based patches via electrophoretic deposition (EPD)
- Bilayer chitosan-based patches are promising mucoadhesive delivery systems for the oral cavity
- The presence of CP does not affect the mucoadhesive properties of chitosan-based patches
- CP, after the release from patches, maintains its biological immunosuppressive activity

Bilayer chitosan-based patches for steroidal drug delivery on the oral mucosa

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Abstract

Clobetasol-17-proprionate (CP) is the most potent, highly lipophilic topical corticosteroid, used for the treatment of immune-mediated muco-cutaneous diseases. No commercial preparations are available for oral cavity and galenic formulations are often ineffective due to the easy displacement by saliva and muscular movements. Here, we developed and characterized ~~a~~ novel mucoadhesive patches for CP delivery on the oral mucosa. Bilayer chitosan (CS)-based mucoadhesive patches (CS-CP) were produced via electrophoretic deposition (EPD)~~,~~ and characterized for physical and biological properties. Bilayer CS-CP patches showed a porous structure at the surface towards the oral mucosa (containing CP) and a more compact occlusive backing layer (without CP). CS-CP patches displayed fast swelling (301.6 ± 0.8%) in PBS, while a sustained CP release was observed over time, both *in vitro* in PBS and using an *ex vivo* model of porcine oral mucosa, with about 40% of CP released after 6 h. CP did not affect the mucoadhesive properties of the patches. Through a 3D model of the oral mucosa, the patches' cytocompatibility and the activity of CP released in regulating immune response-related pathways were evaluated. CS-based patches represent innovative biocompatible biomedical devices for the oral mucosa, able of successfully loading highly lipophilic drugs, including CP.

Keywords: drug delivery, oral medicine, controlled release, mucocutaneous disorders, biomaterials

1. Introduction

Immune-mediated and autoimmune diseases involving the oral mucosa, such as recurrent aphthous stomatitis (RAS), oral lichen planus (OLP), membrane mucous pemphigoid and pemphigus vulgaris, are often associated with painful intraoral ulcers, which can impair the patient oral functions, reducing the quality of life and sociability [1].

Being the etiopathogenesis still largely unknown, all these conditions require only symptomatic treatments, and current management includes the use of immune-modulating drugs, particularly, topical steroids that are the standard of care, aiming at reducing, locally, the chronic inflammation and the exaggerated immune response [2]. Among topical steroids, clobetasol-17-propionate (CP) represents the most potent corticosteroids (Europe: class IV) on the market, available only in form of creams and ointments for dermatological topical application, due to its high lipophilicity. However, literature supports that CP is also effective for the local treatment of oral immune-mediated and autoimmune diseases [3,4], despite the absence of a standard formulation suitable for the oral cavity. To date, galenic formulations at 0.05% (w/w) of CP in 4% hydroxyethylcellulose gel are usually prescribed to treat oral lesions, although showing poor mucoadhesive properties with an easy displacement of the drug and a reduced clinical effectiveness. The clinical success is affected by the difficult application of the drug on the oral mucosa, which is particularly challenging due to the salivary flow, having washing effects, and to the muscular activity, which mechanically displaces the drug during phonation and swallowing [5]. Moreover, the very high lipophilicity of CP can further decrease the drug-to-mucosa contact time, while increasing the unpredictability of drug local distribution, being the oral environment highly hydrophilic, instead. Strategies to improve the CP local effect, by increasing mucoadhesion and the contact time, are currently under investigation with the final aim of obtaining a more effective topical drug delivery system, thus reducing the need of systemic immunosuppressive therapies, often correlated with systemic adverse effects.

The ideal properties of a drug delivery system for the oral mucosa include high mucoadhesion and unidirectional drug release toward epithelial tissue, prolonging the mucosal exposure to the drug, thus increasing drug permeation throughout epithelial layers [6]. From a mechanical point of view, the device

should also show appropriate mechanical flexibility to support any movement within the oral cavity and it should be compatible with the salivary environment that is highly hydrophilic and with an average pH of 6.7 (range 6.2 -7.6) [7]. One of the most challenging aspects, considering that CP is very lipophilic, is related to the need of loading the lipophilic drug into the mucoadhesive system and to release it towards the oral mucosa, into an aqueous environment.

Drug delivery systems for steroidal drugs in the oral mucosa have included polymeric gels, mouthwashes, sprays, particulates, films and patches [8–12], although only few reports focused on CP [13–16]. Low drug loading efficiency, initial burst release, and the possibility of drug expulsion during storage were the main drawbacks. Bilayered buccal patches [13] have been proposed, based on polyvinylpyrrolidone (PVP) and polycaprolactone (PCL), synthetic polymers that require the need of toxic solvents for the preparation, high hydrophobicity and a lack of antimicrobial activity [17,18]. Recently, the Rivelin®- CLO bilayer patches, which are composed of synthetic polymers, i.e. PVP and Eudragit RS100 for the mucoadhesive electrospun nanofibers loaded with CP and PCL for the occlusive backing layer, were tested in OLP patients in randomized placebo-controlled clinical trial, showing promising results [19].

Here, we propose novel bilayer mucoadhesive oral patches based on chitosan (CS), a natural polymer derived from shrimps, to propose an innovative drug delivery system, highly biocompatible and biodegradable, prepared without the need of synthetic polymers or toxic solvents. CS is a biodegradable polysaccharide with intrinsic bio-adhesive [20], anti-microbial and wound healing properties [21]. To date, CS-based buccal patches were obtained by solvent casting technique or electrophoretic deposition (EPD), loading different drugs, such as verapamil, carvedilol or lidocaine, adding additives to improve biomechanical properties, such as polyvinylpyrrolidone (PVP), methylcellulose and pectin [22–25], or functionalizing CS with catechol groups [26].

The aim of this study was to synthetize and characterize innovative CS-based bilayer buccal patches, loaded with CP, via EPD. The patches displayed a bilayer design with an occlusive backing side, not loaded with the drug (towards the oral environment), and a CP-loaded side (towards the mucosa), to promote the unidirectional release of the drug to the target site. The patches were characterized from a morphological

and physical point of view, then the release of CP in physiological solution was assessed. An *ex vivo* model of porcine oral mucosa was used to test the mucoadhesive properties of the patches and assess CP diffusion throughout the epithelial layers. Finally, an *in vitro* 3D model of human oral mucosa was used to verify the patches' cytocompatibility and, via proteomics, the bioactivity of the CP released locally.

2. Materials and Methods

2.1 EPD of bilayer CS-CP patches

All reagents were provided by Sigma-Aldrich® (St. Louis, Missouri). CP (1g L^{-1}, molecular weight = 466.97) was added to a CS (1g L^{-1}, CS medium molecular weight, 75-85 % deacetylation degree) solution prepared with 30% water + 70% ethanol, adjusted to a pH = 4.8 with acetic acid. Titanium plates (Ti, grade 2) were used as cathode in an electrophoretic deposition (EPD) process and two graphite rods used as anodes. The EPD conditions were optimized to obtain bilayer CS-CP patches: EPD was carried out in potentiostatic mode by applying a square waveform (100 - 75 V, duty cycle (DC) = 0.17, t = 5 min) using a power supply (Keithley 2425, Keithley Instruments). The CP concentration in the bath and the EPD conditions were based on previous data about CP loading on monolayer CS-based EPD patches, reported elsewhere [27]. Bilayer patches, in particular, were obtained by a double step EPD deposition: a first deposition was deposited starting from a solution bath, as reported above, containing only CS (backing layer) and, after drying (T = 37 °C for 1.5 h), a second layer was deposited starting the same CS solution with the addition of CP, namely CS-CP (test samples). In case of control samples, the second deposition included only the CS solution (without the addition of CP). The obtained bilayer patches were then oven dried (T = 37 °C for 24 h) and peeled off from the cathode.

2.2 Morphological surface analysis

The morphology of the bilayer porous dried scaffolds was evaluated with optical microscopy and by means of a Scanning Electron Microscope (SEM) at 10 kV (SEM, Stereoscan 360, Cambridge). Pore diameter was measured in triplicate each type of sample, by means of ImageJ software, by analyzing at least 10 pores per image.

2.3 Swelling properties

The water uptake test was performed to investigate swelling behavior of CP-loaded CS patches. The swelling properties of the patches were studied by immersing them in 1 ml of Phosphate Buffer Solution (PBS) (pH=7.4) at room temperature. The dried patches (1 × 1 cm^2) weighed (W_0). At considered time points (up to 8 different time points), patches were removed from the solution and carefully dried using filter paper; free

water was removed, leaving only the interstitial water trapped in the polymer network. The specimens were then weighed (W_t) and put back into the solution. At each time point, the percentage of water uptake was calculated using the following equation:

$(W_t-W_0)/W_0x100$

W_t= the weight of the patch at time t

W_0= the weight of the patch at time zero

2.4 CP loading

CP concentration in CS patches was investigated using liquid chromatography mass spectrometry (LC-MS). The patches (1 × 1 cm^2) were weighed and then dissolved in 1 mL of acetic acid (10%v/v) and 200 µL of ethanol. The solution was than collected and analyzed by LC-MS. LC-MS analyses were performed using a mass spectrometer Orbitrap Q-Exactive Plus coupled with an Ultra High-Performance Liquid Chromatography (UHPLC) Vanquish (Thermo Fisher, Milano).

2.5 CP in vitro release into physiological solutions

CP concentration released from CS patches (1 × 1 cm^2) was analyzed by liquid chromatography mass spectrometry (LC-MS). CP release kinetics was performed in a dissolution medium (0.5M PBS and 0.5% sodium dodecyl sulphate, pH 6.8) at 37 °C: each patch was incubated in 1 ml of solution, and fixed vertically in 2 ml tubes in agitation (100 rpm, 37°C), thus allowing CP release from the patch. The solution was collected at 5, 10, 20, 40 and 60 min and analyzed by LC-MS, using a mass spectrometer Orbitrap Q-Exactive Plus coupled with an Ultra High-Performance Liquid Chromatography (UHPLC) Vanquish (Thermo Fisher, Milano).

2.6 CP release using an ex vivo model of porcine oral mucosa

Samples of porcine oral mucosa were surgically resected from whole porcine cheeks (kindly provided by Prof. Elena Grossini, Laboratory of Physiology – University of Piemonte Orientale), then wetted with PBS solution at physiological conditions. The porcine oral mucosa specimens were prepared with standard dimensions, as squares (2 cm side and 5 mm thickness), then maintained in DMEM/F12 medium at 37°C. Patches were applied, with gentle pressure, onto mucosa samples at the air-liquid interface and allowed to spontaneously release CP for 0.5, 3 and 6 h. At each time-point, mucosa samples were separated from patches, mechanically

homogenized and the released CP was analyzed by LC-MS, using a mass spectrometer Orbitrap Q-Exactive Plus coupled with an Ultra High-Performance Liquid Chromatography (UHPLC) Vanquish (Thermo Fisher, Milano).

2.7 Patches' mucoadhesion using an ex vivo model of porcine oral mucosa

A tack-test was performed using the modular compact Anton Paar MCR302 rheometer and porcine oral mucosa as standard mucosal substrate for testing mucoadhesion, in accordance with the ASTM F2258 standard. The porcine oral mucosa specimens (prepared as squares of 10 mm-side and 5mm-thickness; n=5 per sample type) were deeply washed with PBS and frozen at -20°C for the further mechanical analyses. After being re-equilibrated at room temperature (RT), porcine buccal mucosa pieces were attached to the lower disposable plate of the device. Wet patches (CS or CS-CP) were similarly fixed on the upper disposable plate of the instrument (ø = 25 mm). Mucosa and patches were placed in contact: a compressive preload of 1 N force was applied for 10 s. Then, the upper plate was moved upwards at a constant speed of 50 mm/min until detachment. The release pressure (σ_{adh}= F/S; where F is the Force of detachment and S is the contact area) and the muco-adhesion work (W_{adh}=AUC; where AUC is the area under the σ_{adh} – displacement curve calculated using OriginPro software) were measured as previously reported [28,29].

2.8 Patches' cytocompatibility using a 3D model of human oral mucosa

CP-loaded patch toxicity was investigated *in vitro* by analysing the metabolic activity of the oral mucosa tissue, after a 24 h-direct contact with the patches. Accordingly, wet bilayer patches (both CS-CP and CS) were gently seeded in direct contact with a 3D model of human oral mucosa (SkinEthic HOE, from EPISKIN, Lyon, France), allowed to adhere for 2 h and then maintained for 24h at 37°C at 5% CO_2 , using the maintenance medium as provided by the manufacturer CS patches were used as control considering previous literature supporting the safety both *in vitro* and *in vivo* of this biomaterial [30]. Metabolic activity of cells contained in the oral mucosa was further evaluated by using the colorimetric/fluorometric assay Alamar Blue (Thermo Fisher, Waltham, Massachusetts, USA), following manufacturer's instruction. Briefly, the Alamar blue solution was diluted (10%) and put in contact with the 3D model of oral mucosa for 3h, in the dark. Then, 100µl of solution have been collected, spotted in a black plate and fluorescent signals were evaluated with a

spectrophotometer (Spark multimode microplate reader, Tecan, Männedorf, Svizzera) excitation wavelength 530 nm, fluorescence emission reading 590 nm. Results are reported as Relative Fluorescence Units (RFU).

2.9 Proteomics analysis to test biological activity of released CP on a 3D model of human oral mucosa

The biological effect of CP released from the patch was assessed by proteomics analysis on the tissue in direct contact with the patches. Wet specimens (both CS-CP and CS) were gently seeded in direct contact with the 3D model of human oral mucosa (SkinEthic HOE, from EPISKIN, Lyon, France), allowed to adhere for 2 h and then maintained for 24h at 37°C at 5% CO_2 using the maintenance medium as provided by the manufacturer. Tissue samples were lysed with RIPA buffer and denatured with TFE (Sigma-Aldrich Inc., St. Louis, MO, USA) and then subjected to reduction with DTT 200 mM, to alkylation with IAM 200mM and to complete protein digestion with 2 µg of Trypsin (Sigma-Aldrich Inc., St. Louis, MO, USA). The peptide digests were desalted on the Discovery® DSC-18 solid phase extraction (SPE) 96-well plate (25 mg/well) (Sigma-Aldrich Inc., St. Louis, MO, USA). After the desalting process, the sample was vacuum-evaporated and reconstituted in mobile phase for the analysis [31]. The digested peptides were analyzed with a UHPLC Vanquish system (Thermo Scientific, Rodano, Italy) coupled with an Orbitrap Q-Exactive Plus (Thermo Scientific, Rodano, Italy). Peptides were separated by a reverse phase column (Accucore™ RP-MS 100 x 2.1 mm, particle size 2.6 µm). The column was maintained at a constant temperature of 40 °C at a flow rate of 0.200 mL/min. Mobile phase A and B were water and acetonitrile respectively, both acidified with 0.1% formic acid. The analysis was performed using the following gradient: 0-5 min from 2% to 5% B; 5-55 min from 5% to 30% B; 55-61 from 30% to 90% B and hold for 1 min, at 62.1 min the percentage of B was set to the initial condition of the run at 2% and hold for about 8 min in order to re-equilibrate the column, for a total run time of 70 min. The Mass spectrometry analysis was performed in positive ion mode. The ESI source was used with a voltage of 2.8 kV. The capillary temperature, sheath gas flow, auxiliary gas and spare gas flow were set at 325 °C, 45 arb, 10 arb and 2 respectively. S-lens was set at 70 rf. For the acquisition of spectra, a data-dependent (ddMS2) top 10 scan mode was used. Survey full-scan MS spectra (mass range m/z 381 to 1581) were acquired with resolution R = 70,000 and AGC target $3x10^6$. MS/MS fragmentation was performed using high-energy c-trap dissociation (HCD) with resolution R = 35,000 and AGC target $1x10^6$. The normalized collision energy (NCE)

was set to 30. The injection volume was 3 µL. The mass spectra analysis was carried out using MaxQuant software (version 1.6.14). MaxQuant parameters were set as follow: trypsin was selected for enzyme specificity; the search parameters were fixed to an initial precursor ion tolerance of 10 ppm and MS/MS tolerance at 20 ppm; as fixed modification, carbamidomethylation was set, whereas oxidation was set as variable modification. The maximum missed cleavages were set to 2. Andromeda search engine searched the spectra in MaxQuant against the Uniprot_CP_Human_2018 sequence database. Label free quantification was performed including a match between runs option with the following parameters: protein and peptide false discovery rate was set to 0.01; the quantification was based on the extracted ion chromatograms, with a minimum ratio count of 1; the minimum required peptide length was set to 7 aminoacids. Statistical analysis was performed using MaxQuant software (version 1.6.14) and MetaboAnalyst software (https://www.metaboanalyst.ca/) [32].

2.10 Statistical analysis

Experiments were performed in triplicate. Results were statistically analyzed using the SPSS software (v.20.0, IBM, USA). Groups were compared by the one-way ANOVA using the Tukey's test as a post-hoc analysis. Significant differences were established at $p < 0.05$.

3. Results

3.1 Morphological surface analysis

CS-CP patches were successfully obtained (Figure 1 A and B) via EPD. At optical microscopy, bilayer CS-CP patches showed a complex random macroporosity with a pore dimension similar to bilayer CS patches (Figure 1 C and D).

SEM images showed dried bilayer CS patches with a more compact structure, with the minimum presence of isolated roughness (Figure 2 A and B), while bilayer CS-CP patches displayed higher random microporosity (Figure 2 C and D). The two deposited layers were indistinguishable (Figure 2 D), and appeared stably adhering each other, also after immersion in PBS (data not shown). Moreover, bilayer CS-CP patches also displayed the presence of surface microspheres as observed at a lower magnification, ascribable to superficial CP deposits (Figure 2 E). The latter were no more visible after washing with PBS and ethanol, and a homogeneous and regular microporosity was instead detectable (Figure 2 F). Pore dimensions of CS-CP patches were further characterized using SEM image analysis and showed a mean pore size of 3.0 ± 0.7 µm in the dried state.

3.2 Swelling properties

The swelling data in PBS (pH=7.4) comparing CS patches and CS-CP patches are shown in Figures 3. Both patches displayed a similar swelling kinetics with fast swelling after immersion in the solution up to a stable plateau achieved after 2 h. The mean swelling rate was of 346.0 ± 0.3% for CS patches, while of 301.6 ± 0.8% for CS-CP patches, with no statistically significant difference.

3.3 CP loading and release kinetics

The LC-MS data showed that CP was successfully loaded in the patches at a concentration 0.9 ± 0.2 µg/mg of patch.

At *in vitro* release test, using 1x1 cm^2 patches immersed in 1 mL of PBS, a sustained release over the time could be observed. The amount of drug released was about 1.2 ± 0.8 µg/mL of solution, as achieved after 40 min and corresponding to >30% of the loaded CP (33.0 ± 9.5%). Figure 3A and B summarizes the release profile of CP from CS-CP patches. The sustained release was confirmed at *ex vivo* release test, using porcine

oral mucosa, with about 40% of the loaded CP released within the tissue after 6 h; the released drug amount was of 1.3 ± 0.7 µg/mL (Figure 4 C and D).

3.4 Mucoadhesion using ex vivo model of porcine oral mucosa

No significant differences were observed between CS and CS-CP for both release pressure at detachment (557 ± 213 Pa and 448 ± 8 Pa, respectively) and W_{adh} (745 ± 126 Pa*mm and 699 ± 128 Pa*mm, respectively), and the drug did not affect the mucoadhesive behavior of CS (Table 2).

3.5 Patches' cytocompatibility using a 3D model of human oral mucosa

The 3D model of human oral mucosa was cultivated for 24 h in direct contact with the patches. The cytocompatibility results are shown in the Figure 5A: no significant differences were observed between CS and CS-CP in terms of metabolic activity, after 24 h of contact with the oral mucosa model ($p>0.05$), thus suggesting a favorable cells-friendly behavior of the patches under investigation.

3.6 Proteomics analysis to test biological activity of released CP on a 3D model of human oral mucosa

The analysis of protein extracted from the oral mucosa models in contact with CS or CS-CP patches revealed the presence of 263 protein. Among them, seven were down-regulated and ten up-regulated, as described in Figure 5 C and D, and the map of their interconnection showed consistency in regulating immune response-related pathways, including macrophage migration inhibitory factor (MIF) downregulation and GBP6 and RACK1 up-regulation as shown in Figure 5B.

4. Discussion

Oral mucosa can be affected by painful lesions, which can be ascribed to immune-mediated or auto-immune disorders that severely impact the patient's quality of life, impeding patient's nutrition because of difficulty in chewing and in taking acidic foods, such as some fruits and vegetables.

These lesions are usually managed by topical corticosteroids, although a "gold standard" drug delivery system for the oral mucosa is still lacking. The *in situ*-controlled release kinetics of drugs, on the oral mucosa, is particularly challenging because of the moist mucosal surfaces, salivary flow and muscular forces, which easily displace the delivery system and the loaded drug, decreasing the drug residence time that is pivotal for the topical efficacy of a compound. In an attempt to address these issues, previous works proposed new delivery systems for CP [13–15,19]. In a previous work, we optimized the EPD conditions to obtain monolayer CS patches able to effectively incorporate CP [27]. Here, we further developed and characterized a bilayer mucoadhesive CS patch for CP delivery to the oral mucosa, having an occlusive side toward the oral environment and the opposite side, towards the oral mucosa, containing CP, in order to obtain the unidirectional release of the drug, thus representing a significative improvement in comparison to the previous monolayer model.

This works confirms EPD as a simple, low-cost and fast approach to incorporate water insoluble agents into CS matrix; EPD is available at industrial scale for industrial calcium phosphate coatings on metal prosthesis. The amount of loaded CP (0.9 μg/mg) was comparable with the amount of drug contained in the galenic formulation used in the clinical setting (0.5 μg/mg). The presence of CP within the bilayer CS patches resulted in the presence of micropores, more evident than in our previous work on monolayer CS-CP patches [27].

Several approaches have been reported in the literature for the fabrication of mucoadhesive drug delivery systems for the oral mucosa, such as gels, tablets, films, and patches [33,34]. Oral films and patches usually display superior properties compared to other adhesive systems, being flexible and soft, yet resistant to the mechanical stresses acting on the oral cavity [35]. In this panorama, non-traditional fabrication approaches like EPD and electrospinning have attracted significant attention due to the possibility to one-pot fabricate drug-loaded patches with superior mucoadhesive properties [36]. Both the techniques have been reported

for the fabrication of CS patches [27,33,37,38], even if it is difficult to compare their adhesive performances mainly due to the lack of mucoadhesion tests in most of these studies. Furthermore, the comparison of the drug loading/release is also non-trivial due to the different drugs and different test conditions (*in vitro, in vivo, ex vivo*) used. However, such approaches should be preferred over traditional ones given the possibility they offer to fabricate patches with a high surface-area-to-volume ratio and to load drugs directly in the fabrication process, in a straightforward, cost-effective, and tunable way.

CS-CP samples showed a slightly ($p > 0.05$) lower swelling rate compared to bilayer CS patches. Although not significant, such a difference in the swelling values could be attributed to the lipophilic nature of CP, which could act reducing the overall swelling capacity of the loaded patch. Nevertheless, CS-CP patches preserved satisfactory swelling levels, a crucial aspect for bio-adhesion. Mucoadhesive patches, indeed, are required to display a rapid hydration and subsequent gelation of the polymers on the moist oral mucosal surface, leading to physical and chemical interactions at the biomaterial–mucus interface. Besides hydrogen bonding and hydrophobic interactions, CS can bind mucin mainly via electrostatic interactions, involving positively charged amino groups of CS and negatively charged sialic acid residues of mucin [39,40]. Overall, the presence of CP did not significantly affect mucoadhesive properties. Adequate mucoadhesion is crucial to ensure the prolonged presence of the patches at the intended location that, in turn, leads to a constant release of the drug at the lesion's site.

Considering the release profile, the CS-CP patches slowly released the drug in a sustained manner over 6 h as confirmed by the swine *ex-vivo* model; however, it must be considered that, despite the notable correspondence in terms of permeability between the pig and human oral mucosa [41], the use of a static protocol may represent a limitation in the interpretation of the data. Therefore, for future insights the use of perfusion model such as the modified Franz cell model [42] can be considered to confirm the results in a more physiologically relevant model. By now, we can consider our results in line with previous literature such as Colley *et al.*, who developed patches made of CP-loaded PVP and a PCL backing layer [13].

The cytocompatibility data supported the safety of the CP-loaded patches on the 3D human oral mucosa model, after 24 h from the contact. These results match with the ones obtained by Kumar *et al.* and

Panonnummal *et al*. [43,44], who tested CP activity on human monocytes (THP-1) and human keratinocytes (HACAT) cell lines. Their study demonstrated CP safety at higher concentrations (62.5 and 43.0 µg/mL, respectively) than the one released by the patches here developed (1.2 ± 0.8 µg/mL).

The comparative analysis between the proteins extracted from the 3D model of human oral mucosa in contact with CS patches (used as a control) *versus* CS-CP patches (test samples), performed by proteomics, showed 17 proteins up- or down-regulated by the released CP within the mucosal tissue. Skin equivalents represent a suitable model to study the biological effect of the released drug because the relevance of the results does not strictly depend on the delivery system as demonstrated by Said *et al*. [14], dealing with the release of corticosteroids. However, some elements of the living tissue influencing the drug diffusion, such as the mucus and saliva keeping moist the epithelial surface, are effectively missing in the skin equivalents therefore representing a limitation of the model [45]. Here, the most up-regulated protein was Guanylate-binding proteins (GBP-6): GBPs are factors involved in the defense against cellular pathogens and inflammation [46]. A further up-regulated protein was the Receptor for activated C kinase 1 (RACK1), a scaffolding protein involved in the recruitment, assembly and regulation of a variety of signaling molecules, including those related to immune response [47]. RACK1, in particular, represents an important target for steroids [48]. Further overexpressed proteins include the Three prime repair exonuclease 2 (TREX2), the Macrophage-capping protein (CAP-G) and other proteins involved in keratinization, like Keratin type II (K22E) and Small Proline-Rich 1 (SPR1B). Among the downregulated proteins, the most biologically important one was represented by the Macrophage migration inhibitory factor (MIF), a pro-inflammatory cytokine involved in the innate immune response to bacterial pathogens *in vivo*, acting as mediator in regulating the function of macrophages in host defense [49]. This protein also counteracts the anti-inflammatory activity of glucocorticoids [49]; therefore, its down-regulation, related to CP *in situ* release, confirms the maintenance of bioactivity of the drug from the CS-CP patches.

5. Conclusions

CP, a highly lipophilic drug, can be successfully loaded in double layer CS-based patches via EPD, a simple, low-cost and fast fabrication technique. Bilayer CS-CP patches showed fast swelling, mucoadhesive properties and a porous structure at the surface towards the oral mucosa (containing CP). A sustained CP release was observed over time both *in vitro* in PBS and using an *ex vivo* model of porcine oral mucosa. Patches showed an overall biocompatibility when directly put in contact with a 3D model of human oral mucosa, and the released CP within the tissue maintained its anti-inflammatory effects *in situ*. Our study demonstrated the possibility of obtaining bilayer mucoadhesive patches, loaded with CP and based on the natural polymer CS, highly biocompatible and biodegradable, resulting in a promising topical therapeutic strategy for the treatment of immune-mediated oral diseases.

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Authors contributions

All authors gave their final approval and agree to be accountable for all aspects of the work. EV contributed to conception and design, drafted the manuscript; LA, LDN, LR contributed to design, critically revised the manuscript; LB, FV, MM contributed to acquisition, analysis, and interpretation, critically revised the manuscript; AS contributed to acquisition, analysis, and interpretation, drafted the manuscript; AC contributed to contributed to acquisition, analysis, and interpretation, drafted the manuscript**.**

Declaration of Conflicting Interests

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Data availability

Data will be made available on request.

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Figure legends

Figure 1. EPD apparatus and CS-CP patches' morphology. Image of the EPD apparatus (A), used to obtain CS-CP patches (B). Optical image of bilayer CS patch (C); optical image of bilayer CS-CP patch as (D).

Figure 2. Morphology of the patches at scanning electron microscope (SEM). (A and B) Bilayer CS patches (bottom view and cross-section); (C and D) Bilayer CS-CP patches (top side view of the surface towards the cathode, and cross-section); (E and F) Bilayer CS-CP patches before and after washing with PBS and ethanol (top side view of the surface towards the solution): microspheres were ascribable to CP deposits (E), which could be easily washed (F).

Figure 3. Swelling behaviour of CS patches (green line) and of CS-CP patches (blue line), up to 3 h after the immersion in PSB solution at pH 7.4.

Figure 4. CP release kinetics from bilayer CS-CP patches, at *in vitro* test (PBS solution) (A-C) and *ex vivo* model of porcine oral mucosa (D-F).

Figure 5. Cytocompatibility of the patches and bioactivity of released CP. (A) Metabolic activity (Alamar Blue) of 3D model of oral mucosa in contact with bilayer CS patches versus CS-CP patches, showing no significant difference in terms of cytocompatibility. (B) Map of the down/up-regulated protein coding genes and their interconnection. List of down-regulated (C) or up-regulated proteins (D) upon contact with CS-CP patches vs CP patches used as controls.

Tables

Table 1. Macro-pore diameters of CS and CS-CP bilayer patches.

Type of patch	Mean ± SD
Bilayer CS	1.2 ± 0.3 mm
Bilayer CS-CP	1.2 ± 0.5mm

544 **Table 2.** Mucoadhesion test comparing CS (control) and CS-CP patches (test), expressed as release pressure
545 at detachment and muco-adhesion work.

	CS	CS-CP
Release Pressure (Pa)	557.7±213.4	448.1±8.03
Work of adhesion (Pa*mm)	745.8±126.5	699.4±128.1

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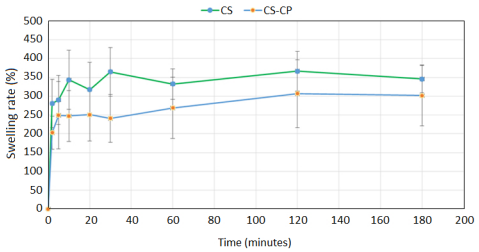

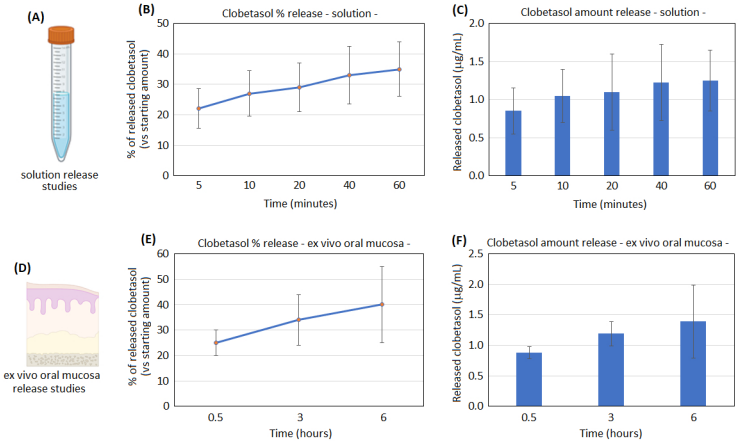

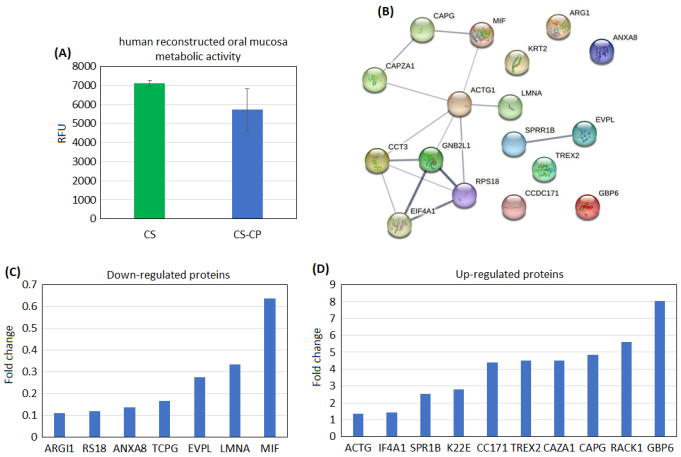

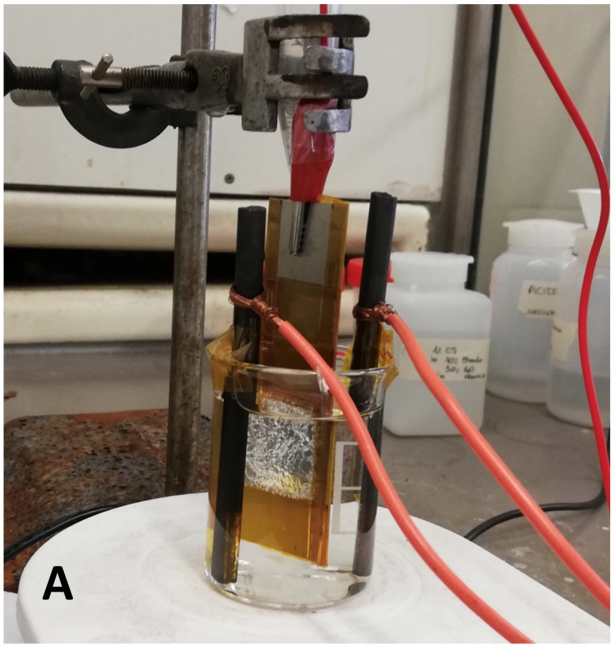
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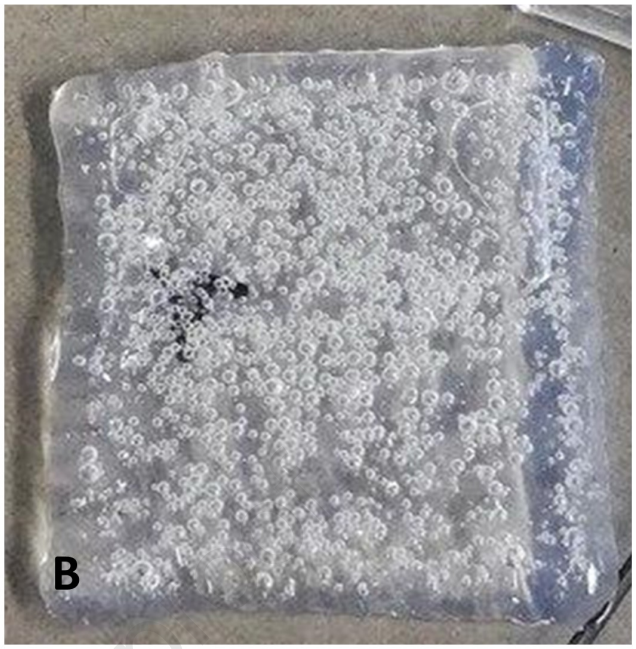
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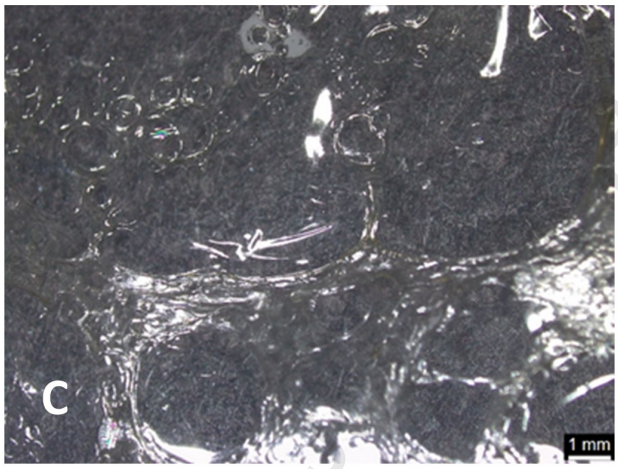
C
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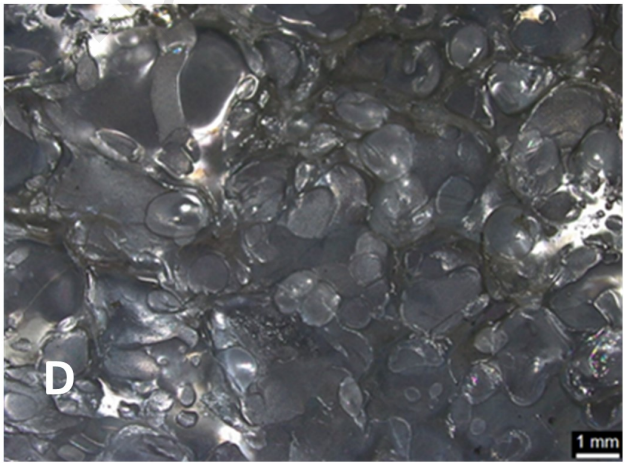
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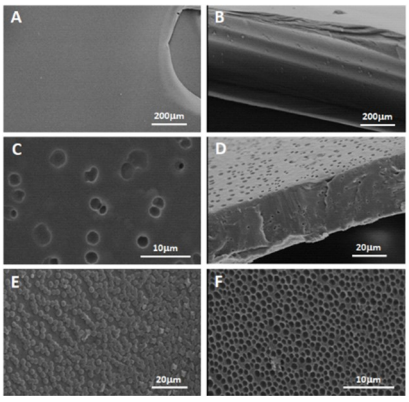

Highlights

- Highly lipophilic clobetasol proprionate (CP) is successfully loaded on chitosan-based patches via electrophoretic deposition (EPD)
- Bilayer chitosan-based patches are promising mucoadhesive delivery systems for the oral cavity
- The presence of CP does not affect the mucoadhesive properties of chitosan-based patches
- CP, after the release from patches, maintains its biological immunosuppressive activity

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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