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Factorial design of *in situ* gelling two-compartment systems containing chlorhexidine for the treatment of periodontitis

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ABSTRACT

Periodontitis is one of the most widespread bacterial infectious oral diseases that affects a significant percentage of the population worldwide. Different bacterial strains are responsible for the chronic inflammation and subgingival plaque that could be effectively treated with prolonged exposure to therapeutic levels of antibiotics and antiseptics in the periodontal pockets. Medicated *in situ* gels of chlorhexidine (CHX), for extended drug release and long-lasting antiseptic effect in the targeted cavities, were prepared in a two-compartment system. One compartment was loaded with sodium alginate solution while other was filled with CHX and calcium solution. The mixing of the solutions during the application resulted in gelation. Two 3^3 full factorial designs were applied in this study in order to optimize the gel formulation. Initially, the effects of concentration of gelling agent, crosslinker, and pH of the system on the dependent variables such as gel formation and structure characteristics were investigated. Then, the concentration of the crosslinker was optimized. Afterwards, the effect of gelling agent, loading of the drug, and pH of the gel system were correlated with the gel characteristics through another factorial design. Optimized formulations were tested for mucoadhesion, *in vitro* drug release, and microbiological investigation. Based on the results of the factorial design, mucoadhesiveness, antimicrobial investigation, and drug release, a 4 % alginate composition can be considered optimal. Overall, the optimized *in situ* periodontal gel was found to be effective with prolonged retention time and desirable outcomes.

1. Introduction

Periodontitis, an infectious disease caused by various strains of gram-positive and gram-negative bacteria, is affecting nearly 3.6 billion people all over the world. The compromised oral health ultimately leads to severe periodontal conditions and dental caries (Vos et al., 2017). Periodontal diseases are a series of pathological conditions affecting the supporting tissues of teeth and are typically initiated by mild infection and inflammation, followed by gingivitis, halitosis, bleeding, swelling, redness, and tenderness of the gums that result in chronic periodontitis and bone loss (Nasra et al., 2017).

In majority of people the polymicrobial biofilm accumulation lead to gingivitis due to improper elimination of dental plaque. As a result of disbiosis of subgingival microbiome, the localized and contained inflammatory response shifts to progressive and destructive periodontitis in susceptible patients (Van Dyke et al., 2020). A pathologically deepened gingival sulcus at the gingival margin and around the tooth is formed, that is called a periodontal pocket. The periodontal pocket has a wide range of pH, varying from 6 to 7 throughout the cavity (Ho et al., 2022). Periodontal pocket size could be 4–8 mm or more in periodontitis. A pocket size over 5 mm leads to an advanced level of periodontal disease that requires deep cleaning and aggressive treatment procedures (Wang et al., 2023). Several clinical studies have revealed statistically significant reduction in pocket depth with the adjunctive use of antimicrobial therapy with scaling and root planning treatment (Bundidpun et al., 2018).

Besides the several surgical and mechanical treatments, local administration of antibiotics and anti-inflammatory drugs at infection site is mandatory. Subgingival instrumentation is considered to be the gold standard in the non-surgical treatment of periodontitis but it has limitation due to several factors (deep pockets, furcation involvement) that result in reduced efficiency of therapy. The use of subgingival

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application of local delivery antibiotics as an adjunct to mechanical non-surgical periodontal treatment may prove to be beneficial and allows for the achievement of long-term minimum concentration of the drug inhibiting periopathogen development (Cosgarea et al., 2022). On the other hand, the currently available dosage forms are not suitable for effective delivery of the medication in the periodontal pocket, resulting in sub-therapeutic efficacy. Moreover, repeated oral drug administration not only leads to inconvenience and eminent side effects but also contributes to antibiotic resistance (Reise et al., 2012). The retention time of the formulation in the periodontal pocket is a critical factor in ensuring treatment efficacy. High clearance rate of gingival fluid form the periodontal pocket is a rate-limiting step in the effectiveness of a drug, as it leads to rapid reduction of the active moiety's therapeutic concentration (Zhao et al., 2020). Owing to the reduced drug retention time, patients have to take frequent medication to maintain therapeutic drug concentration (Petersen and Ogawa, 2000).

A local drug delivery system that can sustainably release the drug at the site of action for a prolonged period of time is favorable for periodontal treatment by subsiding the systemic side effects (Do et al., 2015). Micelles, liposomes, gels, nano-emulsions, and particle delivery systems are getting attention as local drug delivery devices. The chosen candidate dosage form should deliver the drug at the site of infection with the properties of low viscosity, high mucoadhesion, and improved pocket penetration (Mei et al., 2017). For the initial treatment of periodontal disease and the prevention of recurrence and recolonization of microbial agents in the cavity after treatment, commonly used formulations include wafer inserts, implants, films, fibers, and *in situ* hydrogels (Joshi et al., 2016).

Although many new local systems have been introduced in the past period in the indication of periodontitis, none of them can be considered an ideal preparation, as it can be seen in the following examples. For instance, the main drawbacks of wafers include excessive swelling and non-linear erosion release kinetics (Bromberg et al., 2001). Besides wafers, there is a commercially available *in situ* forming implant called Atridox®, composed of a biodegradable matrix. However, it faces the issue of accidental expulsion from periodontal pockets. This may cause a considerable uncertainty regarding the actual drug concentration reaching the site (Wang and Burgess, 2021). Moreover, burst release prior to solidification is another noteworthy issue which may lead to 8–95 % drug removal from the implant during the initial release phase or immediately after insertion (Musmade et al., 2019). As another example, orodispersible films are intended to be applied in the oral cavity but may not release the medicine directly into the periodontal pocket (Scarpa et al., 2017). On the other hand, the thickness and adhesiveness of buccal films pose challenges as preparation parameters in case of oral drug delivery. Some considerable drawbacks, such as poor mechanical properties, deformation, inappropriate and uncontrolled degradation rates, patient discomfort, and drug toxicity related to monolithic electrospun nanofibers are responsible for limiting the application of electrospun nanofibers (Zhao et al., 2022).

In case of hydrogels that can be chemically or physically cross-linked, chemically cross-linked formulations of polymers are prone to incompatibility issues and may involve the use of toxic crosslinkers. That is why scientists are more inclined towards the preparation of physically crosslinked hydrogels under physiological conditions (Van Tomme et al., 2008). Some well-known physiochemical crosslinking methods for gelation include temperature, pH, presence of ions (calcium, potassium, etc.), solvent exchange, and ultra violet irradiation (Kouchak, 2014; Cheng et al., 2014). *In situ* hydrogels are drug delivery systems that form gels at the site of administration in response to different biological stimuli or triggers. They are particularly suitable candidates to overcome the issues associated with several other local drug delivery dosage forms. Rapid absorption due to rich blood supply in the pocket, promotes the enhanced bioavailability of loaded drug. These smart systems could be helpful in reducing the frequency of drug administration and prolonging the drug release in a sustained manner that ultimately ensures patient compliance and cost-effectiveness (Madan et al., 2009). Moreover, *in situ* gelling system has been reported as a biodegradable formulation with efficient spreadability, improved bioadhesion and retention inside the pocket, eliminating the need for remnants removal (Do et al., 2014). In addition, these liquid preparations are easy to administer through microneedles and later harden into a gel under physiological conditions with customized geometry (Yadav et al., 2020).

Sodium alginate, a linear polysaccharide obtained from brown seaweed, is a suitable functional biomaterial due to its nontoxic, biocompatible, and biodegradable nature (Zhang et al., 2019a). The block copolymer polysaccharide consists of α-l-guluronic acid (G) and β-d-mannuronic acid (M) joined by 1,4 glycosidic linkages (Miyazaki et al., 2000). The gelation mechanisms of alginate on addition of divalent ions have been elaborated by the classic egg-box model (Cao et al., 2020; Hu et al., 2012). The gel-forming ability of sodium alginate, which occurs abundantly in physiological fluids of the body, is a result of its interaction with di- and trivalent cations. Due to no discernible flavor and taste, sodium alginate is preferred for prolonged oral drug delivery systems. Additionally, sodium alginate is a non-toxic, non-immunogenic and non-irritating pharmaceutical agent (Jadach et al., 2022). These attributes made sodium alginate the ideal choice for our polymeric gelling system.

CHX is a cationic bisbiguanide broad-spectrum antibacterial and antiseptic agent. It possesses concentration-dependent bacteriostatic and bactericidal activity (Block, 2001). CHX, a primary plaque control agent, serves as a gold standard to measure and compare the antiplaque activity of other agents (Jones, 1997). Tarawneh et al. developed cellulose derivatives-based hydrogel films loaded with CHX, including the crosslinker epichlorohydrin (ECH). *In vitro* release studies demonstrated that the antiseptic effect of CHX-loaded hydrogel formulation extended to two weeks, but this formulation was tested against few bacterial strains (Tarawneh et al., 2021). Yan and colleagues formulated an ultraviolet cross-linked hydrogel systems loaded with chlorhexidine and metronidazole for the intracranial administration to treat root canal infection and exhibited a considerable antimicrobial effects against a couple of bacterial strains (Yan et al., 2021). Lim et al. formulated a dual-action, thin hydrogel film containing CHX with anti-inflammatory and local anesthetic agents to ensure a sustained and slow release of the drugs. This mucoadhesive thin film system led to maximum drug release within 6 h, which implies the need for frequent administration of medicated film (Lim et al., 2020). An acute experimental periodontitis study in rats had already been conducted to evaluate the periodontal healing potential of CHX. In that study, chlorhexidine was found to be effective for mechanical debridement and for accelerating the healing process after mechanical scaling root planing (SRP). This affirms the usefulness of CHX in the periodontal pocket healing process (Prietto et al., 2020).

When studying the literature, we can draw the conclusion that there is no local form that meets all criteria for the treatment of periodontitis. However, by understanding the functions and limitations of the systems, it is possible to design a composition with a favorable therapeutic effect, and factorial experiment design is a valuable tool for this purpose. In our present work, we aimed the optimization of alginate-based *in situ* gelling systems which contain CHX as API. After conducting a thorough literature survey and to prior knowledge, we concluded that in the case of alginate-based *in situ* gelling systems, there are only a limited number of research works available that establish a relationship between the pH of the system, polymer and crosslinker concentration through statistical analysis to determine the strength of the gel and, in particular, the expected water and API loss. The pH of the periodontal cavity can not only affect the gel formation process but also the strength of the gelling system. A weak gel can contribute to excessive water loss, increasing the susceptibility to the loss of the active ingredient. On the contrary, a strong gel may hinder the drug release in timely and sustained manner. Moreover, an optimized amount of crosslinker is needed to assure the formation of a well-structured ion activated gel.

Table 1
Compositions for the first 3^3 factorial experimental design.

Compositions	x_1 Alginate concentration (%)	x_2 pH	x3 $CaSO_4$ concentration (mg/mL)
A1	2	4	5
A2			10
A3			15
A4		6	5
A5			10
A6			15
A7		8	5
A8			10
A9			15
A10	4	4	5
A11			10
A12			15
A13		6	5
A14			10
A15			15
A16		8	5
A17			10
A18			15
A19	6	4	5
A20			10
A21			15
A22		6	5
A23			10
A24			15
A25		8	5
A26			10
A27			15

Our goal was to develop a stable, long lasting *in situ* gelling system with improved mucoadhesion and syringeability that could bear potential antibacterial activities for an extended period of time. For this purpose, a full factorial design approach was applied to evaluate all possible combinations of pH, sodium alginate, and calcium concentration for optimum gel strength and minimum water loss in the first factorial design. A full factorial design is a simple systematic design style for estimation of main effects and interactions between variables.

After acquiring and fixing a suitable concentration range of the crosslinker in the first factorial run, the next factorial design was established to find the effective dose concentration of CHX, using the previously determined calcium concentration. Final optimized formulations with optimum pH were further investigated for their physicochemical and structural properties, as well as their mucoadhesive features, release studies, and *in vitro* antibacterial performance.

2. Materials and methods

2.1. Materials

Sodium Alginate (Ph.Eur. Munucol LKX, FMC, Hungary) G/M ration: 30–40/60–70 with medium viscosity attributes, was a kind gift of the manufacturer. Chlorhexidine digluconate 20 % (CHX) solution was bought from Hungaropharma (Budapest, Hungary). Buffer compositions, such as sodium acetate, acetic acid glacial to prepare 0.1 M sodium acetate with pH= 4, sodium dihydrogen phosphate, and sodium hydroxide (NaOH) to prepare phosphate buffer with pH=6, sodium dihydrogen phosphate, to prepare 0.02 M sodium phosphate buffer solution with pH=8, acetonitrile, triethylamine sodium phosphate monobasic, 85 % phosphoric acid, calcium sulphate were purchased from Molar Chemicals Ltd (Halásztelek, Hungary). All the ingredients and chemicals were of analytical reagent grade.

2.2. Preparation of the gels

Two-compartment formulations were prepared, and the contents of the two compartments were mixed in 1:1 ratio just before application to obtain the final gel formulation. In one of the compartments, various concentrations of sodium alginate (2–6 %) polymeric solution were prepared using different buffer solutions (buffer pH=4.0, buffer pH=6.0, and buffer 8.0) and left overnight for hydration. In the other compartment of the formulations, a solution of chlorhexidine digluconate and calcium sulfate was dispersed in water. To ensure proper homogeneity, the system was continuously stirred during sample preparation.

During the mixing of the two compartments, one syringe was filled with the polymeric solution and the other with calcium sulfate and CHX solution, then the content of two syringes were combined. In the case of the first factorial design; concentration of sodium alginate, $CaSO_4$, and the pH of the formulation were varied. The compositions are described in Table 1.

Regarding the second factorial design; sodium alginate, pH and the concentration of CHX were examined while maintaining a fixed concentration of calcium (Table 2). All formulations were prepared in triplicate and tested for tensile strength and water loss.

2.3. Measurement of water loss

The rate of water discharge was determined by gravimetric method that utilizes the absorbent property of filter paper. The prepared gel in the syringe was dispensed on the paper. It absorbed all the excess water discharged from syringe or present on the surface of the gel. The total water loss percentage was determined by dividing the amount of water remaining on the filter paper (m_{water}) by the total weight of the formulation (m_{total}) (Zussman et al., 2022). This gravimetric analysis aided in assessing the water bounding ability of the gel structure.

$$m_{water\%} = \frac{m_{water}}{m_{total}} * 100 \quad (1)$$

2.4. Gel strength

The magnitude of gel strength was measured with texture analyzer (TA.XT plus, Stable Micro Systems Ltd, Surrey, UK) by transferring the cylindrical gelling body (diameter of 10 mm and height of 10 mm). A

Table 2
Compositions for the second 3^3 factorial experimental design.

Compositions	x_1 Alginate concentration (%)	x_2 pH	x_3 CHX concentration (%)
B1	2	4	0.2
B2			0.4
B3			0.6
B4		6	0.2
B5			0.4
B6			0.6
B7		8	0.2
B8			0.4
B9			0.6
B10	4	4	0.2
B11			0.4
B12			0.6
B13		6	0.2
B14			0.4
B15			0.6
B16		8	0.2
B17			0.4
B18			0.6
B19	6	4	0.2
B20			0.4
B21			0.6
B22		6	0.2
B23			0.4
B24			0.6
B25		8	0.2
B26			0.4
B27			0.6

Table 3
Experimental design, values, and levels of independent variables.

Determinants	Code	Lower level	Middle level	Upper level
Independent				
Sodium Alginate (%)	x_1	2 %	4 %	6 %
pH	x_2	4	6	8
Calcium sulfate (%)*	x_3 *	5 mg/mL*	10 mg/mL*	15 mg/mL*
Chlorhexidine (%)**	x_3 **	0.2 %**	0.4 %**	0.6 %**
Dependent				
Gel Strength	y_1			
Water Loss	y_2			

* 1st factorial design.
** 2nd factorial design.

spherical probe, with a diameter of 50 mm, was penetrated into the sample at the rate of 1 mm/s (Nair et al., 2021). The instrument recorded the force distance curve, and the maximum force of the curve was determined, which corresponds to the force required to destroy the gel structure.

All the developed formulations underwent visual examination to assess their shape retention, uniformity, homogeneity.

2.5. Mucoadhesion

The mucoadhesion of the gels was evaluated through tensile strength measurement. In the following method, the adhesive force and work of adhesion were measured, which involved determining the force required to separate the formulation from the membrane surface.

A texture analyzer (TA.XT plus, Stable Micro Systems Ltd, Surrey, UK) was used with a 5 kg load cell. To mimic the *in vitro* mucosal lining, the filter paper was wetted with 50 mL of 8 % (*w/w*) mucin dispersion in PBS (pH = 7.4). The wetted filter paper was then placed into the mucoadhesive rig. 20 mg of formulation was placed onto the lower surface of the upper cylindrical probe with a diameter of 10 mm (Garg and Kumar, 2007).

By lowering the probe at a rate of 0.3 mm/s, the mucin layer and sample came into contact. After the contact a preload of 2500 mN was applied for 3 min. Then the probe was lifted upward at a rate of 2.5 mm/min that ultimately resulted in the breakage of adhesive bonds. Five parallel measurements were carried out to evaluate the work of adhesion (mN.mm) (Eouani et al., 2001). A force vs. distance curve was plotted, and the adhesive work was calculated by the area under the curve.

Table 4
The 1st factorial design: effect of X_1, X_2 and X_3 on gel strength and water loss.

Samples	Alginate conc. (%) (x_1)	pH (x_2)	$CaSO_4$ conc. (mg/mL) (x3)	Gel strength (g) (y_1)	Water loss (%) (y_2)
A1	2	4	5	10.0 ± 0.0	5.7 ± 0.2
A2			10	10.0 ± 0.0	7.1 ± 1.5
A3			15	10.0 ± 0.0	9.8 ± 0.5
A4		6	5	18.5 ± 3.3	8.1 ± 2.6
A5			10	28.5 ± 9.0	3.6 ± 0.6
A6			15	35.8 ± 9.0	4.1 ± 0.8
A7		8	5	10.0 ± 0.0	8.8 ± 1.7
A8			10	10.0 ± 0.0	6.2 ± 0.7
A9			15	10.0 ± 0.0	7.8 ± 0.3
A10	4	4	5	26.9 ± 7.9	3.4 ± 1.1
A11			10	75.2 ± 19.0	2.6 ± 0.3
A12			15	73.5 ± 20.0	8.5 ± 3.0
A13		6	5	50.0 ± 6.0	4.9 ± 1.2
A14			10	102.1 ± 29.0	1.5 ± 1.0
A15			15	85.8 ± 20.0	1.2 ± 0.4
A16		8	5	10.0 ± 0.0	8.3 ± 0.3
A17			10	10.0 ± 0.0	4.8 ± 0.2
A18			15	10.0 ± 0.0	6.8 ± 0.2
A19	6	4	5	57.1 ± 16.0	2.0 ± 0.3
A20			10	106.4 ± 18.0	2.8 ± 0.1
A21			15	131.0 ± 30.0	1.3 ± 0.3
A22		6	5	35.6 ± 8.0	7.9 ± 1.2
A23			10	68.8 ± 25.0	0.5 ± 0.2
A24			15	10.0 ± 0.0	4.7 ± 0.3
A25		8	5	40.5 ± 5.0	5.2 ± 0.4
A26			10	40.1 ± 5.2	7.2 ± 1.0
A27			15	10.0 ± 0.0	8.2 ± 1.0

2.6. In vitro drug release studies

Franz Diffusion cell system (MicroettePlus system by Hanson Research USA) was utilized to conduct the drug release study. All the optimized samples were evaluated in 3 parallels for 24 h. Approximately 300 mg of each 4 % and 6 % *(w/w)* polymeric gel formulations, containing 0.2 %, 0.4 %, and 0.6 % *(w/w)* of CHX, were uniformly placed on Porafilm membrane filters (width 25 mm and 0.45 mm pore size) in the donor chamber. The assembly temperature was set at 37 ± 0.5 °C, and the rotation speed was predefined to 400 rpm. Before commencing the experiment, the membrane filters were immersed in phosphate buffer solutions for 20 min. The acceptor phase, a capacity of 7 mL, was filled with phosphate buffer (pH = 7.4). In the course of release study, sampling was done at ten time points with three parallel measurements on the six cells. Automatically, 0.8 mL aliquots of the sample was removed from the acceptor phase using an autosampler. After each sampling point, an equivalent volume of PBS was added to the acceptor phase to maintain sink conditions. Later on, the collected samples were analyzed with HPLC (Shimadzu Nexera X2 UHPLC, Kyoto, Japan) at a wavelength of 239 nm for the absorption (Havlíková et al., 2007). The mobile phase consisted of a mixture of acetonitrile and buffer solution in a ratio of 35:65 *(v/v)*. The mobile phase used was prepared by combining a 0.08 M solution of monobasic sodium phosphate dihydrate containing 0.5 % triethylamine. The pH of the mobile phase was adjusted to 3 using phosphoric acid (85 %). BetaBasic™ Phenyl (Thermo Scientific™, 5 µm, 250 mm x 4.6 mm) column was used as stationary phase with temperature set at 40 °C. The solvent flow rate was fixed to 1 mL/min and the injection volume to 10 mL. Time of analysis for each sample was programed to 8 min, and retention time of CHX was recorded at 4.9 min. The obtained data was then analyzed to interpret the release kinetics, and the regression coefficient (r^2) was calculated.

2.7. Antibacterial study

S. mutans ATCC 25175 and *A. actinomycetemcomitans* DSM 11122 strains were used for the examination of the effect of CHX containing gel samples on bacterial growth. A 1 McFarland bacterial suspension of both bacterial strains was prepared in saline solutions, separately that is equivalent to 3×10^8 colony-forming units/mL (Cui et al., 2021). Bacterial suspensions were spread onto Schaedler agar supplemented with 5 % sheep blood (Biomérieux SA, Craponne, France). Gel samples were arranged on each agar plates followed by a 24 h-long incubation at 37 ± 0.5 °C under anaerobic conditions. After incubation time, zone of inhibition was measured for each sample. Subsequently, the gel bodies were removed from previous plates and placed on new Schaedler agar plates inoculated with freshly prepared 1 McFarland suspension of both bacterial strains. Three parallels of the measurements were repeated for two weeks.

2.8. Experimental design

The Quality by Design approach was implied to evaluate the prepared ion-activated *in situ* gel formulations containing the active ingredient. Full factorial design is a well-known simple lattice design of experiment (DoE) (Kurniawansyah et al., 2020). To investigate the interaction and influence of independent variables on the dependent variables, 3^3 factorial design was applied. This statistical techniques by Statistica for Windows (version 13.5, StatSoft Inc., Tulsa, OK, USA) helped us to filter the most significant factors from the trivial determinants (Szalai et al., 2022). The optimization of three independent

Table 5
The 2nd factorial design: effect of X_1, X_2 and X_3 on gel strength and water loss.

Sample	Alginate conc. (%) (x_1)	pH (x_2)	CHX (%) (x_3)	Gel strength (g) (y_1)	Water loss (%) (y_2)
B1	2	4	0.2	25.9 ± 2.5	8.1 ± 0.10
B2			0.4	27.0 ± 1.5	5.3 ± 0.15
B3			0.6	24.7 ± 1.0	4.0 ± 0,15
B4		6	0.2	18.2 ± 0.8	4.4 ± 1.3
B5			0.4	24.2 ± 1.5	0.9 ± 0.08
B6			0.6	22.0 ± 0.2	0.7 ± 0.20
B7		8	0.2	19.7 ± 3.0	1.8 ± 0.3
B8			0.4	17.2 ± 3.0	2.9 ± 2.0
B9			0.6	10.0 ± 0.0	2.2 ± 0.5
B10	4	4	0.2	78.2 ± 16.0	1.5 ± 0.8
B11			0.4	50.3 ± 3.0	1.5 ± 0.5
B12			0.6	71.7 ± 10.0	1.8 ± 0.3
B13		6	0.2	105.9 ± 30.0	0.2 ± 0.9
B14			0.4	112.5 ± 38.0	0.2 ± 0.1
B15			0.6	77.7 ± 7.0	0.5 ± 0.09
B16		8	0.2	28.0 ± 5.0	2.2 ± 0.5
B17			0.4	34.4 ± 6.0	2.2 ± 0.2
B18			0.6	26.4 ± 4.0	2.2 ± 0.3
B19	6	4	0.2	67.7 ± 19.0	0.3 ± 0.01
B20			0.4	74.2 ± 20.0	0.2 ± 0.02
B21			0.6	47.2 ± 10.0	1.2 ± 0.1
B22		6	0.2	93.2 ± 20.0	0.2 ± 0.2
B23			0.4	73.3 ± 16.0	0.8 ± 0.03
B24			0.6	83.1 ± 19.0	0.5 ± 0.1
B25		8	0.2	10.0 ± 0.0	0.5 ± 0.01
B26			0.4	10.0 ± 0.0	0.3 ± 0.01
B27			0.6	56.1 ± 3.0	0.4 ± 0.02

variables, such as the concentration of sodium alginate (x_1), pH (x_2), and concentration of calcium (x_3) was performed for each dependent variable gel strength (y_1) and water loss (y_2) as shown in the Table 1. After the determination of the optimal amount of cross-linking agent ($CaSO_4$), another 3^3 factorial design was conducted with concentration of sodium alginate (x_1), pH (x_2), concentration of CHX (x_3) as independent variables against (y_1) and (y_2). Lower, middle, and upper level values of x_1, x_2 and x_3 are presented in Table 3.

The pH range examined during our experiments was established based on the pH values measured in the oral cavity and periodontal pocket. The concentration range of CHX was determined by the concentration of CHX-containing products (mouthwash, gum cream) on the market. When specifying the concentrations of alginate and calcium, we used our previous preliminary test results as a basis.

2.9. Statistical analysis

The mean ± standard deviation of all the obtained data was calculated. For the purpose of comparison and analysis of the optimized effect on responses, two 3^3 factorial designs by Statistica for Windows (version 13.5, StatSoft Inc., Tulsa, OK, USA) were applied. In the first factorial design, the dependent variables, gel strength and water loss, were evaluated against three independent factors: concentration of sodium alginate, calcium, and pH. In the second factorial design, the dependent variables were recorded based on the responses of concentration of sodium alginate, CHX, and pH. A statistically significant difference between values is indicated when $p < 0.05$.

In the case of tensile strength measurements, Prism for Windows (GraphPad Software Inc., La Jolla, CA, USA) was used to conduct statistical data analysis using the one-way ANOVA variance analysis (Tukey post-hoc test). For the analysis of the results of the drug release curve, two-way ANOVA analysis was applied with Bonferroni post-tests. Differences were regarded as significant if $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ versus the control.

3. Results

3.1. Characterization of in situ gelling systems

After the preparation of the gels, visual observations were carried out. The detailed results are presented in the supplementary materials (Tables S1 and S2, Fig. S1 a–e). The compositions were classified on the basis of stability and homogeneity. The first group showed stable and shape-retaining gel structures (Fig. S1a), which hold the shape even after being pressed out of the syringe. Group 2 was found to consist of elastic and slightly translucent bodies (Fig. S1b). Group 3 also preserved the shape of the syringe when squeezed, but consisted of very soft, sensitive gel pieces that were prone to deformation. Their structure was not homogeneous, as it was covered by a stronger gel layer on the outside around them; however, it was almost fluid on the inside (Fig. S1c). Group 4 showed homogeneous appearance with no lumps (Fig. S1d). Group 5 appeared to possess heterogeneous, uneven, and nodular gel structures (Fig. S1e).

During the gel strength measurement using texture analyzer, it was observed that the gel structure initially showed resistance to the force exerted by the specimen, but gradually, at a certain value of input force, the specimen broke through the gel surface medially and penetrated inside. A 3^3 full factorial design was applied to select the optimal formulations to analyze the interactions between the components (see Chapter 3.2).

Under the influence of crosslinking, a certain amount of water was released from the gel structure that can result in instability of the structure and lead to the immediate elimination of a certain amount of drug. It has been observed that in certain formulations, a significant amount of water eliminated from the gel and liquefied in just a couple of seconds. This might contribute to the loss of active content and the elimination of the formulation from the periodontal pocket.

Formulations containing a lower concentration of sodium alginate appeared to be more elastic, soft, and translucent in appearance. While formulations with higher concentrations of sodium alginate and calcium sulfate resulted in a more stable gelling structure, within a median pH range (Tables S1 and S2). Heterogeneous structures were reported to be stiffer and lumpier in nature with an increased amount of calcium concentration at relatively high pH (Tables S1 and S2). As the calcium concentration raised, the viscosity and gel strength of the system also started to increase, resulting in a more intact system (Tables S1 and S2). Carboxyl groups present on polymer chains are continuously occupied with divalent ions until saturation occurs (Nickerson et al., 2003). Upon further addition of the gelling agent, a reverse behavior of gel strength was reported by Sworn and co-workers due to the dominating repulsive forces between anionic sites and calcium ions (Sworn et al., 1995), or the formation of inordinate number of nuclei at elevated temperature that weaken the gel structure upon cooling (Kasapis et al., 1999).

To attain the most suitable polymeric system, all 27 formulations were assessed with the help of statistical analysis.

3.2. Analysis of gel structure with factorial design

Studies reported that polymer concentration, gelling agents, and pH of the system not only contribute to formulate a stable gel structure, but also alter the characteristics of the formulation. Thus, in our work we applied the polymer and calcium concentration, and the pH of the system as independent variable, and to completely understand the interaction of formulation variables and their impact on dependent variables, two factorial designs were conducted (Tables 4 and 5).

The relationship between the composition (x_1, x_2, and x_3) and the characteristics of the gels (y_1, y_2) is described with Eqs. (2) and (3). Significant factors are highlighted with bold-faced letters in the equations.

$$y_1 = 42.2381 + 22.82x_1 + 5.305x_1^2 - 19.42x_2 + 9.108x_2^2 + 12.61x_3 + 6.39x_3^2 - 17.0x_1x_2 - 3.641x_1^2x_2 - 6.42x_1x_2^2 + 10.07x_1^2x_2^2 + 9.165x_1x_3 + 4.43x_1x_3^2 + 2.604x_1^2x_3^2 - 7.54x_2x_3 + 2.98x_2^2x_3^2 \quad (2)$$

The concentration of sodium alginate (linear L and non-linear Q) is a promising factor that affects gel strength, while the negative coefficient indicates the inverse relationship between dependent and independent determinants. Both calcium concentration (L and Q), as well as pH and sodium alginate, are of significant importance. The pH (Q) and calcium concentration (Q) together enhance the gel strength in a direct relationship (Fig. 1).

$$y_2 = 5.448 - 1.037x_1 - 0.55x_1^2 + 1.255x_2 - 0.650x_2^2 + 1.39x_1x_2 + 0.99x_1x_2^2 - 0.719x_1^2x_2^2 - 0.319x_1x_3^2 - 0.362x_1^2x_3^2 - 0.66x_2x_3 - 1.28x_2^2x_3 \quad (3)$$

In the case of water loss, the concentrations of sodium alginate (L and Q) are inversely related. Moreover, the equation depicted the inverse relation between sodium alginate (L) and calcium concentration (Q) with excessive water loss. The negative sign of the non-linear coefficient indicates minimum functionality in the investigations (Fig. 2).

After careful evaluation of the experimental results obtained from the first factorial design, some *in situ* hydrogel compositions (A14, A15, A23) with better gel strength and less water loss were found to be appropriate for further investigations. The next factorial design was applied to optimize our formulation and establish the relationship between CHX concentration and polymer concentration at different pH values and their impact on dependent responses (Table 5). On the basis of the first factorial design, the optimal calcium concentration was established (middle concentration: 10 mg/mL $CaSO_4$). This value was applied in the composition of the second factorial design.

Regarding gel strength, Eq. (4) establishes a significant interaction between sodium alginate concentration and gel strength. The interaction between sodium alginate (L) and pH (Q) showed a direct relation with gel strength. Sodium alginate (Q) and pH (Q) are responsible for strengthening the gel, while sodium alginate (non-linear) and CHX (linear) have an inverse impact on gel strength (Fig. 3).

$$y_1 = 47.7 + 18.1x_1 + 12.9x_1^2 - 14.1x_2 + 15.04x_2^2 - 6.87x_1x_2 - 3.29x_1^2x_2 + 9.57x_1x_2^2 + 7.66x_1^2x_2^2 - 3.37x_1^2x_3 + 5.25x_2x_3 \quad (4)$$

Less concentrated gel formulations may result in excessive water loss, as clarified by Eq. (5), while linear quadrants of sodium alginate and pH together have a considerable impact on water loss. CHX did not show a significant relationship with water loss (Fig. 4).

$$y_2 = 1.846 - 1.55x_1 - 0.33x_1^2 - 0.636x_2 - 0.655x_2^2 - 0.43x_3 + 0.98x_1x_2 + 0.68x_1^2x_2 + 0.59x_1x_2^2 + 0.86x_1x_3 + 0.39x_1^2x_3 + 0.435x_2x_3 + 0.26x_2x_3^2 \quad (5)$$

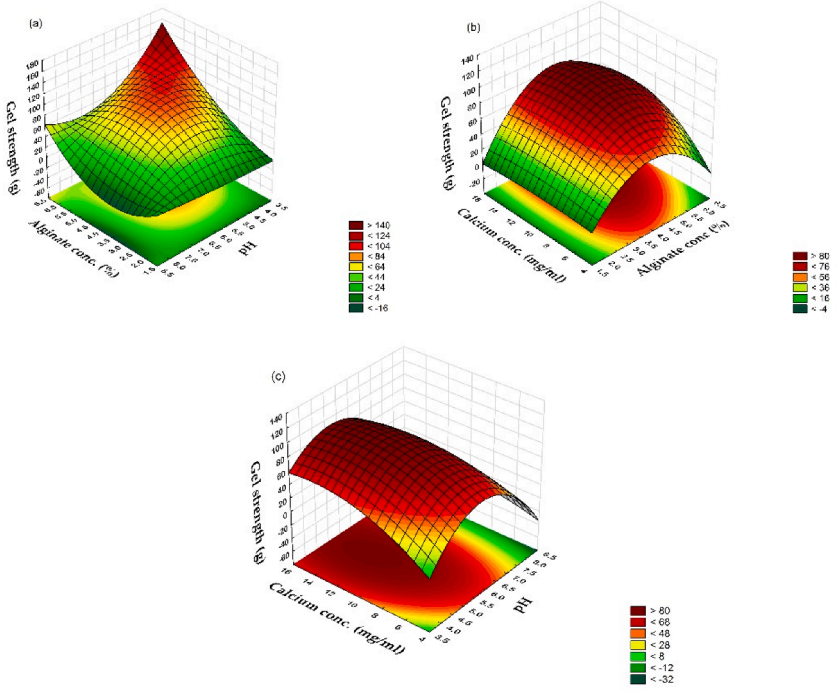

Fig. 1. Response surface graph of gel strength. The effect of the composition on gel strength y_1: (a) concentration of sodium alginate and pH, (b) concentration of sodium alginate and calcium, (c) concentration of calcium and pH.

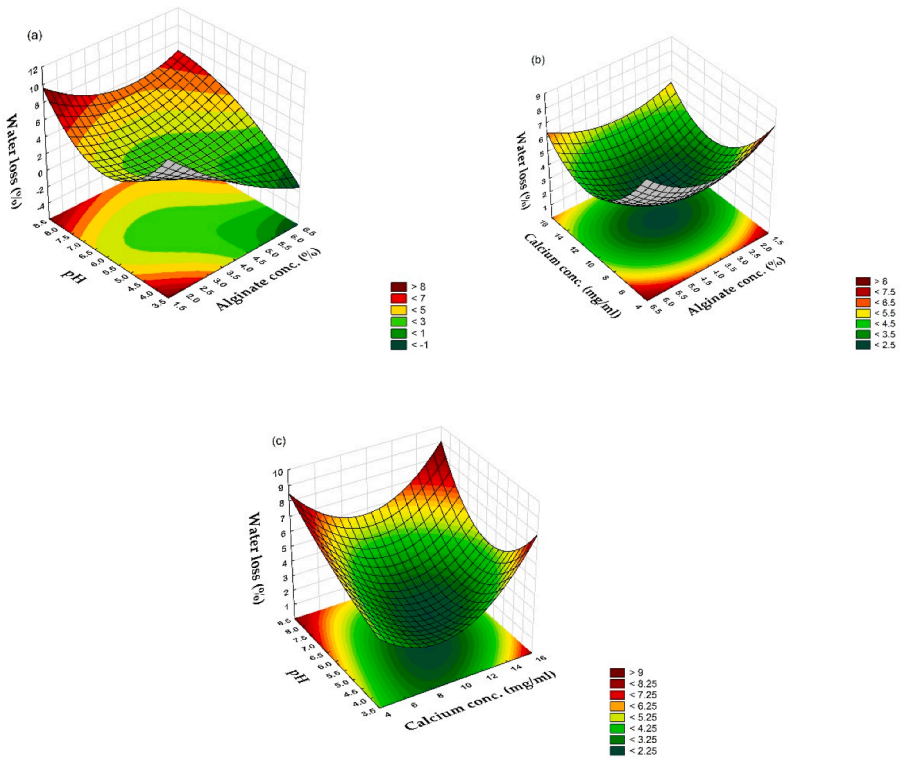

Fig. 2. Response surface graph of water loss. The effect of the composition on water loss y_2: (a) concentration of sodium alginate and pH, (b) concentration of sodium alginate and calcium, (c) concentration of calcium and pH.

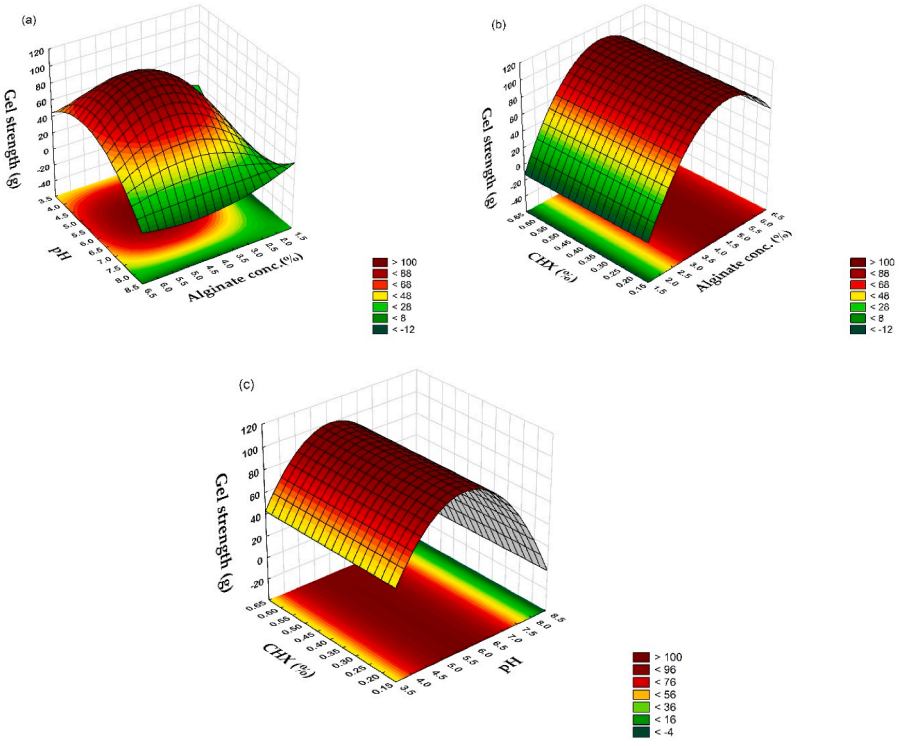

Fig. 3. Response surface graph of gel strength. Effect of the composition on gel strength y_1: (a) concentration of sodium alginate and pH, (b) concentration of sodium alginate and CHX, (c) concentration of CHX and pH.

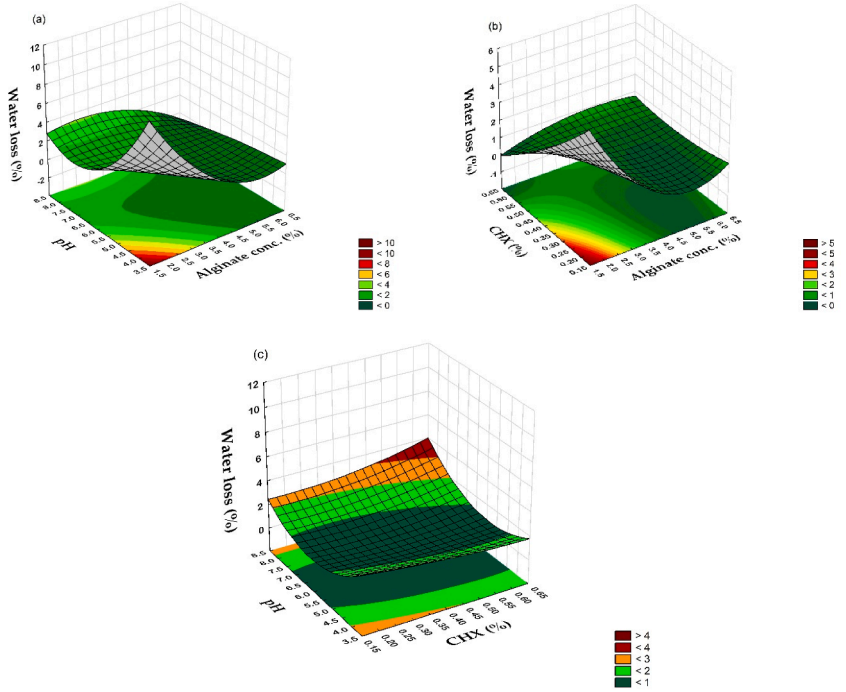

Fig. 4. Response surface graph of water loss. Effect of the composition on water loss y2: (a) concentration of sodium alginate and pH, (b) concentration of sodium alginate and CHX, (c) concentration of CHX and pH.

Table 6
Optimized formulations with compositions.

Formulation code	Polymer concentration (%)	CHX concentration (%)
F1	6	0.6
F2		0.4
F3		0.2
F4	4	0.6
F5		0.4
F6		0.2

Summarizing the above results, it can be concluded that the acidic pH can reduce the strength of the gel, and a weak gel structure is formed at low polymer content in the investigated range. Based on these, a medium pH range and a higher alginate concentration are recommended for the formation of a suitable and stable gel. Based on these statistical analyses, the appropriate formulations (B13, B14, B15, B22, B23, and B24) were chosen for further studies. Furthermore, mucoadhesive, dissolution, and antimicrobial studies were conducted to presume the optimal composition possessing the desirable attributes. Thus, optimized formulations data acquired from both factorial designs, is enlisted in Table 6. These formulations were prepared at pH 6.

3.3. Mucoadhesion

Tensile tests were performed to determine the adhesive force, that is also known as detachment force (the force required to separate the interface), and the adhesive work, calculated from the area under the curve (AUC) of the force versus distance graph (Thirawong et al., 2007). In the initial step, the surfaces were pressed together and then pulled apart, resulting in surface separation.

Duchene et al. reported that mucoadhesion is a gradual process. The

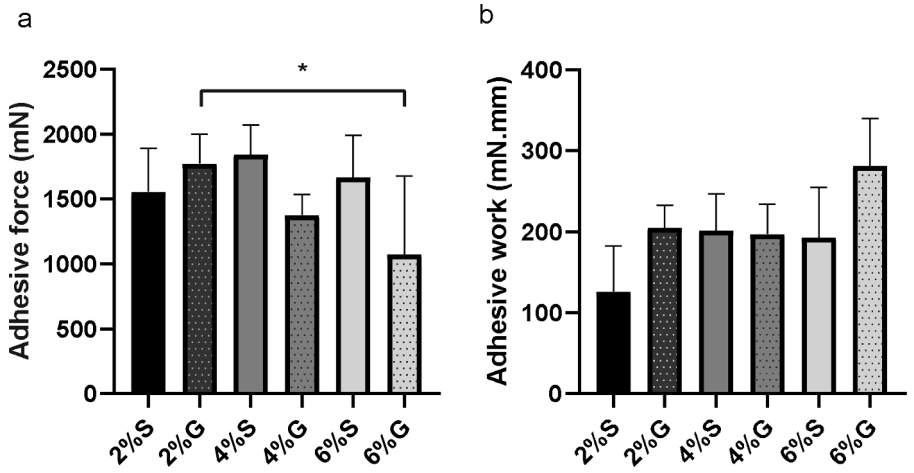

Fig. 5. Tensile strength measurement of polymer solution (S) and gel formulation (G) as a function of polymer concentration: Adhesive force mN (a) work of adhesion mN.mm (b). (Statistical data analysis: one-way ANOVA variance analysis with Tukey post-hoc test * $p < 0.05$ means significant differences).

first step involves the interaction between macromolecules and mucin, followed by swelling, physical bonding, and ultimately non-covalent bonding (Duchêne et al., 1988). In our case, the alginate gel could form both physical and chemical bonds (secondary interactions) with the mucin. The crosslinked gel ("G" formulations in Fig. 5) were compared with non-crosslinked alginate systems (polymer solution, "S" formulations in Fig. 5), and the effect of different alginate concentrations (2, 4, and 6 %, Fig. 5) was investigated.

The peak detachment force showed the highest value at a comparatively lower concentration, while the work of adhesion progressively amplified with the increase in polymeric concentration from 2 % to 6 %. Similar results were reported for peak detachment force with mucoadhesive polymer in the literature by another research group (Ishida et al., 1983). It was established earlier (Park and Munday, 2002) that the mucoadhesive force depends on the formation of chemical interactions between the functional groups of the polymer and the mucin, whereas the work of adhesion depends on the formation of both chemical and physical interactions, including entanglement and interpenetration of the mucin and polymer chains. In our work, the crosslinked and non-crosslinked systems were different. In the case of polymer solution, a maximum adhesive force was observed at 4 % alginate concentration, while in the case of crosslinked polymer structure, the possibility of chemical interaction decreased with increasing polymer concentration. This phenomenon can be explained by the formation of egg-box-type crosslinks, which may reduce the interaction between the polymer and mucin functional groups. It is also a potential explanation for why the crosslinked systems presented lower adhesive force values. In contrast, the results were different for the adhesive work measurement, where increasing polymer concentration resulted in higher adhesive work values and the crosslinked structure presented superior mucoadhesion. This observation suggests that the crosslinked structure promotes the formation of physical bonds, leading to improved mucoadhesiveness.

3.4. In vitro drug release

In vitro drug release studies can provide insights about the mechanism of drug release. Franz cell diffusion system was used for three parallel measurements on 4 % (*w/w*) and 6 % (*w/w*) optimized gel formulations containing 0.2 %, 0.4 %, and 0.6 % of CHX. Fig. 6 displays the cumulative amount of drug released over a period of time.

The cumulative release profile of CHX in all the formulations indicated a sustained release effect over an extended period of time. The drug was released rapidly (burst effect) within the first two hours, followed by a sustained release over a prolonged time period. The initial fast release can ensure that a therapeutic concentration was reached at the beginning of treatment, and later, the continuous drug release can maintain it. The results obtained were as expected, with smaller amounts of CHX incorporated resulted in smaller amounts of drug released. The highest amount was released from the 0.6 % CHX gels. The alginate concentration did not influence the drug release, as no significant differences were detected between the formulations with different alginate concentrations.

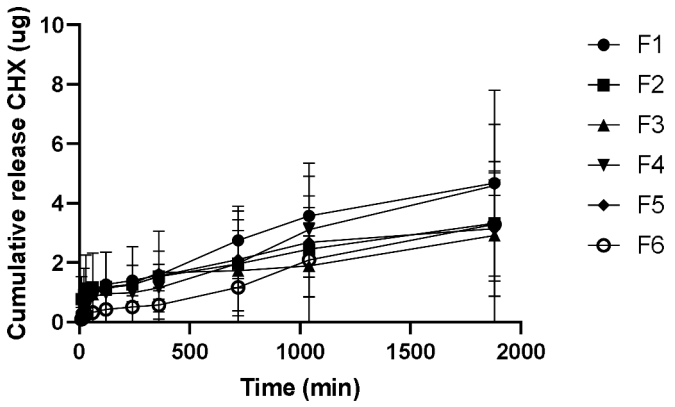

Fig. 6. Release profile of CHX from gels using a Franz diffusion cell.

Table 7
Release kinetics of CHX from the optimized formulations.

Formulation code	Zero order	First order	Higuchi	Korsmeyer–Peppas	
	R^2	R^2	R^2	R^2	n
F1	0.95	0.89	0.98	0.99	0.53
F2	0.98	0.34	0.89	0.90	0.42
F3	0.85	0.27	0.74	0.95	0.54
F4	0.91	0.91	0.93	0.98	0.67
F5	0.50	0.40	0.94	0.98	0.44
F6	0.98	0.98	0.87	0.98	0.90

Furthermore, the drug release data were analyzed using four different kinetic models, such as zero order Eq. (6)), first order (Eq. (7)), Higuchi (Eq. (8)), and Korsmeyer–Peppas (Eq. (9)), with the purpose to analyze the best fitting kinetic equations given by Eqs. (6)–((9).

$$Qt = k_0\, t \tag{6}$$

$$\log Q_t = \log Q_0 - k_1\, t \tag{7}$$

$$Q_t = k_H\, t^{1/2} \tag{8}$$

$$M_t/M_\alpha = Kt^n \tag{9}$$

The detailed release kinetic data of all polymeric systems is shown in Table 4. In order to investigate the release mechanism from hydrogel systems, Korsmeyer–Peppas model assumed to be the best fit model (Korsmeyer and Peppas, 1984), as it was also observed in our results (Table 7).

Korsmeyer et al. established a relationship to elaborate drug release patterns mainly in polymeric systems. M_t/M_α is the drug release or drug diffusion fraction expressed as a power function of time (*t), k* is a rate constant, and n is the release exponent to characterize the transport mechanism of drugs through polymeric matrix systems (Paarakh et al., 2018).

Hydrogel systems absorb water and help the polymer to swell. The dispersed drug started to diffuse into external medium. Molecular relaxation and diffusion describe the characteristic release kinetics of non-Fickian behavior. Regarding the obtained results, the value of *n* is generally greater than 0.5, indicating the expression of anomalous or non-Fickian diffusion behavior. This behavior suggests the involvement of two mechanisms, namely relaxation and erosion (Lee, 1985).

Table 8
Microbial activity of the optimized formulations against *A. actinomycetemcomitans*.

Days	Mean diameter of the inhibition zone (mm)		
	F6	F4	F1
1	13.5 ± 0.5	13.5 ± 0.6	15.2 ± 0.9
2	17.3 ± 0.1	16.7 ± 1.2	19.2 ± 0.4
3	16.9 ± 1.6	17.9 ± 0.5	20.1 ± 0.1
4	18.4 ± 0.9	19.7 ± 0.5	20.9 ± 1.2
5	18.2 ± 1.1	18.9 ± 0.5	19.3 ± 1.0
6	17.6 ± 0.7	19.1 ± 0.5	19.5 ± 0.2
7	17.8 ± 0.9	20.0 ± 0.4	20.4 ± 0.5
8	17.8 ± 1.2	19.2 ± 0.3	20.0 ± 0.9
9	17.7 ± 0.9	19.6 ± 0.2	19.9 ± 0.8
10	17.9 ± 0.8	18.3 ± 0.7	20.3 ± 0.7
11	18.8 ± 0.2	20.2 ± 0.7	19.3 ± 1.0
12	18.3 ± 0.6	20.1 ± 0.7	19.8 ± 0.8
13	17.2 ± 0.6	20.2 ± 0.5	20.2 ± 0.4
14	17.6 ± 0.9	20.1 ± 0.5	20.3 ± 0.9

Table 9
Microbial activity of the optimized formulations against *S. mutans*.

Days	Mean diameter of the inhibition zone (mm)		
	F6	F4	F1
1	19.0 ± 0.6	18.8 ± 0.2	19.0 ± 1.1
2	21.4 ± 0.2	22.4 ± 0.8	22.3 ± 1.4
3	21.1 ± 0.2	21.8 ± 0.5	21.8 ± 1.5
4	21.7 ± 0.2	22.3 ± 0.5	22.2 ± 0.6
5	21.3 ± 0.7	22.3 ± 0.3	21.3 ± 0.9
6	20.3 ± 0.9	21.2 ± 0.9	20.8 ± 0.2
7	23.6 ± 0.4	24.1 ± 0.2	23.2 ± 0.8
8	20.8 ± 0.7	21.1 ± 0.3	20.9 ± 1.0
9	21.2 ± 0.4	21.8 ± 0.1	21.3 ± 1.1
10	23.1 ± 0.1	23.4 ± 0.5	23.3 ± 0.5
11	24.1 ± 0.7	25.0 ± 0.1	24.9 ± 0.3
12	22.3 ± 0.7	23.9 ± 0.1	23.3 ± 0.3
13	21.8 ± 0.6	23.3 ± 0.4	22.3 ± 1.7
14	22.4 ± 0.8	23.6 ± 0.8	22.6 ± 1.8

3.5. Antimicrobial studies

The zone of inhibition of *Streptococcus mutans* and *Aggregatibacter actinomycetemcomitans* was determined by agar diffusion method to verify the effectivity of the prepared gel systems. After incubating the first sample for 24 h, the zone of inhibition was determined before shifting the disk to other agar media. The process was repeated for a duration of 14 days, until the gel started to lose its initial intact shape. After consecutive 14 days of experiment, the mean diameter of zone of inhibition was calculated. The results indicated a significant growth inhibitory effect against both strains. The CHX gel formulations containing 4 % and 6 % gelling agent and 0.6 % active content exhibited a notable antibacterial effect in comparison to the blank polymeric formulation, which was used as control, where no inhibition zone was detected. The zone of inhibition for both strains were calculated in triplet. The diameter of the inhibition zone for 4 % polymeric gel was found to be 23.6 mm and 18.7 mm against *S. mutans* and *A. actinomycetemcomitans* respectively. While in case of 6 % formulation, it was 22.6 mm and 19.3 mm. Results showed slightly different activity against both strains, but the activity was approximately the same for the 4 % and 6 % polymeric formulations containing same amount of active moiety. It is suggested that the 4 % polymeric solution containing 0.2 % CHX had similar results in the case of *S. mutans* in comparison with other two formulations. But in relation to *A. actinomycetemcomitans*, the zone of inhibition started to decrease after a week (for the formulation that contained a lower amount of CHX), whereas the formulations with 0.6 % CHX maintained their effectiveness, as shown in Tables 8 and 9.

Overall, a significant and prolonged antimicrobial effect was recorded for two weeks, which supported the results of the *in vitro* drug release, where slow and continuous release was observed. Antimicrobial activity for a longer period of time is advantageous over commercially available mouth rinse and gels that typically require daily administration for 4–6 weeks in periodontal treatment (Zhang et al., 2019b).

4. Discussion

Alginate, a biodegradable natural polymer, is extracted from brown algae through several steps using sodium, aluminium hydroxide or sodium carbonate in an alkaline medium (Goh et al., 2012). Sodium alginate is a polyanionic copolymer composed of two uronic acids, β-D-manuronic acids (M) and α-L-glucuronic (G), linked by glycosidic bonds. The ratio and length of M and G units in the alginate structure depends on the source species of brown algae from where it is obtained (Lee and Mooney, 2012).

Alginate is considered as a mucoadhesive polymer with pseudoplastic rheological properties. The presence of free carboxyl and hydroxyl groups facilitate quick and permanent adhesion to the mucosal surface (Khan et al., 2015). Besides, alginates have favorable spreading, wetting, and swelling capabilities. They are biocompatible, viscoelastic, and have no any reported toxic or irritating effects.

CHX has an established antibacterial and healing effect in case of periodontitis. A clinical study was conducted in 40 periodontal pockets of 10 patients for the evaluation of bacteriocidal activity of CHX, stannous fluoride, and amine fluoride gels. A considerable reduction of microbial count was reported with CHX gel and stannous fluoride gel. It was documented that 2 % CHX gel was responsible for 99 % reduction in the microflora present in periodontal pockets. While the researchers were able to achieve the desired bactericidal effects with CHX gel but a disadvantage of this study was the frequent irrigation of periodontal pockets with the gel. After every 3 min the gel was applied in the cavity to ensure its maximum contact time and pocket irrigation (Oosterwaal et al., 1991). It is plausible from the above stated clinical study that CHX therapy is a practical solution for periodontitis. Another study speculated the significant healing effect with CHX gel after consistent application of CHX for two months, in clinical setup (Mohammad et al., 2023).

Ji et al. formulated a thermosensitive hydrogel loading 0.1 % chlorhexidine (CHX) for periodontal treatment. The hydrogel was prepared from chitosan, quaternized CS, and α, β-glycerophosphate. Researchers found that the gelling time was 6 min, while the release duration extended to over 18 h (Ji et al., 2010). Furthermore, 1.5 % CHX was incorporated into gum based gel formulation for chronic periodontitis. The results showed the gradual degradation of gel within 10–30 days in the periodontal pocket (Jain et al., 2013).

Recently, Zhou D and co-workers formulated thermosensitive hydrogel for sustained release of CHX as well as better cytocompatibility. Desirable therapeutic effect on periodontal pathogens were confirmed by antibacterial studies on *Staphylococcus aureus* (*S. aureus*) and *Escherichia coli* (*E. coli*) (Zhou et al., 2021).

CHX polymeric nanoparticles were loaded into *in situ* gel-forming using a design of experiment (Box–Behnken design) to optimize the nanoformulations for size, PDI, and zeta potential. Later on, the optimized of CHX-NPs were loaded into a thermosensitive ISGF and evaluated for physical appearance, gelling temperature, gelling time, and viscosity. The optimal formulations reported a prolonged release of active agent (7 days) along with potential antibacterial activity (Suriyaamporn et al., 2023).

Periodontal pockets act as a natural reservoir filled with gingival crevicular fluid (an inflammatory exudate). Upon contact with gingival fluid in the periodontal cavity polymers swell to form a strong gel for prolonging the residence time of loaded substances. There are various mechanisms involved for the instant gelation of the polymeric solution in the pockets. In case of ionic polymers like sodium alginate, electrolytes are inevitably found in the fluids of the oral cavity such as Ca^{2+} that are held responsible for ionic strength of the resultant gel (Baranov et al., 2021). This is the reason that we select calcium concentration as an independent factor in our first factorial design to evaluate the gel strength and to determine the amount needed to administer along with polymeric solution in two-compartment syringe. Although the saliva in oral cavity has calcium ions but to ensure an instant gelation, a sufficient supply of calcium solution will be provided externally.

In our research work we incorporated an antiseptic drug CHX into an optimized *in situ* gelling system of considerable bloom strength (firmness and softness) and negligible water expulsion on gel formation to contribute for a long-lasting (minimum 1 week) effect. The main prerequisites of our formulation are gelling strength and water loss. For easy instillation at the affected site, *in situ* gelling system must possess optimum strength and bioadhesivity to stay intact and longer in the cavity, which undergoes a rapid sol-gel transition due to ionic interaction. The ionic sol to gel transformation occurs in the presence of cations, in our formulations the calcium was a source of cations for internal ionotropic gelation of sodium alginate.

The bioadhesivity of the formulation on the mucous surface is a crucial parameter to ensure the retention of optimized formulations

(Nair et al., 2021). The gel strength is an important physicochemical property that needs to be evaluated to maintain the structure integrity within the periodontal cavity. It has been observed that the gel strength increases with the rise in mucoadhesive polymer concentration (Bansal et al., 2009).

Although the gel strength and mucoadhesivity of gelling system are frequently investigated and reported in literature, while the elimination of water (and thus the API) from gelling systems during the gelation process along with gel strength at the time of gelation, are not widely studied. Water loss is an important factor to consider when determining the total drug amount in the system. As a result of polymer crosslinking with ions, a certain amount of water with API escapes from the gel structure immediately, potentially leading to the formation of an unstable structure. Excessive water loss may cause the loss of active ingredients from system and decreasing bioavailability. On the other hand, negligible water loss is not a critical factor to take into consideration.

In our work two full factorial statistical designs were applied to assess the key factors that presumably strengthen the gel structure and water bounding ability (lower the water loss). Thorough examination of the absolute coefficient values revealed a clear picture of the effecting factors. On the basis of the two factorial designs, it was determined that alginate, calcium concentration, and pH of the systems are significant factors influencing the gel strength and water loss, whereas the CHX concentration did not affect the gel characteristics. Based on the findings, it could be interpreted that the most important factor was sodium alginate concentration, while pH took the second place. Considering the pH tolerance of the system, a neutral pH can be the most appropriate, the strongest gel structure could be observed at 6–7 pH, which correspond to the pH of the periodontal pocket. The factorial model indicated that sodium alginate at a medium concentration resulted in better gel strength and showed a higher potential for forming a strong gel in the presence of electrolyte, thereby contributing to less water and API loss.

Formulations that possess good mucoadhesive properties are more prone to adhere to the periodontal pocket, facilitating the prolonged release of the active ingredient from the local gel structure over an extended period of time. Another advantage of this local delivery system is the reduction in the possibility of drug elimination, leading to enhanced bioavailability. All of these characteristics ultimately contribute to improved patient compliance.

If the gel structure has low viscosity, it tends to remain in the pocket for a shorter period of time, resulting in a reduced bioavailability. However, the chances of (gel and mucin containing layer) interaction and longer residence time are higher when the gel is closer to the epithelial layer. Free carboxyl and hydroxyl groups in sodium alginate play a vital role in mucoadhesion properties. Electrostatic repulsive forces occurred between sialic acid and sulfate groups of mucin and carboxylic groups of sodium alginate under physiological environment that lead to intra- and intermolecular hydrogen bonding between mucosa and alginate. Initially, wetting and swelling of the polymer occur upon contact with mucin, and later, interpenetration of polymeric chains and polymer through hydrogen bonding facilitates the mucoadhesion. This mucoadhesive nature of sodium alginate contributes to increase contact time, gel adhesion and bioavailability of active compound in the periodontal cavity. Moreover, the additional need of mucoadhesive agent can be dismissed. Mucoadhesivity of our gelling system was determined in the laboratory by texture analyzer with the use of artificial mucin membrane (Frent et al., 2022).

Hence the local *in situ* gelling system not only allows the release of the drug when placed in the periodontal pocket but also maintains relatively constant concentration of the drug. Such bioadhesive formulations are difficult to remove by salivary secretion and, therefore, cannot be swallowed by the patient as well.

In our work the adhesive work was directly dependent on the concentration of polymer, while the adhesive force reached to its maximum level with just 2 % alginate concentration. At lower polymer concentrations, non-covalent bonds are formed between the polymeric functional groups and surface glycoproteins. Due to the egg-box type cross-linking, it occupied almost all the available free groups and reached saturation level even at lower concentrations. As the concentration increased, a gradual decrease in mucoadhesive force was observed which supports the theory of chemical bonding between surface groups and the absence of interpenetration (Horvát et al., 2015; Thakre and Singh, 2018). In contrast, with the increase in polymer concentration, the adhesive work continued to rise due to the involvement of other physical phenomena alongside chemical bonding. This indicated that the formation of crosslinks promoted the occurrence of physical interactions. Ponchel and co-workers suggested that physical entanglement and interpenetration play significant roles as crosslinking mechanisms in mucoadhesion. As the concentration of polymer increased, the swelling of system amplified, that contributed to a higher degree of physical interaction between the mucus and the polymeric system. Consequently, an augmented work of adhesion was observed (Ponchel et al., 1987).

A literature survey revealed multiple mechanisms of drug release from hydrogels, namely, diffusion, swelling, physical, and chemical response mediated release (Abasalizadeh et al., 2020; Danafar et al., 2014). The pores of hydrogels absorb the water and release the drug through diffusion (Kamata et al., 2015). This biphasic pattern of the drug release promote the initial burst effect of hydrated polymer that could be controlled and considerably reduced with the concentration of gelling agent (Londhe and Sharma, 2022). Initially, the drug was released from the less dense or crosslinked part of the gelling system, while the tightly bound part of the gel structure hampered the rapid drug release. This mechanism ensured the sustained release of the active ingredient. It was also observed from the curve that the system with a high concentration of CHX released the most part of active ingredient over time, which was consistent with the findings reported by Babickaite et al. (2016). Moreover, it could be perceived that a higher concentration of polymer led to a tighter gel structure, which directly correlated with the concentration of the gelling agent. This finding was in agreement with the results published by Ahuja et al. (2008), Mekkawy et al. (2013). It was apparent form the *in vitro* release study that the 4 % and 6 % alginate systems formed a tighter structure and exhibited almost identical release profiles that aligned with our antimicrobial study measurements. Harish and colleague researchers mentioned that increasing the concentration of polymer led to higher viscosity in the formulation system. The chemical crosslinking of gelatin generated 3D polymeric hydrogel structure with water filled spaces (Ghobril and Grinstaff, 2015). A relatively higher concentration of polymeric solution provoked the formation of hydrogel in a comparatively shorter time. This was attributed to the increased availability of additional bonding sites per chain and a reduction in free space, as reported by Normand et al. (2000). These properties contributed to the formation of a stable and stiff gelling system. On the contrary, although a higher content of sodium alginate ended in a more viscous gel, but it posed difficulties during injection. While gels with low viscosity are more flowy in nature and also contribute to excessive water loss, such systems are not ideal for *in situ* gels (Harish et al., 2009). Thus, more weightage was given to the 4 % sodium alginate formulation which appeared to be appropriate for achieving the same effect of sustainability as obtained from the 6 % alginate formulation that is evident from our experimental results. In order to limit the use of higher concentration of gelling agent and to enhance the ease of administration, the 4 % alginate system was considered as the optimized formulation.

Based on the microbiological test results, it was vivid that the release of the active ingredient from the complex system could be controlled for a longer period of time as compared to conventional rinses and endodontic irrigants. Moreover, it can be concluded that CHX mouthwash is not effective for moderate and severe periodontitis as it lacking the ability to penetrate deep into the cavities (Bescos et al., 2020; Brookes et al., 2020). Conventional dosage forms are required to be administered

multiple times a day, whereas medicated gelling systems provide a long lasting antiseptic effect against harmful bacteria (Babickaite et al., 2016). Major challenges linked with CHX mouth rinse are (i) a large amount of formulation needed in repeated manner, (ii) lack of penetration into deep periodontal pockets, and (iii) inefficient to provide persistent and prolonged antibacterial and antiseptic effect at the site of action.

The current *in situ* gelling system containing CHX indicated a prolonged antimicrobial effect for up to 2 weeks; thereafter, it gradually disintegrated into smaller fragments leading to an easy elimination from the periodontal pocket. Full factorial design optimization and experimental results are encouraging to formulate sol to gel CHX local delivery system.

5. Conclusions

Knowing the *in situ* gelling nature and the limitation of alginate systems, we have named all the processes/properties that impair the effectiveness of the preparation. In our factorial design, we tried to minimize these, so by examining the different variables and clarifying the interactions between them, we were able to provide an optimized composition, which may be suitable for creating a favorable local therapeutic effect in periodontitis according to the specified criteria.

CRediT authorship contribution statement

Rabia Ashfaq: Software, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization. **Bianka Sisa:** Investigation, Formal analysis. **Anita Kovács:** Methodology, Writing – review & editing. **Szilvia Berkó:** Methodology, Writing – review & editing, Resources, Funding acquisition. **Mária Szécsényi:** Methodology, Investigation. **Katalin Burián:** Methodology, Investigation, Resources. **Péter Vályi:** Conceptualization, Methodology, Writing – review & editing. **Mária Budai-Szűcs:** Conceptualization, Methodology, Formal analysis, Writing – original draft, Writing – review & editing, Supervision, Project administration.

Data availability

Data will be made available on request.

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Supplementary materials

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