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Mucoadhesive alginate pastes with embedded liposomes for local oral drug delivery

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ABSTRACT

Oral cancers are extremely common among adults with increasing incidences due to human papillomavirus, while treatment modalities are limited. This study aims to develop a new oral mucoadhesive delivery system based on the combination of alginate and liposomes. The polymer provides adhesion properties and induces local release of the drug-loaded carriers, while the liposomes protect the drug from degradation and improve its absorption into the cells. Three hybrid alginate/liposomes delivery systems were investigated: a hybrid paste, which presented excellent adhesive capabilities, yet fast burst release of 90% after 2 h; a hybrid hydrogel, demonstrating controllable release rates of 5%, 30% or 60% after 2 h but poor mucoadhesive properties. These findings led to the development of a hybrid cross-linked paste. Polymer retention studies demonstrated that 80% of the crosslinked paste was retained on tongue tissue compared to 50% retention of the non-cross-linked pastes, verifying its superior mucoadhesion. The hybrid cross-linked paste presented controllable release rate of 20% after 2 h. Alginate paste incorporating doxorubicin loaded liposomes presented similar release rates and were highly effective in promoting cancer cell death. Thus, our innovative formulation, including both desired characteristics of mucoadhesion and sustained liposomes release, is an important milestone in the development of a new potential treatment for oral cancer.

1. Introduction

Oral cancer is the sixth most prevalent cancer worldwide. Specifically, squamous cell carcinoma (SCC) accounts for over 90% of all head and neck cancers, and the overall survival rates are only 40–50% [1,2]. Over the past decade, oral cancers incidences have risen by 35%, with limited treatment modalities [3]. Administering anti-cancer agents in close proximity to cancerous lesion has proven to be clinically effective when dealing with head and neck tumors [4]. However, no drug delivery system of anti-cancer agents, for controlled administration to the oral cavity, exists in the clinic [5,6]. Currently, the main treatment for oral cancer involves aggressive treatments by surgery and radiotherapy [7]. There is a significant need for the development of a local drug delivery system to reduce cell dissemination and tumor size, and to minimize surgical resection, which results in loss of facial morphology followed by reconstructive surgery.

Drug delivery through the oral mucosa, owing to its close proximity to the diseased tissue, could potentially serve as a promising route for oral cancer treatment. *Trans*-mucosal drug delivery involves transport of therapeutic agents through the mucus, a moist gel layer that lines organs which are exposed to the outer surface of the body, yet not covered with skin [8]. This mode of delivery offers multiple benefits over oral or intravenous administration, especially when dealing with lesions of the oral cavity. For example, mucoadhesive drug delivery facilitates rapid circulation of drugs in the local capillaries. In addition, it enables enhanced bioavailability, resulting from partial avoidance of the body's natural defense mechanisms and first-pass metabolism [9–12].

Mucoadhesive polymers are proficient attaching substances to the mucosal surfaces, providing prolonged residence time of drugs at the application site [9,10,13,14]. Mucins, the main component of mucus, are glycoproteins responsible for the adhesion phenomena by their ability to form electrostatic, disulfide, hydrogen bonding, and hydrophobic interactions with other substances such as polymers [15]. The polymer's mucoadhesive properties can vary depending on the molecular weight, flexibility of the polymeric chains, hydrogen bonding capacity, cross-linking density, charge, concentration, or hydration degree of the

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polymer [16]. Widely investigated mucoadhesive polymers are hydrophilic macromolecules e.g., poly(acrylic) acid, cellulose, alginate and chitosan, capable of creating multiple non-covalent bonds with the mucin glycoproteins.

Alginates are a series of natural unbranched polyanionic polysaccharides composed of β-D-mannuronic acid (M) and α-L-guluronic acid (G) linked by a 1 → 4 linkages and arranged in M- and G-block regions combined with alternating regions (MGMG) [17]. Alginate has shown biocompatible and non-irritant properties, denoting its applicability for various biomedical applications, specifically as a drug release carrier [18,19]. Alginate is known for its ability to create hydrogen bonds with mucin-type glycoproteins through carboxyl–hydroxyl interactions resulting in mucoadhesive properties. Additional biomedical applications of alginate are: dental impression material, wound dressings, cell culture and tissue regeneration with protein and cell delivery [17]. Another major advantage is its crosslinking ability; it can be crosslinked by positively charged ions, thus achieving a controlled drug release rate from the polymer matrix.

Motivated by the need to advance the development of improved vehicles of local oral cancer treatment, we aimed to fabricate and characterize a novel bio-adhesive, controlled drug release system based on alginate and liposomes. Liposomes are vesicles with an internal aqueous core surrounded by a lipid bilayer/s, and are widely used as drug carriers [20]. They are biocompatible and biodegradable, and hold the ability to incorporate both hydrophobic and hydrophilic compounds [21]. Thus, by combining mucoadhesion with the advantages of liposomal drug delivery, e.g., sustained release rate, protecting pharmaceuticals from chemical and enzymatic degradation, and improving drug bioavailability, the way is paved for a new approach for non-invasive hybrid alginate/liposomes drug delivery vehicles [22–25]. To this date, limited formulations based on natural polysaccharides are in routine clinical use [26,27], however none of them utilizes both liposomes and polysaccharides.

The administration of sustained release formulation to the oral cavity requires it to be stable under dilution, shear flow and physiological conditions of the saliva fluids. Herein, pastes and hydrogels of hybrid alginate/liposomes systems were thoroughly investigated. Based on these studies, a novel hybrid alginate formulation was developed and designed to adhere to the oral mucosa surface and to release an anticancer drug loaded liposomes as a potential treatment for oral cancer. This formulation was further evaluated by in vitro drug release, mucoadhesion assays, stability and toxicity experiments under physiological condition of simulated saliva buffer and shear flow conditions.

2. Materials and methods

2.1. Materials

Sodium alginate HF120RBS, molecular weight of $3 \cdot 10^5$ g/mol, with G content of ~50%, was generously supplied by FMC-Biopolymer (Norway). 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) was purchased from Tzamal D-chem (Israel). Fluorescein isothiocynate (FITC), cholesterol, tetrazolium salt-3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), Dulbecco's Modified Eagle's Medium (DMEM), Dulbecco's phosphate buffered saline (PBS), glucono delta-lactone (GDL), 2-(N-morpholino) ethanesulfonic acid (MES) and N-hydroxysuccineimide (NHS) were purchased from Sigma-Aldrich (Israel). Sodium chloride (NaCl) was purchased from S.D. Fine-Chem (India). 32% Hydrochloric acid (HCl) and citric acid were purchased from Frutarom (Israel). 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC) was purchased from Lipoid (Germany). 1,2-dimyristoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl) (ammonium salt) (14:0 Liss Rhodamine PE) was purchased from Avanti Polar Lipids Inc. (USA). Calcium chloride ($CaCl_2$) was purchased from J.T. Baker (USA). Cyanoacrylate glue was purchased from ZAP (Taiwan). Barium chloride ($BaCl_2$) was purchased from Alfa Aesar (England). Ethanol (EtOH), methanol (MeOH), chloroform, acetone and ethylene glycol were purchased from Bio-Lab ltd. (Israel). Ethylene glycol tetra acetic acid (EGTA) was purchased from Strem Chemicals (USA). Potassium chloride was purchased from Nile Chemicals (India). Potassium phosphate monobasic, sodium phosphate dibasic and potassium thiocyanate were obtained from Merck (Germany). Potassium bicarbonate was purchased from Loba Chemie (India). L-glutamine, penicillin, streptomycin, trypsin and EDTA were purchased from Biological Industries (Israel). Doxorubicin was generously provided by Teva Pharmaceutical Industries Ltd. (Israel). Porcine tongue was supplied by the Preclinical Research Authority at the Technion (Israel).

2.2. Liposomes preparation

Liposomes, either empty or containing doxorubicin, were prepared using the thin film hydration method. DMPC and cholesterol in molar ratio of 60:40 were dissolved in chloroform. For empty liposomes, fluorescence detection was enabled by the addition of 1% v/v of a 2 mg/mL 14:0 Liss Rhodamine PE stock solution to the chloroform solution. Then the solvent was evaporated using a rotary evaporator (BUCHI Labortechnik AG, Postfach, Switzerland) resulting in a thin lipid film. The film was hydrated with 5% dextrose (w/v) while rotating at 50 °C. The dispersion became milky indicating the spontaneous formation of lipid vesicles. Nanoscale vesicles were formed by stepwise extrusion through polycarbonate membranes (GE healthcare, Wisconsin, USA) using 400 and 200 nm pore size membranes in a 10 mL extrusion system (Northern Lipids, Vancouver, Canada) at 50°C. Liposome size was validated by dynamic light scattering (DLS).

2.3. Preparation of doxorubicin (DOX) loaded liposomes

Active liposomes encapsulation of DOX was performed by an ammonium sulfate gradient according to Haran et al. [28]. Liposome composition and thin film preparation was as described above in Section 2.2, without the addition of rhodamine. The hydration of the lipid film was performed with ammonium sulfate solution at 50 °C, followed by extrusion five times, as described above. Dialysis exchanges with 5% dextrose were used to remove the external ammonium sulfate from liposomes. After dialysis, DOX was added to the liposomes for 1 h at 55 °C while shaking. In order to remove non-encapsulated DOX the liposomes were ultra-centrifuged (150,000 × g, 1 h, 4 °C). The liposome pellet was resuspended with 5% dextrose. The percentage of encapsulated DOX within the liposomes was determined by diluting them 100 fold in methanol and measuring fluorescence (excitation 488 nm, emission 570 nm). The fluorescence was compared to a calibration curve of doxorubicin (0.01–25 μg/mL) in methanol.

2.4. Synthesis of alginate-fluorescein

FITC (100 mg, 0.256 mmol) was reacted with an excess of ethylene diamine (100 μL, 1.5 mmol) in 1 mL EtOH for 15 min at room temperature. The solvent was evaporated under reduced pressure by rotary evaporation. The crude product was re-dissolved in MeOH and filtrated through a short silica pad. The solvent was evaporated and the primary amine-conjugate to FITC was isolated as an orange-red solid. The product was verified by NMR (Figure SI1 and SI2, supplementary information).

Alginate (0.1 g) was dissolved in 10 mL of 50 mM MES buffer pH = 6.5, EDC (78 mg, 0.5 mmol) and NHS (29 mg, 0.25 mmol) were dissolved in 2 mL MES buffer, added to the alginate solution and stirred for 1 h. After 1 h, the primary amine-conjugated FITC (112 mg, 0.25 mmol) was dissolved in MES buffer and added to the reaction. The reaction mixture was stirred for 24 h at room temperature protected from light. The labeled alginate was precipitated with acetone, dissolved in 100 mL DDW and dialyzed against 1% HCl (v/v) and 1% NaCl for 1 week (fresh solution was introduced 3 times a day),

followed by freeze-drying (Labconco, Kansas, USA). Finally, a yellow-orange solid was isolated and stored at 4 °C until further use. The labeled polymer was characterized by NMR, FTIR, UV–Vis absorbance and fluorescence (Figure SI3, SI4 and SI5, respectively).

2.5. Preparation of hybrid alginate/liposomes pastes

Alginate pastes were prepared by dissolving 30, 40 or 50 mg alginate-FITC in 0.4 mL DDW and 0.6 mL of liposome solution. The mixtures were stirred until homogenous viscous solutions were obtained.

The preparation of cross-linked hybrid alginate paste was the same however after spreading the paste on the porcine's tongue tissue it was cross-linked by dropping 50 μL Ca^{+2}/Ba^{+2} cross-linking solution (mixture of 760 μL of 100 mM Ca-EGTA and 40 μL 100 mM $BaCl_2$) on it.

2.6. Mucoadhesion and liposomes release rate characterization of hybrid alginate pastes

Hybrid alginate paste (50 μL) was spread on porcine's tongue tissue ($1cm^2$) which was glued with cyanoacrylate glue to a weight and placed in a vial with 10 mL simulated saliva buffer, pH = 6.8. Simulated saliva buffer was prepared as was previously reported [29]. The vials were immersed in a shaking water/ethylene glycol bath at 37 °C and 25 rpm. An aliquot (200 μL) was withdrawn and used for quantification; it was then replaced with fresh buffer. The release of alginate-FITC and liposomes were quantified by measuring the fluorescence (alginate-fluorescein λ_{ex} = 420 nm, λ_{em} = 530 nm, liposome-λ_{ex} = 530 nm, λ_{ex} = 586 nm) of the release media at different time points. In order to correct for the influence of impurities of unreacted FITC contained in the above pastes, hybrid alginate-FITC paste without liposomes was spread on a piece of tissue and inserted into a dialysis bag. The absorbance of this sample was used as a blank for the fluorescence readings.

2.7. Mathematical modeling of liposomes release

Liposomes release from non-cross linked and cross-linked alginate paste was studied by fitting various mathematical models to the experimental data using a least-square analysis implemented in Microsoft© Excel software.

2.8. Preparation of hybrid alginate/liposomes hydrogels

Hybrid alginate hydrogels were prepared by mixing alginate solution with liposome solution and subsequently crosslinking the alginate as previously reported [30] with minor modifications. Alginate (40 mg) was dissolved in 1.8 mL DDW and 1 mL of 100 mM liposomes solution was added, followed by the addition of Ca-EGTA solution (800 μL, 100 mM) and GDL solution (400 μL, 20 mM) and stirring for 1 min. The resulting solution was placed onto a silicon ring mold (600 μL) and allowed to cure for 24 h at room temperature in a closed petri dish to avoid evaporation. Ca^{+2}/Ba^{+2} cross-linked hybrid alginate hydrogels were prepared in a similar manner by replacing the Ca-EGTA solution with a mixture of 780 μL of 100 mM Ca-EGTA and either 20 μL 100 or 200 mM $BaCl_2$.

2.9. Liposomes release rate from hybrid hydrogels

A hybrid alginate hydrogel (600 μL) was placed in a vial containing 10 mL simulated saliva buffer pH = 6.8. Next, the vial was immersed in a shaking water/ethylene-glycol bath at 37 °C and 100 rpm. An aliquot (200 μL) of the medium was used for quantification; this volume was replaced with fresh buffer. The fluorescence values of the release media at different time points were recorded at 586 nm, by using an excitation wavelength of 530 nm, attained by Tecan Infinite 200 Pro, multimodal micro plate reader, Männedorf, Switzerland.

2.10. Polymer retention study

Mucoadhesion studies of alginate and cross-linked alginate pastes were performed on porcine tongue mucosa using a home-made flow apparatus, as previously described [31]. The chamber consists of a channel, half a pipe, which was anchored on a stand at 45° angle. Frozen porcine's tongue tissue was used as a substrate. The tissue was thawed for 5 min in 100% humidity and a temperature of 37 °C prior to the measurement. 3% (w/v) Alginate-fluorescein paste or cross-linked 3% (w/v) alginate-fluorescein (50 μL) were placed on the piece of tissue (1.5 cm × 3.0 cm) and allowed to incubate in the dark at 37 °C and 100% humidity for 30 min. Next, simulated saliva buffer (pH = 6.8) was dripped onto the substrate at a constant rate of 2.25 mL/min (total volume of at least 17 mL), using a syringe pump. The collected liquid, aliquots of ~1 mL each, were fluorescently measured using a Tecan plate reader

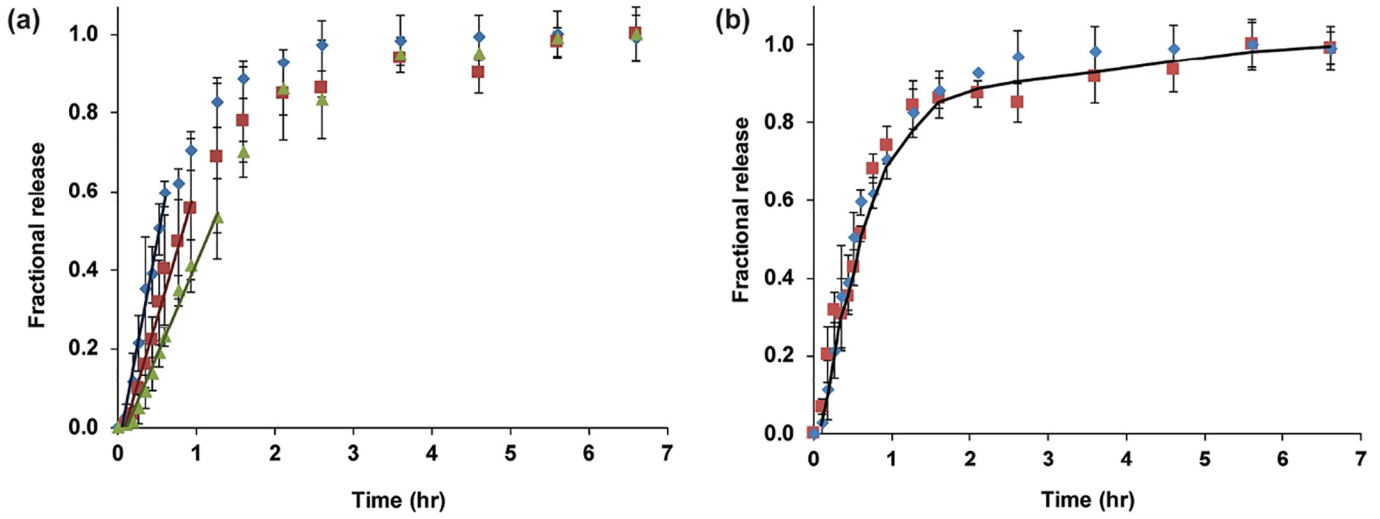

Fig. 1. (a) Fractional release of liposomes from alginate pastes with different alginate concentrations in simulated saliva buffer vs. time, pH = 6.8, at 37 °C. (♦) 3% (w/v) alginate paste; (■) 4% (w/v) alginate paste and (▲) 5% (w/v) alginate paste. The solid lines are best fits to the Korsmeyer-Peppas power law. (b) Fractional release of (♦) liposomes and (■) polymer chains from 3% (w/v) alginate paste into saliva simulated buffer vs. time, pH = 6.8, at 37 °C. The line was added as a guide to the eye.

($\lambda_{em} = 530$ nm, $\lambda_{ex} = 420$ nm). The labeled polymer concentration was calculated using a calibration curve of the labeled polymer in simulated saliva buffer following precise measurement of its volume. All measurements were performed in triplicates.

2.11. Dynamic light scattering (DLS)

Liposomes size distribution was measured by DLS using a Malvern ZSP. Stability of the liposomes was evaluated by measuring the particles size during liposomes release experiment at different time points. DLS data of liposomes samples were compared to measurements of stock liposomes solution. The reported particles size is based on number distribution.

2.12. Small angle x-ray scattering (SAXS)

SAXS experiments were performed as previously described by Josef et al. [32] using a Molecular Metrology SAXS system equipped with a sealed microfocus tube (MicroMax −002 + S) emitting CuKα radiation. The scattering patterns were recorded by a two dimensional position-sensitive wire detector (Gabriel). The studied solutions were sealed in thin-walled glass capillaries and measured under vacuum at 37 °C. The scattered intensity I(q) was recorded where q is the scattering vector defined as $q = 4\sin(\theta)/\lambda$, 2θ the scattering angle, and λ the incident wavelength. The examined samples were: 100 mM liposomes in 5% dextrose, 3% (w/v) alginate hydrogel solution with and without liposomes (25 mM) which was cured overnight within the capillary.

2.13. Cell culture

Human tongue squamous cell carcinoma cell line, CAL-27 was kindly provided from Prof. Israel Vlodavsky's lab originally purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in DMEM supplemented with 10% (v/v) FBS, 2 mM L-glutamine, 100 Units/mL penicillin and 100 μg/mL streptomycin in a humidified atmosphere containing 5% (v/v) CO_2 and 95% (v/v) air at 37 °C. Cells were detached by a solution of 0.25% (w/v) trypsin and 0.05% (w/v) EDTA and split every 2–3 days to maintain cell growth.

2.14. Toxicity of drug loaded hybrid polymer/liposome system to cells

Hybrid alginate gels incorporating either empty or DOX loaded liposomes were prepared as described above, in a volume of 100 μL each with liposomes proportion of 6.7%, 16.7% and 33.3% (v/v). The final concentration of DOX in each gel was 30, 70 and 130 μg/mL, respectively. In preliminary experiments this concentration range of DOX promoted cancer cell death (results not shown). In order to determine the toxicity of this drug loaded hybrid systems to CAL-27 cells, cells were initially plated in 24-well plate in density of 50,000 cells per well in a total volume of 500 μL medium. After 24 h, the medium was replaced and alginate gels (control, with empty liposomes, or with DOX loaded liposomes) were placed in the cells for 24 and 48 h. Alginate pastes with DOX loaded liposomes were applied by smearing onto the side walls of the wells with a spatula (50 μL paste on each well) and then 100 μL of medium was added. Viability was measured using the MTT assay. After 24 or 48 h of treatment, the medium was aspirated, and 100 μL of MTT 1 mg/mL in PBS were added to each well. After MTT addition, the plates were covered and returned to the incubator at 37 °C for 2 h, the optimal time for formazan product formation. After two hrs of incubation, the formazan product was dissolved by adding an amount of MTT solubilization solution, and the plate was incubated for 1 h for dissolution enhance. The absorbance was measured at 570 nm and the background absorbance measured at 690 nm. All experiments were done in quadruplicates.

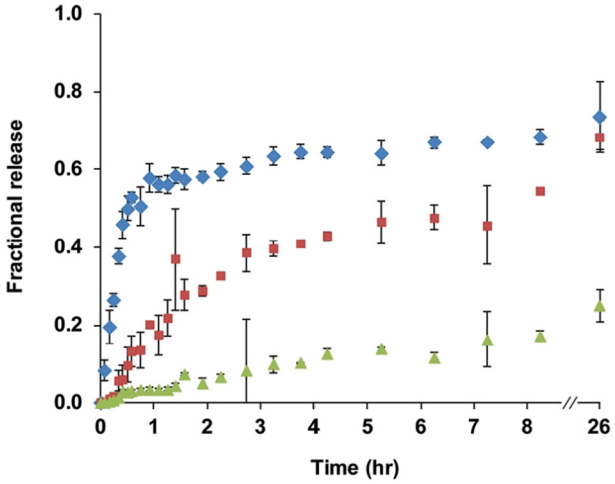

Fig. 2. Fractional release of liposomes from hybrid alginate hydrogels with different cross-linker compositions: (♦) (19.5 mM) Ca^{+2}; (■) (19.5 mM) Ca^{+2}/(0.5 mM) Ba^{+2} and (▲) (19.5 mM) Ca^{+2}/(1 mM) Ba^{+2} cross-linked alginate hydrogels in simulated saliva buffer vs. time, pH = 6.8, at 37 °C.

2.15. Statistical analysis

Data from independent experiments were analyzed for each variable using Microsoft© Excel. Comparisons were made with analysis of variance (ANOVA). A p-value of <0.05 was considered to be statistically significant. Standard errors of the mean were calculated and presented for each treatment group.

3. Results and discussion

3.1. Hybrid alginate pastes

The goal of this research was to develop an innovative, stable hybrid alginate/liposomes paste system for controlled drug release in the oral cavity. For this purpose, we selected a mild preparation process which does not include drying and/or compression molding, assuming that it will retain the liposomes' structures due to the high water content of the pastes in comparison to dry systems such as tablets and films. Our

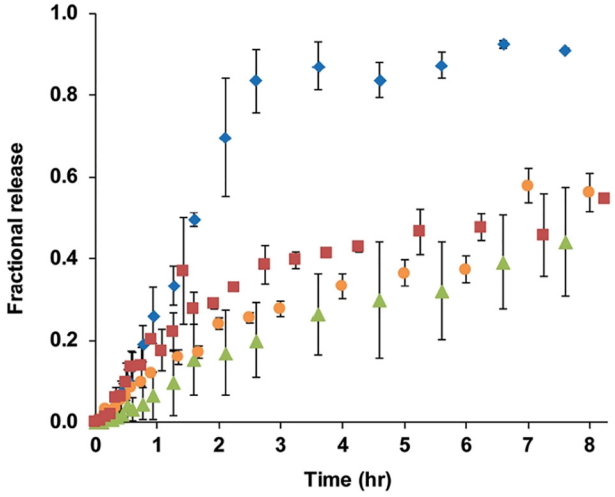

Fig. 3. Fractional release of different liposomes from 3% (w/v) hybrid alginate pastes: (♦) empty liposomes from paste, (▲) empty liposomes from (19.5 mM) Ca^{+2}/(0.5 mM) Ba^{+2} cross-linked paste, (●) Dox loaded liposomes from (19.5 mM) Ca^{+2}/(0.5 mM) Ba^{+2} cross-linked pastes and (■) empty liposomes from (19.5 mM) Ca^{+2}/(0.5 mM) Ba^{+2} cross-linked hydrogel in simulated saliva buffer vs. time, pH = 6.8, at 37 °C.

Table 1
Summary of liposome release obtained from different alginate systems.

Alginate system	Liposome release	Release index (hours to 50% liposome release)
Hydrogel:		
(19.5 mM) Ca^{+2}	Fast release	0.5
(19.5 mM) Ca^{+2}/(0.5 mM) Ba^{+2}	Sustained release	8
(19.5 mM) Ca^{+2}/(1 mM) Ba^{+2}	Negligible release	>26
Paste	Fast release	1.7
Cross-linked paste	Sustained release	8

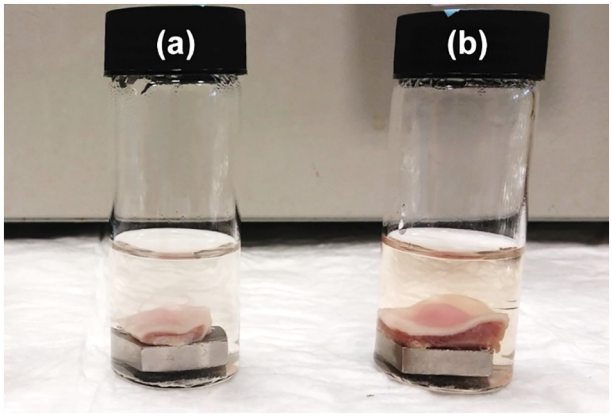

Fig. 5. The different hybrid alginate systems with Rhodamine labeled liposomes (pink) on porcine tongue: (a) paste and (b) cross-linked paste after 2 h of release experiment.

hypothesis was that these formulations will be able to adhere to the oral mucosa by formation of intermolecular interactions between the polymer and mucin glycoproteins due to high chain mobility and penetration into the mucosal membrane [33,34]. As a first step, different concentrations of alginate pastes were prepared to characterize the influence of the paste's viscosity on the release manner. Visually, all pastes adhered well to the tissue and remained adhered throughout the experiment, but gradually dissolved (data not shown).

Fig. 1a depicts fractional release of liposomes from pastes with different alginate concentrations. Most of the liposomes were released in the first two hours of the experiment. While reducing the alginate concentration in the paste accelerated the liposome release rate, this effect was insignificant (Anova, $p > 0.9$ for all three samples). Next, the experimental release profiles were fitted to an appropriate model to clarify the liposome release mechanism. Various kinetic models have been tested, leading to the conclusion that the well-known Korsmeyer-Peppas power law [35] is the most suitable one. This model is described by:

$$M_t/M_\infty = kt^n \quad (1)$$

where M_t and M_∞ are the absolute cumulative amount of drug released at time t and at infinite time, respectively, k is a constant incorporating structural and geometrical characteristics of the device, and n is the release exponent, indicative of the drug-release mechanism. The value of the release exponent (n) was found to be 1, presenting a linear correlation between fractional release and time, and a release mechanism termed super case-II transport mechanism that involves relaxation process of the polymeric chains with solvation [36]. According to this mechanism, the solvent diffusion rate inside the polymeric matrix is faster than the polymeric chain relaxation. The relaxation process also involves polymer disentanglement [37]. Further insight into the release mechanism, and support to the conclusion drawn based on the model fitting, was obtained by labeling the alginate and the liposomes with different florescence dyes which allowed detecting their release separately. Fig. 1b demonstrates that the rate of liposome release from 3% hybrid alginate paste is similar to the rate of polymer chains release from it (Anova, $p = 0.4863$). Similar behavior was observed for 4% and 5% hybrid alginate pastes (Supplementary information, Fig. SI6). Taken together, our findings suggest that the rate determining process of liposome release is the detachment of the polymer chains from the paste, meaning that the liposomes are released from the paste due to its dissolution within the buffer medium. Apparently, the polymeric network limits diffusion of liposomes due to their size, which is much larger than the mesh size of the entangled chains.

3.2. Hybrid alginate hydrogels

The turnover rate of the oral mucosal epithelium ranges from 2 to 6 days [38–40]. Therefore, our objective is to design a system that will release most of the active compound before major turnover occurs, namely within the first day after application. The experiments presented in the previous section revealed profound adhesive capabilities

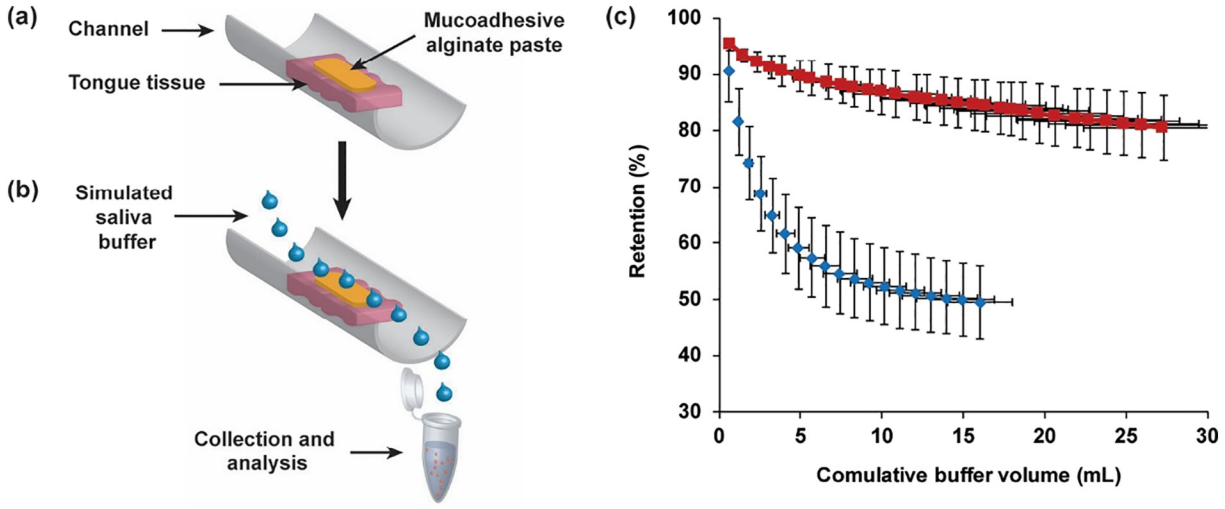

Fig. 4. Flow-through experiment for assessing mucoadhesion: (a) Preparation of the experiment, (b) Buffer flow over the sample, (c) Mucoadhesion evaluation of 3% (w/v) alginate-fluorescein (♦) paste; (■) cross-linked paste on porcine tongue.

Table 2
DLS results of liposomes size distribution after releasing from alginate hydrogels during release experiment.

	Ca^{+2} cross-linked alginate hydrogel		(19.5 mM) Ca^{+2}/(0.5 mM) Ba^{+2} cross-linked alginate hydrogel	
Time (hr)	Size (d.nm)	PDI	Size (d.nm)	PDI
stock liposome solution	136 ± 44	0.075	136 ± 44	0.075
2	126 ± 47	0.119	124 ± 62	0.221
5	134 ± 48	0.131	137 ± 40	0.080
7	124 ± 51	0.196	122 ± 52	0.167
26	130 ± 46	0.109	128 ± 46	0.118

of alginate pastes however insufficient sustaining of liposome release was observed; therefore, further studies were performed to achieve an applicable formulation for controlled drug release. Alginate hydrogels are suitable as drug carriers due to their biocompatibility and ease of cross-linking [30,41,42]. Thus, the release of liposomes from hybrid alginate hydrogels incubated in simulated saliva buffer was examined. Fig. 2 shows fractional release of liposomes from hybrid alginate hydrogels with different cross-linker compositions. Calcium ions are the most common crosslinker for alginate and therefore were examined first. We found that Ca^{+2} cross-linked hybrid alginate hydrogels presented a burst release where 60% of liposomes were released in the first hour of experiment. This rate is considered to be too fast for the suggested application [43–45]. Since the release from alginate pastes is induced by its dissolution rather than liposome diffusion, it stands for reason that this mechanism will be also valid for hydrogels that are characterized by even denser polymeric network. Thus, in order to sustain the release, delaying the degradation rate of the polymeric matrix is required. Different compositions of alginate gels were prepared according to this principle and liposomes release rate was measured over time. All hydrogel formulations were cross-linked mainly with Ca^{+2}, while adding minute quantities of Ba^{+2} to the Ca^{+2}/GDL pre-gel solution (Fig. 2). Ba^{+2} is a metal ion with high affinity to alginate resulting in a stronger binding complex compared to Ca^{+2} [46,47]. Ca^{+2}/Ba^{+2} cross-linked hydrogels slowly degraded over time, thus resulting in decreased liposome release rate in comparison to Ca^{+2} cross-linked hydrogels, denoting that the liposome release rate can be regulated by the addition of diverse concentration of Ba^{+2} (Fig. 2). Indeed, hydrogels cross-linked with (19.5 mM) Ca^{+2}/(0.5 mM) Ba^{+2} and (19.5 mM) Ca^{+2}/(1 mM) Ba^{+2} cross-linked hybrid alginate hydrogels presented sustained release where 20% and 3% of the liposomes were released in the first hour of experiment, respectively. During the first 8 h the release from the Ca^{+2}/(0.5 mM) Ba^{+2} cross-linked gel was significantly lower than the release from the Ca^{+2} cross-linked gel (Anova, $p = 0.0459$).

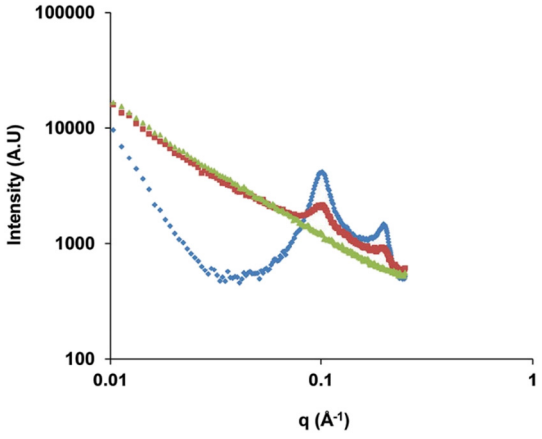

Fig. 6. SAXS data collected for: (♦) 100 mM liposomes solution; (■) 3% (w/v) hybrid alginate hydrogel with 25 mM liposomes; (▲) 3% (w/v) alginate hydrogel.

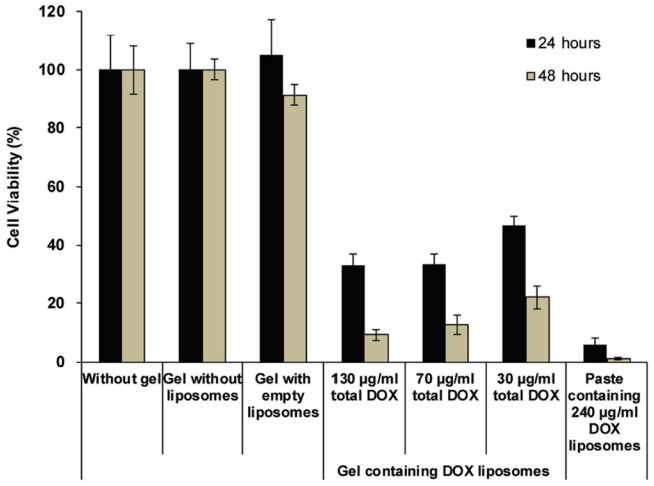

Fig. 7. Cell viability of CAL-27 cells in the presence of alginate hydrogel or paste with and without DOX loaded liposomes. The viability was determined after 24 and 48 h of incubation with alginate hydrogels or paste containing different amounts of drug loaded liposomes, and measured using the MTT assay.

However, the liposomes were fully released from these two formulations after 26 h. The release from the Ca^{+2}/(1 mM) Ba^{+2} cross-linked gel was significantly lower than both the Ca^{+2} cross-linked gel (Anova, $p = 0.0001$) and the Ca^{+2}/(0.5 mM) Ba^{+2} cross-linked gel (Anova, $p = 0.0005$) throughout the experiment. The use of higher Ba^{+2} ions concentration slowed the liposome release rate and controllable liposome release rates were achieved. This could be ascribed to the addition of some binding points with higher affinity into the alginate-Ca^{+2} network without interrupting the homogeneous gelation of alginate by Ca^{+2}/GDL mixture. Moreover, Ca^{+2}/Ba^{+2} cross-linked formulations delayed the degradation rate of the hydrogel by Ca^{+2}-phosphate ions chelation and prolonged the release rate of the liposomes.

It should be mentioned that although the release rates from hybrid alginate hydrogel were controllable and acceptable for the intended applications, the hydrogels displayed limited mucoadhesion properties, as only mild pressure was enough to detach an alginate hydrogel placed on a tongue tissue. Therefore, the combination of paste and hydrogel systems, termed in the following section cross-linked paste was suggested.

3.3. Cross-linked hybrid alginate pastes

In order to combine the beneficial mucoadhesive properties of the pastes with the sustained release rate of the hydrogels, cross-linked hybrid alginate pastes were designed. The procedure for their preparation included spreading a paste on porcine tongue tissue surface followed by in situ cross-linking by dropping Ca^{+2}/Ba^{+2} solution onto the top layer of the paste. In real life the in-situ crosslinking can be performed in the same manner as it was done in our experiments: spreading the paste and then dripping aqueous solution containing the ions. The mouth cavity is accessible to the physician. This strategy allows the polymer chains in contact with the tissue to interpenetrate into the mucus layer and interact with the mucin glycoproteins, while the top layer is cross-linked and decelerates the water diffusion inside the paste and hence the degradation of the polymer. Further, based on our results with the hydrogels, we expected that crosslinking could sustain the release of liposomes from the polymeric matrix. Immediately after preparation of cross-linked hybrid paste, the tissue assembly was immersed in simulated saliva buffer and liposome release rate was determined (Fig. 3). To further examine the suitability of the cross-linked pastes as a sustained release delivery system, liposomes were loaded with DOX, a clinically approved chemotherapeutic drug [48], and applied on the tissue at the same manner. Fig. 3 shows that the fractional release of the

cross-linked hybrid paste displays a gradual profile in comparison to the non-cross-linked hybrid paste (Anova, $p = 0.0003$). Further, the release from the crosslinked paste is similar to that of the hydrogel (Anova, $p = 0.8907$). Moreover, similar release rates of DOX loaded liposomes and empty liposomes in the Ca^{+2}/Ba^{+2} cross-linked hybrid alginate pastes were obtained, indicating that drug loading did not affect the release rate (Anova, $p = 0.6712$). This result is expected since the release rate is determined by the polymeric matrix degradation and not by the liposome cargo. Its practical implications are important since the cargo can be changed without the need to design a new carrier. Summary of liposome release obtained from different alginate systems is presented in Table 1.

3.4. Adherence: Polymer retention study

Flow through study [49] was used to quantify the mucoadhesion properties of alginate pastes. Fig. 4a and b demonstrate a typical flow through experiment. Fig. 4c compares between the adhesion properties of 3% alginate-fluorescein paste and 3% cross-linked alginate-fluorescein paste.

The retention of alginate paste reached a steady state after washing with ~10 mL of eluting buffer. On the contrary, the retention of cross-linked paste constantly decreased and reached a steady state after washing with 27 mL of eluting buffer. The retention at long times was ~50% for the paste and 80% for the cross-linked paste (Anova, $p = 0.0036$). The clearance of cross-linked alginate paste is slower than that of alginate paste, and its retention is higher, indicating on the stronger adhesion of the cross-linked paste. Visual inspection further confirms the improved adherence of the cross-linked hybrid alginate paste onto the tissue surface (Fig. 5).

3.5. Liposome size and stability

Samples collected from the release experiments were characterized using DLS to evaluate liposome stability. Table 2 summarizes liposome size distribution after releasing from alginate pastes. Liposome size distribution depicts particle size range of 122–137 nm, similar to that of the stock liposome solution, characterized by particle average diameter of 136 ± 44 nm. Further, the size distribution of the liposomes in the release medium was constant throughout the experiment, suggesting the overall liposomes stability under the experimental conditions.

Next, SAXS measurements were carried out to further examine the structural stability of the trapped liposomes in alginate hydrogel. Fig. 6 shows two distinctive peaks specific for liposomes in the stock liposomes solution, which can be attributed to the lamellar structure of the lipid membrane [50]. Similar peaks, at the same position, can be seen for liposomes entrapped within hybrid alginate hydrogel, revealing the liposomes retain their structural morphology in the polymeric matrix.

3.6. Biological activity: Toxicity of hybrid system to oral cancer cells

The toxicity of the drug loaded hybrid alginate/liposomes system was examined in vitro using a human cell line derived from a tongue SCC (CAL-27). Alginate gels and paste incorporating DOX loaded liposomes were exposed to cells grown in tissue culture plates. After 48 h, alginate gel and gel incorporating empty liposomes had no effect on cells, while the DOX loaded liposomes released from alginate gels promoted significant decrease in viability to an average of 38% and 15% after 24 and 48 h, respectively (Fig. 7). Alginate pastes incorporating DOX loaded liposomes were the most effective system in promoting cancer cell death. Alginate paste promoted significant decrease in viability to 6% and 1% after 24 and 48 h, respectively. These results indicate that the liposomes released from the polymer systems (either gel or paste) remain active and effective. This feature is an essential characteristic for the development of the proposed oral cancer treatment.

4. Conclusions

This study demonstrates the development of a hybrid alginate/liposomes system for local drug delivery. The release kinetics of liposomes can be fully controlled by the degradation rate of the polymeric matrix which is determined by the cross-linker concentration and composition. The achievement of both mucoadhesion and sustained liposome release is an important milestone toward the development of an efficient delivery system. This system allows sustained release of anti-cancer drugs over an extended period of time upon lingual administration. In the future, we will be applying this approach as a potential treatment of oral cancer.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijbiomac.2017.12.137.

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