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HPMC-based liposomal mucoadhesive films with model peptide as target API

Diploma Thesis

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I hereby declare this thesis to be my original work. All sources used are listed and properly cited in the bibliography section. This thesis has not been misused for obtaining an equivalent or any other academic degree.

Prague, 1. 5. 2018 Hedviga Belcakova

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ABSTRACT

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Diploma thesis title: HPMC-based liposomal mucoadhesive films with model peptide as target API

This thesis describes the preformulation stage of mucoadhesive films intended for liposomal peptide delivery via buccal membrane. The evaluation consisted of thickness, maximum tensile strength, strain, moisture content, *in vitro* swelling and liposome integrity measurements. The chosen polymer (hypromellose, HPMC) was found to perform optimally in concentrations of 10 % with PEG 400 (5 %) acting as plasticizer and liposome concentration of 2 %. The developed preparation method showed good reproducibilty with room for improvement in the homogenization area. The choice of medium (H2O vs. PBS) showed strong influence on formulation's mechanical properties resulting in significant loss of elasticity and mucoadhesive strength. The addition of liposomes in the third stage had been carried out successfully with only occassional effect on their integrity after dissolution.

ABSTRAKT

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Názov diplomovej práce: Lipozomální mukoadhezivní filmy na bázi HPMC s modelovým peptidem jako cílovým API

Predmetom diplomovej práce je preliminárna formulačná fáza mukoadhezívnych bukálnych filmov určených k transmukozálnemu podaniu peptidových liečiv. Skúšobné metódy zahŕňajú hodnotenie hrúbky, pevnosti v ťahu, elongácie, reziduálnej vlhkosti, *in vitro* 'swelling' parametru a podisolučnej integrity lipozómov. Zvolený polymér (hydroxypropyl methylcelulóza) preukázala optimálne vlastnosti v koncentrácii 10 % v kombinácii s polyethylenglykolom (5 %) ako plasticizérom. Použitá koncentrácia lipozómov bola 2 %. Vyvinutá metóda prípravy vykazovala uspokojivú reprodukovateľnosť s priestorom pre optimalizáciu v oblasti homogenizácie.
Výber solventu (H2O vs. PBS) preukázal silný vplyv na mechanické vlastnosti formulácie vedúce k značnej strate elasticity a mukoadhezívnej sily. Prídavok lipozómov v tretej fáze bol úspešný. Následná disolúcia mala len mierny vplyv na ich integritu.

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1. Aims and objectives of the study

The aim of this study was to prepare a preliminary formulation of hypromellose-based mucoadhesive film allowing the entrapment of liposomes. The objectives were to:

• perfect the formulation in terms of mechanical properties and durability

• develop preparation method with good reproducibility

• provide baseline testing protocol for key parameters of the film

2. List of abbreviations

API	Active Pharmaceutical Ingredient
GUVs	Giant Unilamellar Vesicles
HPMC	Hydroxypropyl methylcellulose
LUVs	Large Unilamellar Vesicles
m	weight
MLVs	Multilamellar Vesicles
Mw	Molecular weight
PBS	Phosphate Buffer Saline
PDI	Polydispersity Index
PEG	Polyethylene glycol
RMC	Residual Moisture Content
s.c.	subcutaneous
SUVs	Small Unilamellar Vesicles
V	Volume
VPG	Vesicular Phospholipid Gel

1. Introduction

The large number of biological medicinal products (e.g. peptides, proteins, etc.) emerging create an incentive for alternative routes of administration.

The advance in technology allowed development in the area of biologicals which often enable treatment of (often very specific) conditions with no cure prior to their existence. However, their instability in harsh gastric environment and susceptibilty to enzyme degradation creates room for novel dosage form development as the current routes of administration (i.v, s.c., i.m.) are often associated with decreased patient compliance.

Oral cavity mucosa with its rich blood perfusion and direct drainage to jugular veins (and thus to vena cava superior) present drug delivery option with good absorption prospects and no subsequent first-pass effect. [1] Furthermore, its mucinous upper layer can be utilised as mucoadhesion site thus opening doors for prolonged contact time when considering systemic administration. [2]

Following the trend of patient comfort upon drug administration, non-invasive buccal films as dosage form of choice are resilient (compared to buccal tabltes), easy to apply and provide control over dose administered (as opposed to oral gels and pastes).

Considering the presence of factors limiting peptide stability in oral cavity (pH fluctuations, enzyme degradation) and its penetration through mucosal epithelium (barrier-like quality of the structure), [3] an additional delivery element in the form of liposomes has been introduced in order to ensure peptide's stability and efficacy.

In terms of polymer matrices suitable for buccal film formulations, hydroxypropylmethylcellulose is a non-toxic cellulose derivate used extensively in pharmaceutical industry partially due to its low-cost profile. It possesses mucoadhesive properties and is capable of gel-formation, which makes it an ideal candidate. [4]

In regards to peptide stability in oral cavity, a combination of a polymer matrix and liposomes as drug-carrier system was used as perspective solution to protein-nature-related obstructions.

4. Theory

4.1 Oral films

Oral films present a fairely recent addition to the dosage-form palette, gaining general recognition through the success of Listerine® Pocket Packs® (Pfizer) in 2001.
Although initially developed for OTC-drugs such as local anaesthetics (Chloraseptic® Relief Strips, Prestige brands), their potential has been quickly utilised in formulation of new dosage forms for already approved prescription drugs (e.g. Suboxone®Sublingual Film, Reckitt Benckiser Pharmaceuticals; SildeHEXAL SF, HEXAL Sandoz).

Oral films can be divided into two classes in accordance with their influence on drug's release profile and retention time in the oral cavity.

4.1.1 Orodispersible films

Orodispersible films are currently defined as single- or multi-layer sheets of suitable material, to be placed in the mouth where they disperse rapidly. [5]

They are often composed of hydrophilic polymers with molecular weight below 9 000 Da. Following disintegration they are intended to be swallowed and absorbed into systemic circulation via gut mucosa. [6]

There is a number of patented technologies in FDA-approved commercially available products including Pharmfilm® (MonoSol Rx LLC) [7], RapidFilm® (Labtec GmBH/ APR Applied Pharma Research) [8], SmartFilm® (Seoul Pharma Co., Ltd.) [9]s, etc.

4.1.2 Buccal films

Buccal films, characterised as single- or multi-layer sheets that adhere to buccal mucosa and may dissolve [5] are designed to deliver the drug through transmucosal permeation and therefore are suitable for both, immediate and prolonged drug delivery.

Following subchapters are specific to buccal films but can also be applied to orodispersible films to a certain degree.

4.1.2.1 Benefits of buccal films

Advantageous traits of the dosage form in question stretch out across three focal domains of pharmaceutical care: drug safety, patient compliance and economical optimalisation.
Buccal films utilise mucous membrane as drug delivery site thus providing protection from the acidic gut environment and enzyme degradation along with diminishing possible fluctuations due to differences in gastric health and content, intestinal flora and eliminating the effects of first- pass metabolism on achieving therapeutical concentrations.

Furthermore, they provide an alternative to tablets and syrups in patient groups with swallowing difficulties (generally children below the age of twelve, elderly patients [10] and individuals with corresponding medical condition).

Along the same lines, they can be considered as substitutes for intraveous and subcutaneous route of administration with impact on patient comfort and reliability as no additional mechanical device is required. Moreover, their higher resilience puts them ahead of buccal tablets.

Lastly, the design novelty ensures new possibilities for currently widely-discussed*, drug- repurposing'* along with, *life-cyle management'* as the developement of a new dosage form is generally recognised as lower-cost (estimated difference of 40 mil. USD) and less time-consuming (4–5 years) compared to new API-developement. [11]

4.1.2.2 Limitations of buccal films

When it comes to limiting factors, buccal films provide, depending on the particular polymer and formulation used, a restricted loading capacity since film size and thickness play an important role in patient compliance. This factor can be balanced out by the absence of first past effect in comparison with peroral formulations.

Moreover, it is crucial that the target API is capable of transmucosal permeation and/or possesses a transport structure in the mucous membrane, downsizing the selection predominantly to non- polar molecules of low molecular weight.

4.1.2.4 Physical properties

Considering their novelty, the European Pharmacopoeia is yet to expand the corresponding monography thus leaving space for wider interpretation of optimal values when it comes to buccal film qualities. These should provide sufficient mechanical resilience preventing breakage and crumbling upon handling. [5]

When addressing the issue of physical properties, investigation of following characteristics in early stages is recommended.

Film thickness and weight variation for their correlation with dosage form uniformity. Quoting Anroop B. Nair et al.: *„In general, an ideal buccal film should exhibit a thickness between 50 and 1000 µm.* [12]

To explore film's physical integrity, tensile strength and tear resistance are measured, whereas study of elastic properties employs elongation at break and/or percent elongation. These are crucial especially during formulation optimization due to the effects of different polymer and plasticizer type, concentration and ratio. When it comes to the devices used Texture Analyzer TA.XT Plus (*Stable Micro Systems*) [13] is a frequent choice as it is capable of measuring aforementioned attributes as well as providing stress-strain curve, which can be utilised for further characterisation of film deformation. In the test, uniform film cut-outs are placed between two clamps, which then move apart at a set rate. The device records data until the film breaks or the pre-defined track ends.

Furthermore, scanning electron microscopy (SEM) can be used to gain infomation on structure, porosity and surface morphology of the film [14], which are particularly valuable in later stages (e.g. for polymer – drug interaction studies, API release from the dosage form, wettability, etc.).

In attempt to acquire a rough estimate of film's performance in the oral cavity, residual moisture content (RMC) and swelling capacity are determined as they are directly associated

with mucoadhesion strength and drug release from dosage form. According to Anroop B. Nair et al., RMC should not exceed 5 %. [12] In swelling tests, a film patch of defined size is placed in phosphate buffer saline (pH 7.0–7.4) or model saliva and weighed in regular intervals. The percentwise increase in weight is than calculated. [15] RMC values on the other hand are easily obtained from infrared scale measurements.

To ensure drug stability and potency whilst keeping tissue irritation to minimum, surface pH measurements (using a pH meter [16] after suffiecient incubation in destilled water) play a key role and consequent adjustment should therefore be taken into consideration from the start.

Strength of mucoadhesion is measured in conditions more or less simulating oral cavity but are predominantly carried out either *in vitro* or *ex vivo*. Strength upon separation counts to the methods most relevant to the formulation stage. In the experiment force necessary for breaking the bond between tested mucoadhesive and a model membrane is noted using Texture Analyzer. [17] Depending on the direction in which the breaking is tested, detachment, shear or rupture strength can be quantified. A mucin disc is commonly used as a model membrane in *in vitro* tests, whereas animal mucus membrane (e.g. porcine, rat mucosa) is an option in *ex vivo* studies. [18] This concept works best for solid formulations but has been utilised in semi-solid systems as well. [19]

Another possible approach focuses on shear strength measurement between two parallel glass slides covered in mucus and tested mucoadhesive. [20] A slight modification (glass plate being attatched to microforce balance and lowered into mucus solution) known as Wilhemy's model [21] has been suggested although it does not provide an accurate representation of *in vivo* conditions.

The *in vitro* dissolution with its pertinence to residence time in oral cavity is tested via various systems, simulating salivary composition, ph and flow rate. Starting with simple designs in laboratory conditions, buccal patch of defined size (usually size containing therapeutic dose) are submerged in suitable medium (phosphate buffer, model saliva fluid [22]) and the outer container is subjected to mechanical stress (mild shaking, rotating) simulating enviroment in mouth. The more advanced techniques implement one of the USP dissolution apparatus. [23] The dissolution medium used in USP methods can be switched

for one simulating the environmental conditions more accurately. In both instances, period necessary to film's complete dissolution is measured.

4.1.2.3 Preparation methods

Choice of preparation technique is influenced by two factors: polymer properties in regards to dosage form as well as equipment at hand. The result combination may consequently require process adjustments when trying to achieve optimal values.

In the solvent-casting technique polymer is dissolved or dispersed in solvent volume corresponding to desired end concentration and mixed intensely [24] (one of the devices used is SpeedMixer as it does not incorporates air bubbles during the proccess [25]). After the rest of the components (e.g. plasticizers, drug) have been added, the mixture is once againg homogenized and poured onto an appropriate surface (glass plate, Petri dish). Finally the film is left to dry at room temperature (which can generate reproducibilty issues due to humidity fluctuations) or in cabinet dryer.

Alternatively, industrially used hot-melt extrusion technique is an efficient counterpart. In the initial stage polymer is melted and mixed with API (Active Pharmaceutical Ingredient) ensuring high level of homogenity. [26] The film then forms during cooling stage. The absence of solvent and therefore drying period renders this approach more time-efficient. Som literature also states that it increases drug bioavailability [27].

4.2 Mucoadhesion

Mucoadhesion presents a form of bioadhesion and can therefore be characterized as material's ability to adhere to biological surface.[28] The distinct trait setting mucoadhesion apart from other forms of adhesion on organic tissues is the presence of mucus on the epithelial lining.

The usual sites of mucoadhesion include eye [29], ear [30], oral [31] and vaginal [32] tissue.

It is implemented in drug delivery technologies when prolonged residence time is expected to increase drug permeation.

4.2.1 Oral cavity

Oral cavity (cavum oris) provides multiple sites for drug delivery originating from its anatomy.

The inner space is framed by hard palate (palatum durum) adjacent to soft palate (palatum molle) superiorly, floor of the mouth inferiorly, the cheeks (buccae) laterally and by the lips (labia) anteriorly.

Other major anatomical structures include tongue (lingua), gums (gingiva) and teeth (dentes).

Of all the above-mentioned structures, it is gum, buccal and floor of the mouth mucosa that are generally recognised as significant mucoadhesion sites. However, they all exhibit different characteristics, which has profound effect on their permeation ability in respect to the drug administered.

Whilst buccal mucosa with its 500–800 μm (comp. 100–200 μm in gum and floor of the mouth mucosa) [3] poses the biggest challenge in terms of mechanical barrier, it also possesses the highest blood flow rate with 20,3 ml/min (comp. 19,5 ml/min and 12,2 ml/min in gum and floor of the mouth mucosa respectively) [33], which is a contributing factor in absorption increase. Moreover, in contrast to sublingual mucosa, buccal mucosa is not submitted to a constant saliva flow, which makes any drug dosage form application problematic. When comparing additional epithelial modifications, keratinization – a protein-based layer acting as protective barrier against microorganisms and small molecules (such as drugs)- can only be examined in gingival epithelial tissue [1].

All of these factors result in buccal mucosa being the preferred site for transmucosal drug delivery. [34][35]

4.2.2 Mucus

Mucus is a gel-like substance forming a 40–300μm thick layer on top of epithelial cells in oral mucosa. It exhibits viscoelastic properties due to its composition (95–99 % water, 1– 5 % glycoproteins, minerals, lipids and free proteins). [33]

The most prevalent glycoproteins bear the name mucins with molecular weight ranging between 0.5 and 20 MDa. They contain oligosaccharide (5–15 monosaccharide long) chains bound to the peptide scaffolding. The most frequented monosaccharide types are *N*- acetylglucosamine, *N*-acetylgalaktosamin, fucose, galaktose and sialic acid [1], where the latter along with sulfate residues is responsible for the molecule's negative charge at physiological pH. The consequent intramolecular repulsion is further supported by hydrophobic interactions and disulphide bonds between protein chains resulting in the final structure of mucus. The three-dimensional network adheres strongly to the cell surface playing a crucial role in mucoadhesion.

4.2.3 Theories of mucoadhesion

The process of mucoadhesion can generally be divided into two stages: contact stage (characterised by wetting and swelling of the polymer and formation of non-covalent bonds) and consolidation stage, where the outer polymer chains are freed during continuing hydration and interpenetrate mucus layer forming secondary bonds. [36]

Should mucoadhesion prove to be successful, both steps must be completed, whereas the varying degree of intensity upon doing so has direct influence on its strength.

The precise mechanism on molecular and/or subatomic level has been explained through various theories contributing to a complex picture of the actual process.

Electron theory is based on the premise that attractive forces between bioadhesive material and biological structure (both of them having opposite electric charge) lead to electron exchange forming an electrical double layer. This exchange is only possible when relevant chemical groups are present on either side. [17]

When explaining mucoadhesion, adsorption theory uses secondary chemical interactions such as van der Waals and hydrogen bonds along with electrostatic and hydrophobic interactions. [36] The consequent mucoadhesive strength vastly depends on their count as cumulative effect plays a big role in this case. Moreover, polymer types quantitatively

superior in terms of polar chains tend to form van der Waals and hydrogen bonds, which are generally recognised as stronger than other secondary chemical interactions.

Wetting theory represents a concept mainly applicable to liquid systems as it explains the increasing affinity of the adhering substance as a function of decreasing contact angle. This notion is thus directly related to the wettability of the surface. [36]

The aforementioned chain interpenetration presents the corner stone of diffusion theory. Currently used predictor of the final stability is the penetration depth l (*Fig. 4.2 (a)*). It is a function of contact time (t) and diffusion coefficient (D_b), the latter being influenced by chain nature, mobility and flexibility. [2]

$$l = \sqrt{t.D_b}$$

Fig. 4.2 (a)

Another commonly used explanation is the fracture theory. In experiments based on this understanding of mucoadhesion, force needed for breaking apart two surfaces after completed adhesion S_m is measured. It is then calculated as the ratio of maximal detachment force (F_m) and area of adhesion (A_0) (*Fig. 4.2(b)*).

$$S_m = \frac{F_m}{A_0}$$

Fig. 4.2 (b)

4.2.5 Extrinsic factors influencing mucoadhesion

As the subject of this thesis primarily focuses on mucoadhesion in oral mucosa majority of extrinsic factors is oral-cavity-related.

The tissue lining of oral cavity is consistently being washed by saliva produced in there major and numerous minor salivary glands. The three major glands are the submandibular, the sublingual and, to buccal mucosa most relevant, the parotid gland. Their secretion depends on the time of day, external stimulation (including drug-induced), age and eventual pathologies. Saliva as dilution medium poses a problem to the initial (contact) stage in particular as it makes attachment of polymer to buccal mucosa more difficult. On the other hand, it is a major force contributing to its detachment, which is a quality bioadhesive drug delivery forms with prolonged release come to depend upon.

The salivary pH lies between 6,0 and 7,5 with minor deviations predominantly due to food consumption. [37] The main significance of oral pH lies in its ability to affect ionisation of functional groups leading to change in charge. This can result in polymer having a decreased ability to engage in secondary molecular interactions as well as the disruption of stability in some.

As mucus layer is not a static system, it is constantly being renewed in a process called mucus turnover at a rate named the turnover time. This parameter has been experimentally measured in humans and amounts to 12–24 hours in average. [38]

Finally, as with any other biological system there is a distinct possibility of tissue pathologies having an effect on mucoadhesion sites. [2]

4.2.6 Mucosal permeability in drug delivery

Buccal (along with sublingual) mucosa is non-keratinized type of oral mucosa, which accounts for the absence of acylceramides as well as only residual amounts of ceramides and neutral polar lipids present. [39]

Three distinct layers are discernible upon microscopical inspection: epithelium, basement membrane and connective tissue. [1]

The most serious issue pertaining to buccal membrane permeability comes in form of so- called Membrane Coating Granules (MCGs) [40]. These are spherical objects with diameter of 100–300 nm. MCGs are located in the outermost one fourth to one third

of epithelium producing intercellular material via exocytosis, which contributes to cell adhesion. They are considered to be the main reason for barrier-like traits of oral membranes. (*Fig. 4.2 (c)*) On the other hand, buccal membrane shows 4–4000 times higher permeability compared to skin. [41] This phenomenon can be partially explained through lower to no levels of keratinization compared to skin.

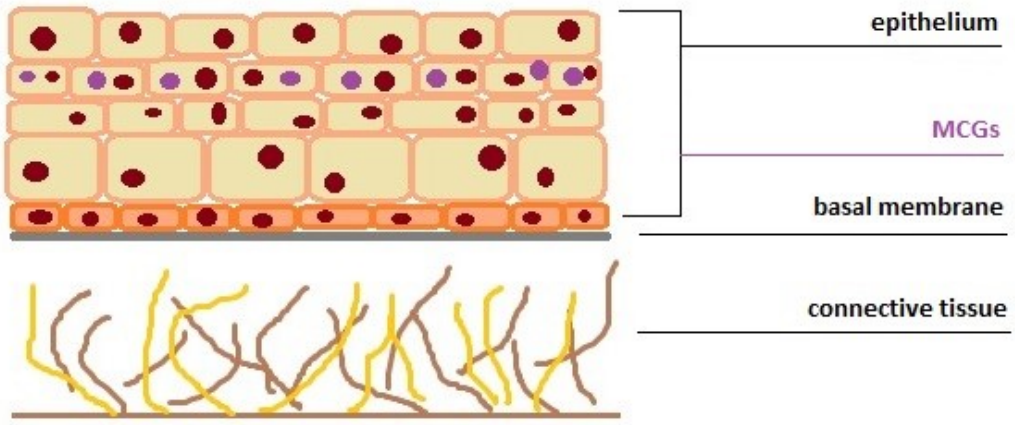

Fig. 4.2 (c) – drawing by the author

In terms of drug delivery obstacles, one additional point to be considered is enzymatic degradation. The ever-present saliva contains esterases, carbohydrases and phosphatases. Proteases, however, are not present. These enzymes are responsible for degradation of susceptible drugs. Several approaches (such as drug-containing nanoparticles or non- soluble protective layer in dosage form) have been suggested in attempt to counter this debilitating effect.

There are two types of transport available for substance or drug penetration through the membrane: paracellular and transcellular pathways. When discussing specifics, vast majority of molecules suitable for membrane permeation are transported through simple diffusion while only a small percentage has been proven to utilise carrier-mediated transport or endocytocis. [2] Other possibilities such as active transport or membrane filtration are not typical for the tissue in question. The way of transport utilised by a particular molecule depends on its geometry, lipophilicity, charge and/or polarity, and pK_a.

In search for a solution to overcoming permeability barriers penetration enhancers present a certain possibility. These substances increase permeation efficacy through decrease in mucus viscosity (e.g. cationic L-lysine, positively charged chitosan), increase in lipid bilayer fluidity (e.g. fatty acids), enzyme inhibition (e.g. carbomera binding Zn, Ca or EDTA- modified polymers) or increase in drug's solubility (cyclodextrins). [41] Dextran- protamine coating has been proposed as prospective permeation enhancer for lipid- based nanostructures. [42]

It is apt to mention that mucoadhesive polymers (some more than others) increase drug penetration through the opening of tight junctions as they absorb water (swelling) causing the cells to shrink. [43]

4.3 Mucoadhesive polymers

4.3.1 Characterization

Mucoadhesive polymers are macromolecules of natural, semi-synthetic or synthetic origin capable of mucoadhesion. That can occur either in dry state or, which is more usual, in the form of hydrogels forming upon contact with fluid (e.g. saliva, intestinal or vaginal fluid).

An ideal polymer should carry strong hydrogen-bond groups, either negative or positive charge, it should have surface properties favouring good wettability and spreading on the mucus layer as well as sufficient molecular weight. [36]

However, substance intended for therapeutic use is expected to possess a few additional qualities such as compatibility with both hydrophilic and lipophilic drugs and affinity specific to target areas or cell structures. Moreover, specimen promoting absorption or stimulating endocytosis are held in high regard for their ability to increase drug permeation. [44]

4.3.2 Polymer-related factors influencing mucoadhesion

The overall mucoadhesive strength (and consequently mucoadhesion) is affected by various elements related to the type of polymer used.

Molecular weight provides a baseline for forming a web-like structure between the chains of both parties, polymer and mucus. When discussing the optimal range, 10^4–$4x10^6$ Da is the interval cited in research papers [36] as the increasing mucoadhesive strength does not stretch out infinitely with growing length of polymer chains.

In the initial step (contact stage) wettability and subsequent hydration of the polymer play a big role in the release of side chains. They allow the chains to move and form hydrophobic interactions with mucus structures building a foundation for the second stage. It is also necessary to mention contact time as it correlates with the amount of liquid absorbed upon contact.

As for the consolidation stage, polymer branching is one of the two key characteristics. It is the premise of favourable geometry for chain entanglement and is closely related to cross- linking, which then has direct effect on chain flexibility [45]. Both are linked to the overall molecule mobility and binding groups coming together, which is crucial to the consolidation stage. High-density branching and intense cross-linking are limiting to the polymer trying to establish a connection to the mucin layer. Cross-linking also influences the polymer swelling and as such also chain release in the first stage of mucoadhesion [45]. However, due to the differences between various polymers and their molecular structure no generally applicable values for cross-linking and branching grade have been established. The use of a specific polymer (and polymer subtype) requires individual approach depending on the dosage formulation.

As the consolidation progresses, secondary chemical interactions (and occasionally covalent bonds e.g. in the case of thiolated polymers [46]) are formed. In order for the polarity-based interactions to occur, both polymer charge and hydrogen-bond capacity need to be complementary.

Electric charge provides a base for electrostatic interactions, which can also be interpreted as such that non-ionic polymers fail to exhibit the same level of mucoadhesion as their ionic

equivalents. [2] This polymer-related factor operates on the premise that there is a charged functional group present in the polymer molecule. Moreover, out of the two, cation polymers are superior to anionic ones when it comes to adhesion strength.

Hydrogen-bond capacity, on the other hand, comes to play when corresponding functional groups contain a highly electronegative atom (e.g. O, N, F) creating an electrostatic field. This field is a site of attraction for the hydrogen atom. Poly(vinylalcohol), hydroxylated(methacrylate) and poly(methacrylic acid) are among polymers with pronounced hydrogen capacity. [47]

Lastly, polymer concentration has an underlying influence on the prominence of all the factors due to their cumulative effect. Similar to molecular weight, continuous increase in polymer concentration loses its positive effect after surpassing the critical concentration (the concentration limit after which no additional effect on the speed of film formation and thickness can be observed) – once again specific to polymer type.

4.3.3 First generation of mucoadhesive polymers

This group contains hydrophilic polymers binding to mucin glycoproteins via non-covalent bonds. This binding is non-specific rendering the group slightly obsolete especially in face of latest development focusing on targeted drug delivery. On the other hand, their short retention time might be advantageous in cases where the aimed duration of stay is under 12– 24 hours (mucus turnover time in humans). [38]
Depending on their charge first-generation polymers can be divided into three subclasses: cationic, anionic and neutral polymers.

Cationic polymers take advantage of mucin's negative charge and therefore adhere to the surface predominantly on the basis of electrostatic interactions. The most typical and currently extensively studied example is chitosan. [48] [49]

Polymers bearing negative charge include polyacrylic acid (Carbomer) and its cross-linked derivates (Carbopol). [50] While mucoadhesive properties of polyacrylic acid are mainly

used in particle coating, Carbopols form mucoadhesive gels. The latter has enzyme-inhibiting properties linked to their ability to bind bivalent cations leading to co-factor inactivation. [51]
Similar approach is implemented in sodium alginate where different molecular weight and submolecular composition (β-D-mannuronate and α-L-guluronate ratio) affect their behaviour.
Carboxymethylcellulose (CMC), sodium CMC and hyaluronic acid also carry negative charge, which puts them in the same class as the two previous ones.

Finally, neutral polymers include mainly cellulose derivates, namely methylcellulose, hydroxyethylcellulose, hydroxypropylcellulose and hydroxypropyl methylcellulose.
Cellulose derivates contain hydroxyl groups which are essential to polymer solubility in polar solutions as well as sites of various modifications further adjusting polymer's qualities.
Their mucoadhesive properties have already been implemented in delivery forms of commercially available products such as in case of hydroxyethylcellulose (eye drops Trusopt, Tobradex) [53] and hydroxypropylcellulose (Corsodyl oral gel) [54].

4.3.4 Second generation of mucoadhesive polymers

Members of the second generation are known for stronger bioadhesion and structure-specific binding. The former has been rendered possible through covalent bonds due to presence of sulfhydryl groups. Furthermore, the term mucoadhesion is no longer adequate as some of the second-generation polymers bind to cell structures (cytoadhesion).

Lectins, plant glycoproteins binding to glycosylated membrane structures of cells, have been investigated for their potential application to ocular therapeutic systems. [55] What makes them extremely interesting is their ability to stimulate cell receptors initiating lectin internalisation, which holds a promise for facilitated drug absorption.
Their main disadvantage resides in them being recognised by the immune system, which prompted the development of so-called lectinomimetics. These synthetic derivates should combine high binding selectivity of lectins while reducing their immunogenicity. [56]

Along the same lines, antibodies, naturally-occurring molecules known for their high binding specificity, are being examined for their promising application in tumor targeting.

Another subgroup is represented by polymers with sulfhydryl groups attached to their chains allowing them to form disulphide bridges with cysteine in mucin glycoproteins. Polyacrylic acid, polyvinylalcohol, carboxymethylcellulose, chitosan, alginate, polycarbophil are among polymers with the corresponding modification. [57] Recently, a real application of thiolated polymers in drug delivery has been reported (Leu-enkephalin as model pentapeptide has been successfully delivered via buccal membrane utilising polycarbophil-cysteine conjugated polymer). [58]

Since the relatively strong covalent binding poses a valid question regarding tissue irritation and possible toxicity [59], the use of thiolated polymers must await closer evaluation before being extensively used in therapy.

Table 1
Mucoadhesive polymers in buccal delivery

Criteria	Categories	Examples
Source	Semi-natural/natural	Agarose, chitosan, gelatin Hyaluronic acid Various gums (guar, hakea, xanthan, gellan, carragenan, pectin, and sodium alginate)
	Synthetic	***Cellulose derivatives*** [CMC, thiolated CMC, sodium CMC, HEC, HPC, HPMC, MC, methylhydroxyethylcellulose] ***Poly(acrylic acid)-based polymers*** [CP, PC, PAA, polyacrylates, poly(methylvinylether-co-methacrylic acid), poly(2-hydroxyethyl methacrylate), poly(acrylic acid-co-ethylhexylacrylate), poly(methacrylate), poly(alkylcyanoacrylate), poly(isohexylcyanoacrylate), poly(isobutylcyanoacrylate), copolymer of acrylic acid and PEG] ***Others*** Poly(*N*-2-hydroxypropyl methacrylamide) (PHPMAm), polyoxyethylene, PVA, PVP, thiolated polymers
Aqueous solubility	Water-soluble	CP, HEC, HPC (water<38 °C), HPMC (cold water), PAA, sodium CMC, sodium alginate
	Water-insoluble	Chitosan (soluble in dilute aqueous acids), EC, PC
Charge	Cationic	Aminodextran, chitosan, dimethylaminoethyl (DEAE)-dextran, trimethylated chitosan
	Anionic	Chitosan-EDTA, CP, CMC, pectin, PAA, PC, sodium alginate, sodium CMC, xanthan gum
	Non-ionic	Hydroxyethyl starch, HPC, poly(ethylene oxide), PVA, PVP, scleroglucan
Potential bioadhesive forces	Covalent	Cyanoacrylate
	Hydrogen bond	Acrylates [hydroxylated methacrylate, poly(methacrylic acid)], CP, PC, PVA
	Electrostatic interaction	Chitosan

Fig. 4.3 (a) (Smart, 2005 [36])

4.3.5 Hypromellose

Hypromellose (hydroxypropylmethylcellulose, HPMC) is a semi-synthetic biopolymer derived from cellulose. During manufacture process cellulose fibers are treated with caustic soda, methylchloride, and propylene oxide and then submitted to purification process. [60] The end product bears both methoxy and hydroxypropyl groups in different ratios depending on the hypromellose type.

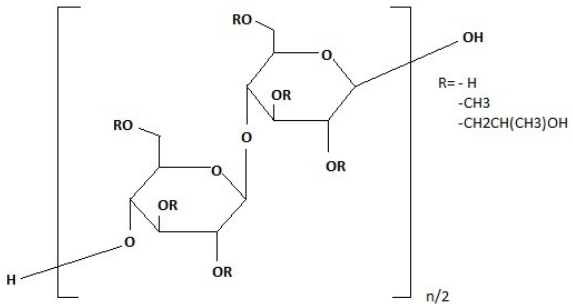

Fig. 4.3 (b)

There are four HPMC types:

HPMC type	**-OCH3 [%]**	**-CH2CH(CH3)OH [%]**	**gelation T [°C]**
1828	16,5-20,0	23,0-30,0	60-70
2208	19,0-24,0	4,0-12,0	70-90
2906	27,0-30,0	4,0-7,5	62-68
2910	28,0-30,0	7,0-12,0	58-64

Fig. 4.3 (c) ([61])

Hypromellose is described as white powder (can exhibit yellow or gray undertones) without a taste or scent. Its molecular weight (Mr) in polymerisation grades (n) 50–70 lies

in the range between 10 000 and 150 000, whereas viscosity of HPMC solutions (3– 100 000 mPa.s) is a function of substitution type. [61]

It forms colloid solutions in cold but is insoluble in hot water where it can be dispersed under vigorous stirring. It is a subject to reversible thermal gelation (Fig. 4.3b). This phenomenon can be explained as follows: the water cage surrounding hydrated polymer chains is lifted (water evaporates) when the gelation temperature is reached allowing functional groups and hydrophobic chains to interact and form seconadary chemical interactions forming a film. When exposed to higher temperature HPMC starts to soften at 130°C. The temperature of degradation accounts for 260–265°C. [61]

American Food and Drug Administration (FDA) lists hydroxypropylmethylcellulose in their GRAS-database (Generally Recognised As Safe), which rules out any potential for allergy, irritation or toxicity. [62] As a result, HPMC is counts among most widely used ingredients in pharmaceutical industry. Fields of its application include tablet granulation (wet and dry), tablet coating (utilizing predominantly low-viscosity types) and retarding ingredient in matrix tablets. Certain eye-preparation and hydrogels use HPMC as a viscosity increasing agent. [4]

However, usage of hypromellose is bound by certain limitations such as its incompatibility with cationic agents and certain degree of sensitivity to salt content (although certain publications report its lesser significant compared to methylcellulose). [63]

4.4 Liposomes

4.4.1 General characterization

Liposomes are nanoparticle defined as spherical structures composed of lipid bilayer enclosing an aqueous core. [64] As liposome diameter may differ depending on the lipid composition and preparation method used, there are three terms discerning liposomes according to their size: small ($d < 0{,}1$ μm), large ($d \sim 0{,}1–1$ μm) and giant ($d > 0{,}1$ μm). When discussing their lamellarity (number of lipid bilayers), three cathegories have been described: unilamellar, oligolamellar and multilamellar. [65]

Owing it to their amphiphilicity, liposomes are capable of incorporating both hydrophilic (inside aqueous core) and lipophilic (in lipid bilayer) drugs into their structure [66] thus making them perspective delivery systems for peptides [67], drugs with unfavourable profile in their current dosage form, drug repurposing [68] and gene therapy[69].

4.4.2 Preparation methods

The liposome preparation process has undergone considerable development in the past forty years since their first appearance in the industry. The methods recognised at present can be characterised and divided based on the force propelling liposome formation.

I. ***Thin film hydration*** employs lipid's dissolution in organic solvent, its subsequent evaporation and addition of aqueous phase. [70] The change in solvent polarity ensures lipid bilayer formation and represents the original approach to the problem. The end products are characterised as MLVs (Multilamellar Vesicles) and the method provides only low encapsulation efficiency. [71]

   Similarly, ***reverse evaporation*** operates on the same principle of medium polarity change. The selected lipids are dissolved in mixture of organic and aqueous solvents and the organic portion is then evaporated under low pressure. The method shows higher encapsulation efficiency and the end-product falls under the category of LUVs (Large Unilamellar Vesicles) and GUVs (Giant Unilamellar Vesicles). [72]
   In order to produce the so-called SUVs (Small Unilamellar Vesicles) the mixture of lipids dissolved in organic medium is injected into aqueous phase. This approach is known as ***solvent injection***. [73]

   A more sophisticated ***membrane contactor technology*** forces the lipids in ethanol to pass through a membrane with aqueous phase flowing on the opposite side. The subsequent ethanol dilution through the aqueous phase results once again in liposome formation. [74]

II. Whilst all of the above-mentioned methods face the issue of organic residue the ***supercritical fluid technology*** takes advantage of unique qualities a suitable substance gains when subjected to critical temperature and pressure. A widely used carbon dioxide exhibits low viscosity (similar to that of a gas) and density corresponding to that of a liquid when subjected to supercritical conditions. Similarly to reverse evaporation lipid content is dissolved in carbon dioxide, the aqueous phase is added and the pressure is gradually decreased, which induces liposome formation. [75]

III. Finally, a fairly new ***dual asymmetric centrifugation*** (DAC) employs two counter-rotating movements generating shear forces, which with the help of ceramic beads, are responsible for liposome formation and homogenization (an example of such device is the Speedmixer). [76]

A related method of ***dual centrifugation*** consists of two coordinate rotating movements (with no asymmetry to the process) and operates on the friction and sheer force basis. This approach can be observed in the commercially available Zentrimix dual centrifuge series. Zentrimix R 380 had been used for liposome preparation in this project as it is highly efficient at producing larger batches of liposomes (500 mg per batch comp. to 100 mg per batch in DAC's Speedmixer). [77]

4.4.3 Liposome interaction with biological membranes

Since liposomes' ability to interact with membranes and to initiate permeation are the main corner stones of successful drug delivery, there have been intensive research into their behaviour at delivery site, the best described structure being the skin. As such, liposomes undergo one of the following four transitions upon contact with skin's structures:

a) they penetrate intact [78]
b) their vesicles disintegrate and only individual lipid come into contact with lipids in stratum corneum [79]
c) liposomes are absorbed and undergo fusion whilst mixing with cellular lipids takes place, whilst the drug is directly transferred into the stratum corneum. [80] Nowadays, it is generally believed that such occurrence requires presence of fusion proteins or peptides, not unlike to those of viruses. [81]
d) liposome vesicles exhibit occlusive effect. [82]

The actual path is decided by liposomes' physiochemical properties (phospholipid type combination as well as ratio, charge, size) [81].

Lower levels of keratinization in buccal mucosa compared to that of skin lead to presume that liposome entrapment of drug will result in better permeation. This effect has been observed in hydrogels where the free substance showed inferior distribution profile than substance in liposomes. [83] The same study shows, however, that liposomes carrying model substance did not manage to overcome permeability barrier composed of MCGs [83], which can be interpreted as such that liposomes increase drug concentration on site while decreasing its systemic concentration. [84] This occurrence can be explained by the absence of permeation enhancing elements. Supporting this argument, results of a study comparing silymarin permeation in neutral, positive liposomes and liposomes with Tween 20 incorporated in their structure proved increased absorption of silymarin in formulation using Tween 20 as permeation enhancer. [85] Similarly, PEG-coated liposomes showed five to seven times higher penetration rate in mucus-covered tissue than their unsubstituted counterparts. [52] Buccal mucosa therefore remains a perspective site of systemic drug delivery whilst noting the need for sophisticated delivery systems at the same time.

5. Materials

5.1 Chemicals

Substance		Manufacturer	Specification
Mantrocel® E5	Hydroxypropyl methylcellulose 2910, HPMC 2910	Mantrose-Haeuser Co., Inc.	n.a.
Lutrol® E 400	PEG-8, Macrogol 400, Polyethylene Glykol 400	BASF SE, Ludwigshafen, Germany	n.a.
Dulbecco's Phosphate Buffer Saline	DPBS	gibco® by life technologies™	STERILE A
Cholesterol	Cholesterin	SIGMA-ALDRICH Chemie GmbH, Steinheim, Germany	≥99%
LIPOID E PC	Phosphatidylcholine from Egg, PC	Lipoid GmbH, Ludwigshafen, Germany	≥99%

5.2 Equipment

Name	Type	Manufacturer
Hot Plate	RSM - 10HS	PHOENIX Instrument
Analytical Balance	ABJ-NM/ABS-N	Kern&Sohn GmbH, Germany
Laboratory Balance	1216 MP	Sartorius Lab Instruments GmbH & Co.KG, Göttingen, Germany
Glass Beaker	SIMAX® 250 ml	KAVALIERGLASS, Prague, Czech Republic
Petri Dish (large)	92x16 mm	-
Petri Dish (small)	32x12 mm	-
Drying Chamber	B6	Heraeus Instruments GmbH, Hanau, Germany
Dual Asymetric Centrifuge	Zentrimix 380 R	Andreas Hettich GmbH & Co.KG, Tuttlingen, Germany
Centrifuge	Heraeus™ Biofuge Primo R	ThermoFisher Scientific™
Ceramic Beads	SiLibeads® Ceramic Beads Type ZY-E; 1,0-1,2 mm „Premium“	Sigmund Lindner GmbH, Warmensteinach, Germany
Particle Size Analyzer	Zetasizer Nano-ZS	Malvern Panalytical GmbH, Kassel, Germany
Disposable Cuvettes	PS	Sarsted AG & Co., Nümbrecht, Germany
Thickness gauge	Digital Outside Micrometer B 302-003 (range = 0–25 mm, read-out = 0,001 mm)	TOOLCRAFT

Texture Analyzer	TA.XT Plus	Stable Micro Systems Ltd., Godalming, UK
Infrared Moisture Analyzer	MLS	Kern&Sohn GmbH, Germany
pH Test Strips	McolorpHast™ (range = 4.0–7.0)	MerckKGaA
Ultrasonic Bath	Bransonic® 72	Branson Ultrasonics, Danbury, USA

6. Methods

6.1 Liposome preparation

Liposomes were prepared in 500 mg batches.

Egg phosphatidylcholine and cholesterol were weighed in 60:40 ratio into injection vials along with ceramic beads (1g beads per 100mg lipid) and suspended in 750 µl PBS. The blend was mixed using dual centrifuge (Zentrimix 380 R) at 2340 rpm for 15 minutes producing a VPG (Vesicular Phospholipid Gel). The vial was then put into a centrifuge (Heraeus™ Biofuge Primo R) to transfer the concentrated VPG to the vial's bottom in order to prevent any losses upon opening. In order to produce liposomal suspension two subsequent dilutions (1250 µl of PBS) and mixing phases (2340 rpm, 5 minutes) followed concluded by one final addition of 1750 µl PBS completing the preparation process. Each liposome batch was subjected to particle size testing using Zetasizer (1 µl of suspension in 1 ml PBS, disposable cuvette). The suspension (5 ml with liposome concentration of 100mg/ml) was stored at 5°C with shelf-life of 14 days (liposomes stored for a longer period of time were always re-tested prior to their use).

6.2 Film preparation

The initial experiments also included HPMC type E4M but type E5 became the preferred option after the initial E4M films exhibited unsuitable properties.

The experiment has been devided into three stages:

1. 10% HPMC E5 + 5% PEG 400 in H2O
2. 10% HPMC E5 + 5% PEG 400 in PBS
3. 10% HPMC E5 + 5% PEG 400 + 2 % non-loaded liposomes in PBS

For each formulation stage 3 batches were prepared for reproducibility confirmation.

Preparation of films devoid of liposomes (Stage 1 and 2)

Total batch size was 45 grams. One third of demineralised water/ PBS (preheated to 65– 70°C, *comp. gelation temperature of HPMC 2910*) were poured into a beaker followed by 5 % of PEG 400. HPMC (10 %) was then added along with remaining water (PBS). The mixture was left stirring with a magnetic stirrer (400 rpm) for 15 minutes on a hot plate heated to 70°C (using HPMC's insolubility in hot water to create a dispersion). The beaker was then left to cool down to approximately 35°C while being stirred continuously (400 rpm). Aqueous medium content lost through evaporation was replaced. The formed hydrogel had been placed into an ultrasound bath for 1.5 hour to get rid off of air bubbles. The mixture was then poured into a Petri dish (16 x 92 mm), 20 grams each. Both discs were placed in a drying chamber without ventilator set to 30°C for 39 hours (these conditions proved to be the most effective ones in terms of future liposome and peptide stability compared to 35°C used in preliminary tests). The dried films were taken out and left to equilibrate overnight and peeled off before analyzing.

Preparation of liposome-containing films (Stage 3)

The steps from Stage 1 and 2 up to ultrasound bath apply with one correction: the addition of the liposomal suspension was substracted from the aqueous phase used for the formation of the gel.

The liposome suspension (100 mg/ml lipid) is pipetted into the mixture devoid of air bubbles. The beaker is placed onto a magnetic stirrer and stirred at 200–250 rpm for 3 minutes. The final product is then poured onto a Petri dish, 20 grams per one dish.

Once again, corresponding steps from Stage 1 and 2 apply.

6.3 Film analysis

6.3.1 Thickness uniformity

Two cut-outs (2.5 x 5 cm) were carved in each film disc (*Fig. 6.3 (a)*) and their thickness was measured in five places (*Fig. 6.3 (b)*) using a digital outside micrometer. The average film thickness was then calculated.

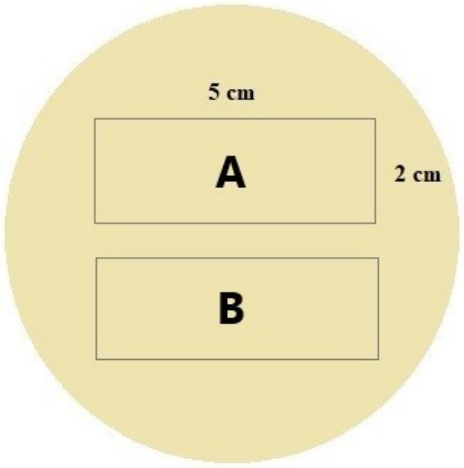

Fig. 6.3 (a)

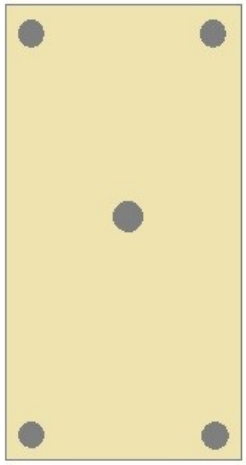

Fig. 6.3 (b)

6.3.2 Mechanical properties

To explore films' mechanical properties, Texture Analyzer TA.XT Plus using drag-strain module (described in section 4.1.2.3) had been employed. Cut-outs (2.5 x 5 cm) with defined average thickness were subjected to pulling generated by 5 kg cell with return distance set to 30 mm, return speed to 10 mm/s and contact force to 1 g.

To characterize films' resilience to tension, ultimate tensile strength as well as stress (both obtained at breaking point) were noted with respect to sample's stress area. Stress- strain curve (see) generated by TA. XT Plus had been used for further characterization of liposomal plasticizing effects.

Films with uneven or ripped break line were excluded from further processing.

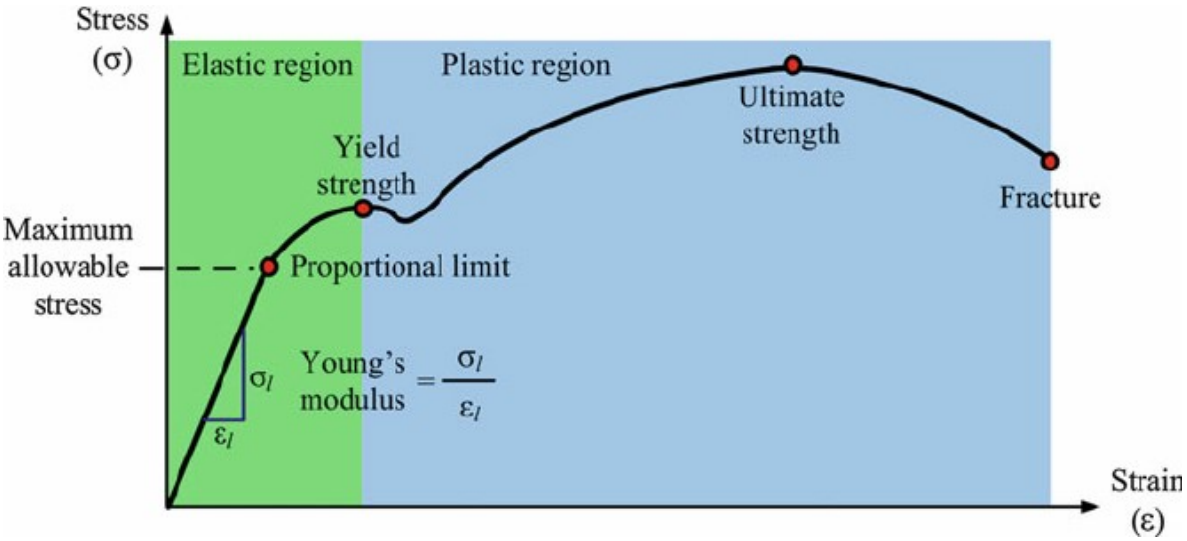

Fig. 6.3.2 - [86]

6.3.3 Residual moisture content

A sample (1g) consisting of a cut-out (processed in previous steps with additional film material added when its weight didn't amount to 1 g) was placed on a metal pan and into the moisture analyser set to temperature range of 21–121°C. The RMC after cycle completion was noted.

6.3.4 In vitro swelling

A patch of 1 cm^2 was weighed and placed into 7 ml of PBS in a small Petri dish (12 x 32 mm) heated to 37°C. The patch was then taken out, dried off using a light absorbent tissue and weighed. These steps were repeated in 5-, 10- and 15-minute intervals.

6.3.5 Liposome integrity after dissolution

Film patch used for *in vitro* swelling analysis was left to dissolve completely. 116.4 µl of the solution were then pipetted into a disposable cuvette filled with 1 ml of PBS and their size and size distribution was analysed using dynamic light scattering (Zetasizer).

7. Results

7.1 Film formulation and method optimization

The final formulation consisted of 10% (w/w) HPMC and 5 % (w/w) PEG 400 as plasticizer.

Liposome concentration had been based on the premise that the switch from the usual s.c. delivery for peptides and proteins to buccal administration route shall require dose increase in order to achieve equivalent plasma concentrations:

Out of the 3 tested liposome concentrations (1 %, 1.5 % and 2 %), the 2 % had been chosen.

1 disc = 20 g of suspension with liposome concentration of 2 %
1 dose (1x1cm patch) = approx. 0.5 g therefore contains 10 mg of liposomes

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Loading of the liposomes with model peptide (encapsulation efficiency of approximately 25%) would deliver 2.5 mg (per 100 mg liposomal suspension) of the calculated peptide.

As each film patch contains 10 mg of liposomal suspension the expected amount of encapsulated model peptide is approximately 0.25 mg.

7.2 Appearance

Films containing pure polymer in H2O were clear and transparent with considerable flexibility when bended or twisted (*Fig. 7.2 (a)*). Second stage films with PBS as solution medium lost this transparency and became translucent with a hint of white (*Fig. 7.2 (b)*). In addition to that, some of the flexibility had been lost resulting in resilience decrease. Liposome inclusion in the third stage caused the films to become opaque white (*Fig. 7.2 (c)*) and lowered their flexibility further but not on the same scale as in the second stage.

Experiments with different drying chambers showed that one without a ventilator is the only suitable option as it did not produce a ripple effect on the films' surface. The drying cycle consisted of 39 hours at 30°C – 2 film discs per cycle.

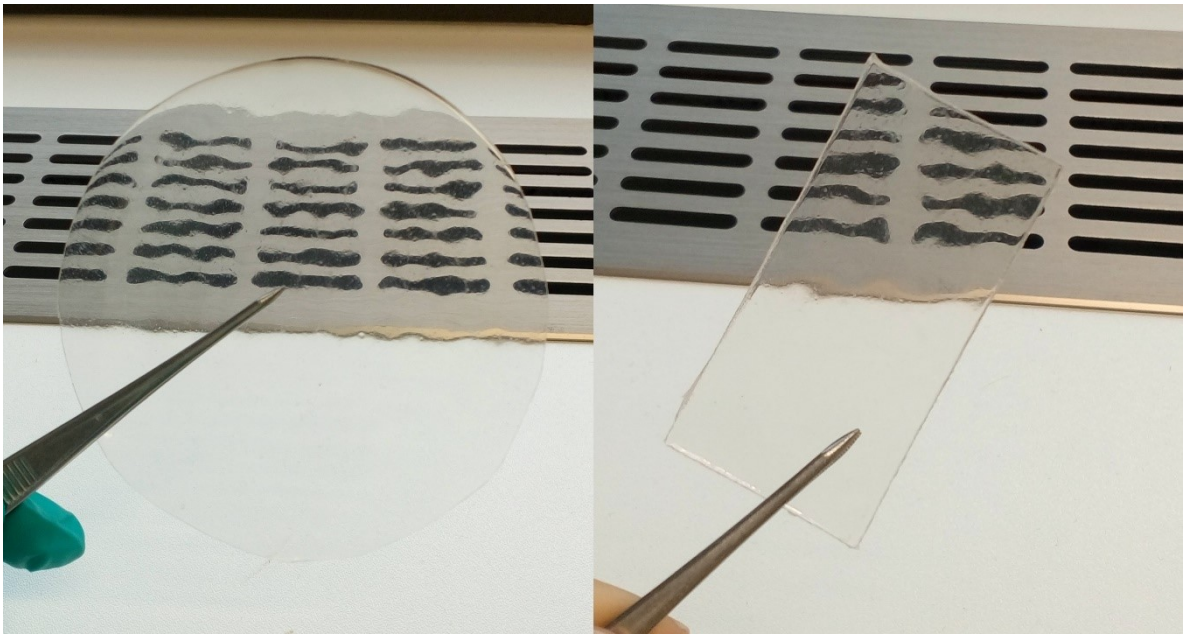


Figure 7.2 (a)

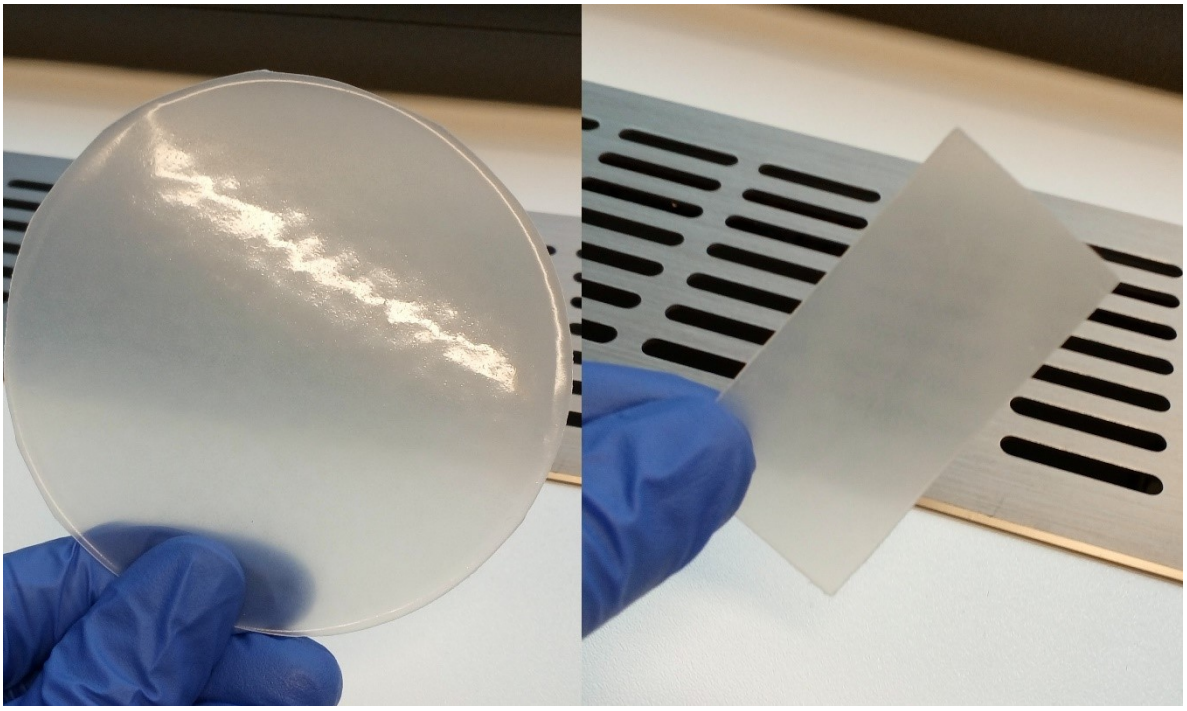


Figure 7.2 (b)

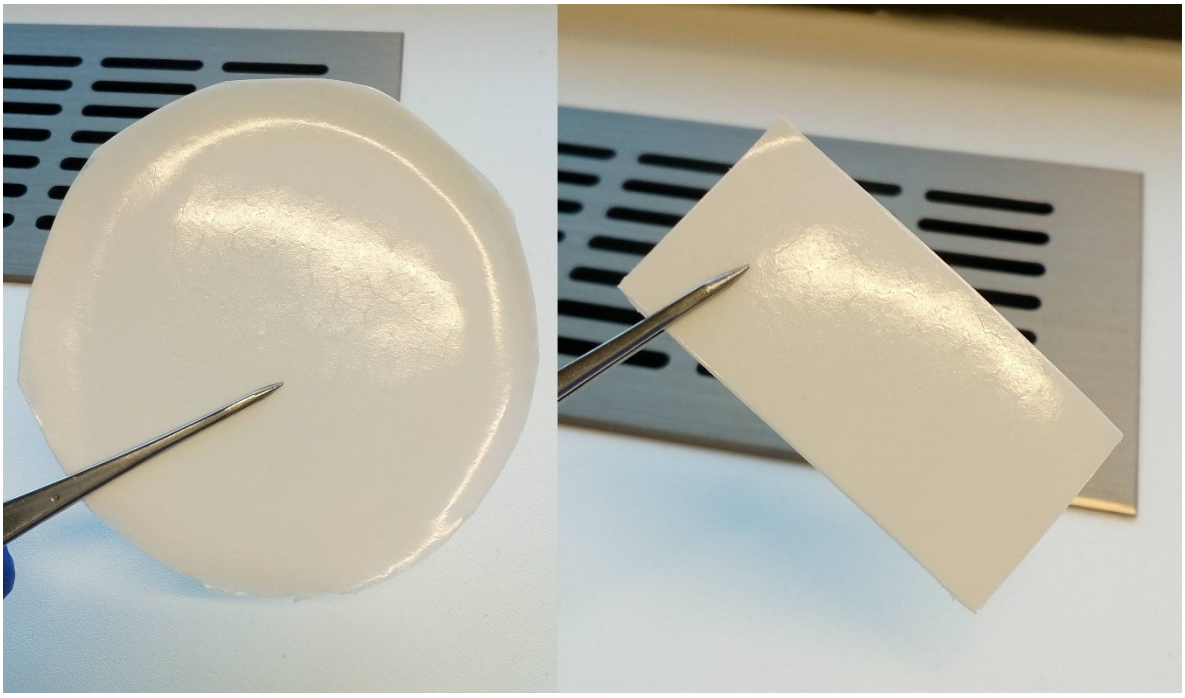


Figure 7.2 (c)

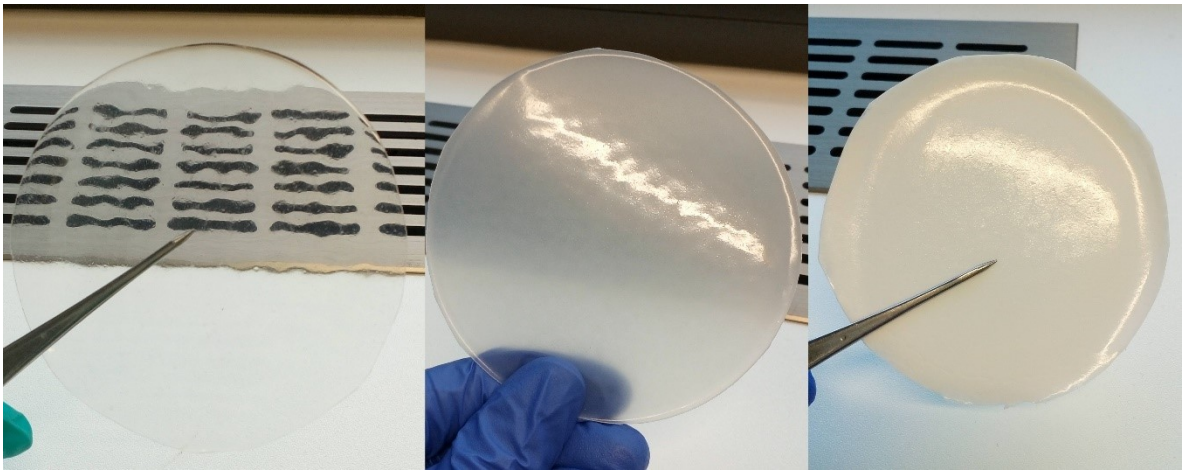


Figure 7.2 (d) (A side-by-side comparison of film formulation in all three stages. From left to right: first stage – H2O, second stage – PBS, third stage – liposomes.)

7.3 Thickness

In terms of formulation thickness films from H2O stage showed average thickness of 448,05 µm (RSD=2,41%), those in PBS stage 483,47 µm (RSD=5,90%) and 552,35 µm (RSD=3,08%) (*Graph 7.3 (a)*).

For batch variability data see *Graph 7.4 (b)*.

H2O	A1	A2	B1	B2	**Batch Average [µm]**	**RSD [%]**	**Formulation Average [µm]**	**RSD [%]**
1st batch	460,00	443,80	460,00	442,00	**451,45**	**1,90**	**448,05**	**2,41**
2nd batch	449,00	448,20	427,20	442,00	**441,60**	**1,98**		
3rd batch	449,00	470,60	440,00	444,80	**451,10**	**2,59**		

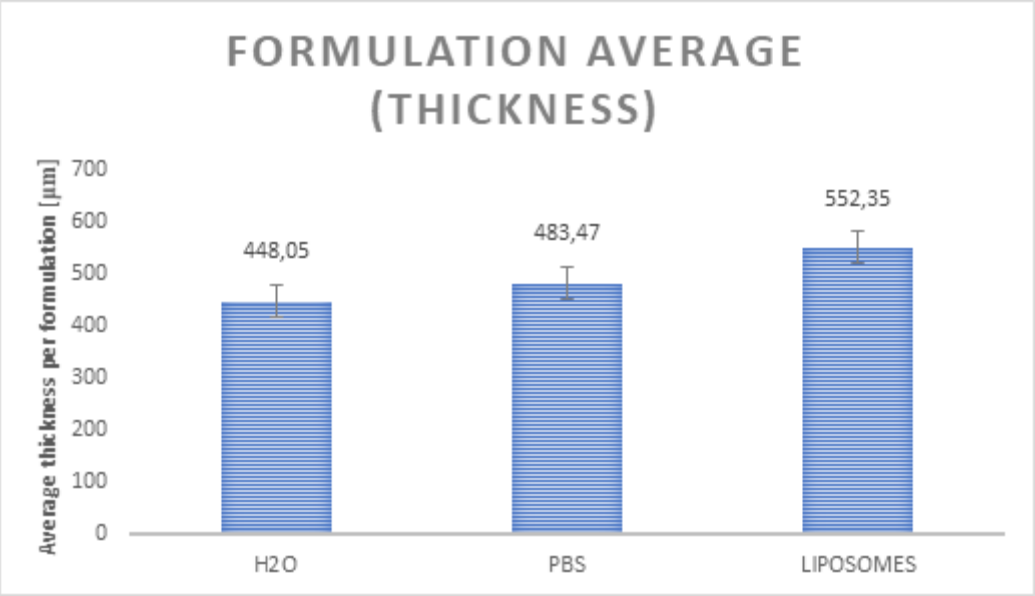
Table 7.3 (a)

PBS	A1	A2	B1	B2	**Batch Average [µm]**	**RSD [%]**	**Formulation Average [µm]**	**RSD [%]**
1st batch	492,20	480,20	472,60	478,00	**480,75**	**1,49**	**483,47**	**5,90**
2nd batch	491,00	483,80	455,80	485,40	**479,00**	**2,85**		
3rd batch	456,60	552,80	437,40	515,80	**490,65**	**9,39**		

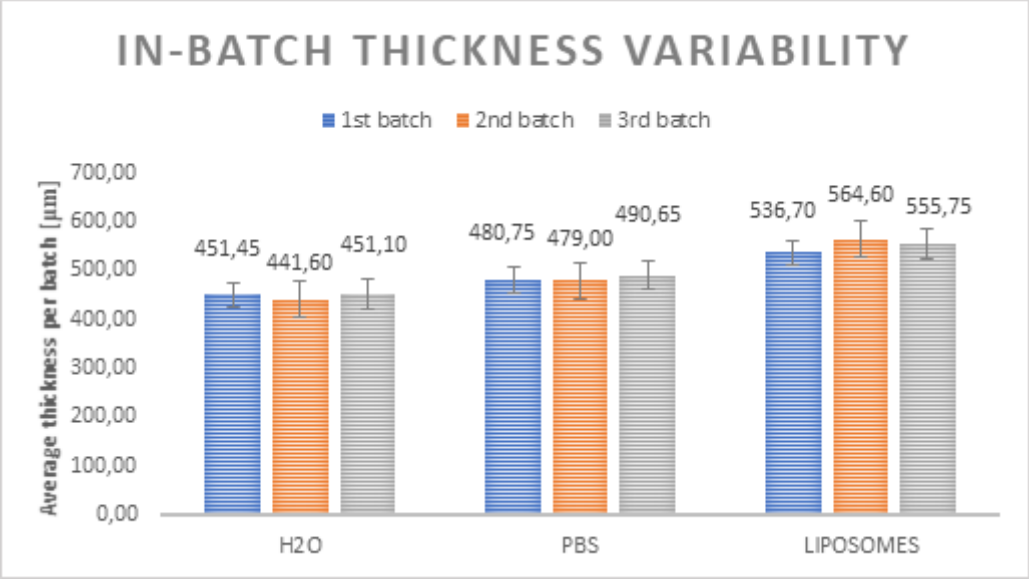
Table 7.3 (b)

LIPO-SOMES	A1	A2	B1	B2	**Batch Average [µm]**	**RSD [%]**	**Formulation Average [µm]**	**RSD [%]**
1st batch	537,80	540,20	542,40	526,40	**536,70**	**1,15**	**552,35**	**3,08**
2nd batch	572,80	542,00	557,20	586,40	**564,60**	**2,95**		
3rd batch	540,20	547,80	566,00	569,00	**555,75**	**2,18**		

Table 7.3 (c)



Graph 7.3 (a)



Graph 7.3 (b)

7.4. Maximum tensile strength

Ultimate (maximum) tensile strength measurements yielded values (at breaking point) as follows: 154,58 N for H2O stage (RSD= 8,92%), 166,29 N in PBS stage (RSD= 5,56%) and finally 120,83 N in liposome-containing formulation (RSD= 4,68%) (*Graph 7.4 (a)*).

The results obtained from the texture analyser are considered of significance if RSD < 10% (empirically established value).

For batch variability data see *Graph 7.4 (b)*.

H2O	A1	A2	B1	B2	**Batch Average [N]**	**RSD [%]**	**Formulation Average [N]**	**RSD [%]**
1st batch	168,77	147,35	154,83	154,24	**156,30**	**4,98**	**154,58**	**8,92**
2nd batch	143,39	121,12	140,40	168,13	**143,26**	**11,66**		
3rd batch	168,44	164,06	161,37	162,90	**164,19**	**1,60**		

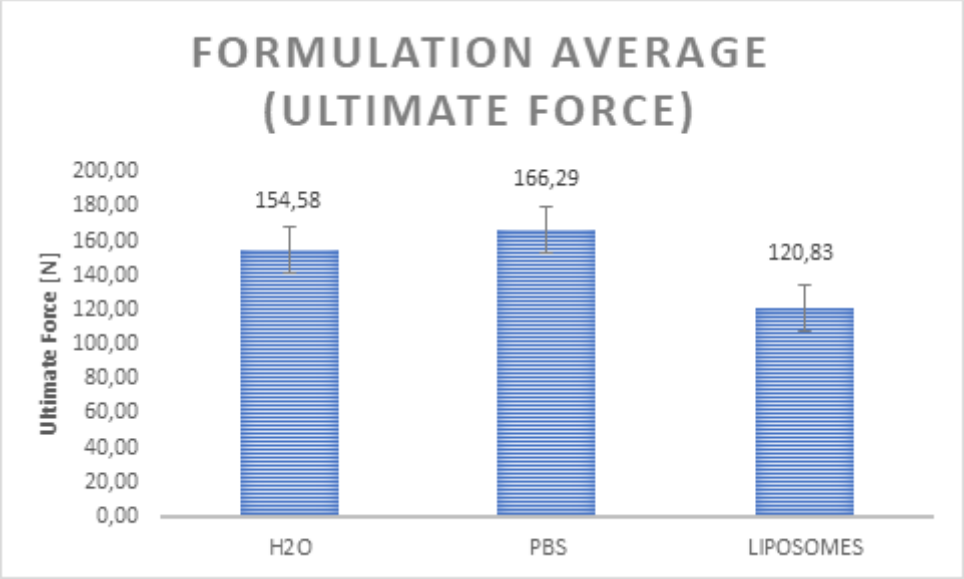
Table 7.4 (a)

PBS	A1	A2	B1	B2	**Batch Average [N]**	**RSD [%]**	**Formulation Average [N]**	**RSD [%]**
1st batch	167,48	161,84	174,14	171,66	**168,78**	**2,76**	**166,29**	**5,56**
2nd batch	163,37	166,03	166,77	172,10	**167,07**	**1,90**		
3rd batch	150,62	181,68	147,52	172,25	**163,02**	**8,82**		

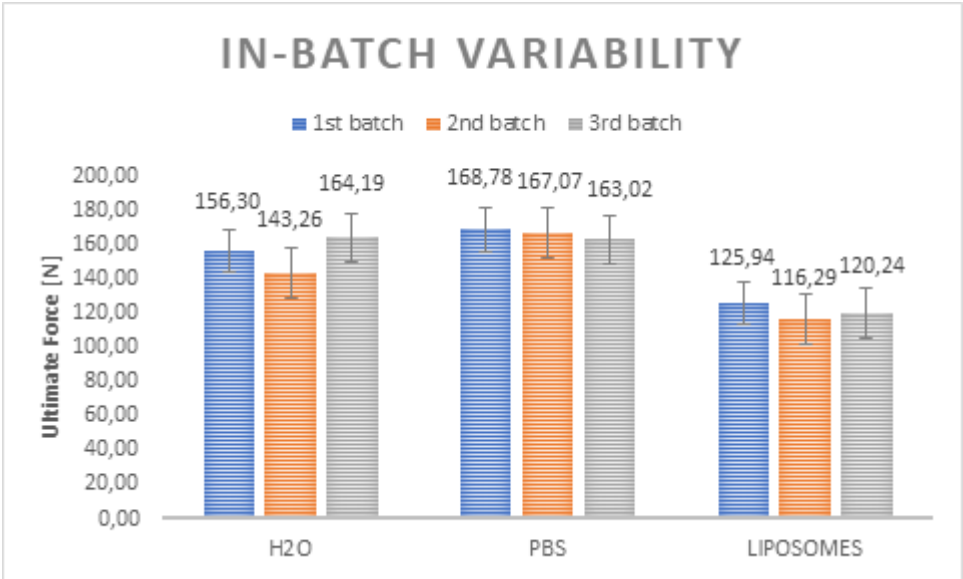
Table 7.4 (b)

LIPO-SOMES	A1	A2	B1	B2	**Batch Average [N]**	**RSD [%]**	**Formulation Average [N]**	**RSD [%]**
1st batch	124,11	123,05	131,69	124,91	**125,94**	**2,69**	**120,83**	**4,68**
2nd batch	122,75	115,07	112,68	114,67	**116,29**	**3,30**		
3rd batch	112,92	119,60	125,84	122,62	**120,24**	**3,97**		

Table 7.4 (c)



Graph 7.4 (a)



Graph 7.4 (b)

7.5 Strain

The strain values were 13,89 % with RSD= 14,53 %, 11,35 % with RSD= 12,92 % and 6,53 %, RSD= 14,30 % in H2O, PBS and liposomal stage respectively and continued with the addition of liposomal suspension (once again indicating shift in polymer chain network (*Graph 7.5 (a)*). For batch variability data see *Graph 7.5 (b)*.

$$strain = \frac{change\ in\ length}{original\ length} \times 100\%$$

Fig. 7.5 (a)

H2O	A1	A2	B1	B2	**Batch Average [%]**	**RSD [%]**	**Formulation Average [N.mm^{-2}]**	**RSD [%]**
1st batch	14,46	14,71	12,61	14,91	**14,17**	**6,46**	**13,89**	**14,53**
2nd batch	9,58	12,76	12,72	16,20	**12,82**	**18,26**		
3rd batch	18,01	14,37	12,70	13,65	**14,68**	**13,69**		

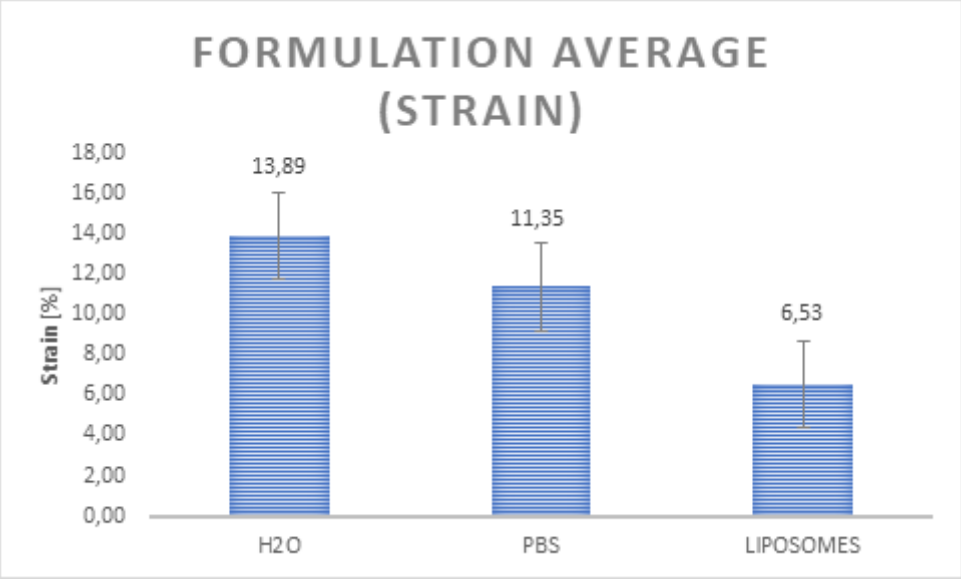
Table 7.5 (a)

PBS	A1	A2	B1	B2	**Batch Average [%]**	**RSD [%]**	**Formulation Average [N.mm^{-2}]**	**RSD [%]**
1st batch	10,65	10,42	13,30	10,42	**11,20**	**10,87**	**11,35**	**12,92**
2nd batch	10,87	11,17	12,33	11,53	**11,48**	**4,74**		
3rd batch	8,22	12,05	11,02	14,20	**11,37**	**18,93**		

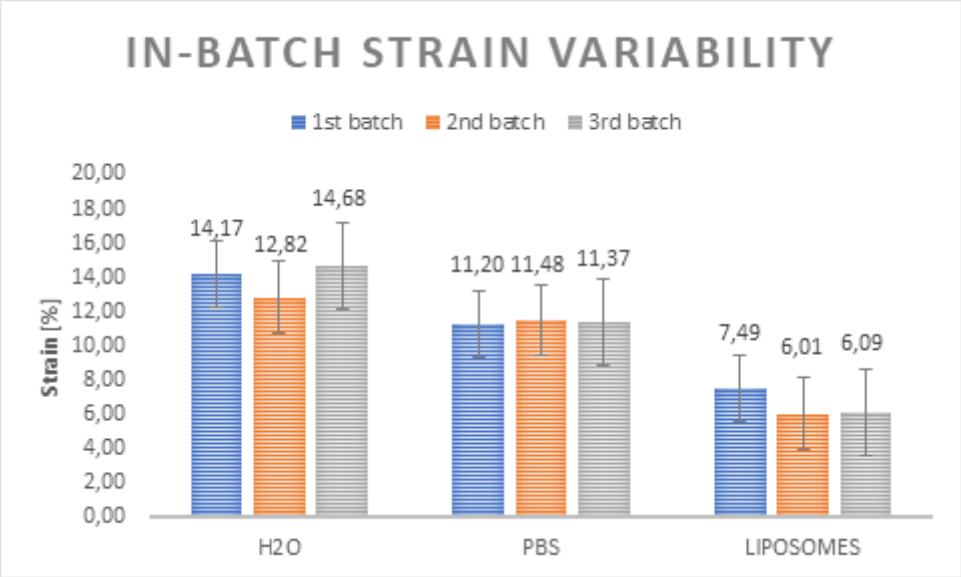
Table 7.5 (b)

LIPO-SOMES	A1	A2	B1	B2	**Batch Average [%]**	**RSD [%]**	**Formulation Average [N.mm^{-2}]**	**RSD [%]**
1st batch	8,67	6,43	8,25	6,60	**7,49**	**13,14**	**6,53**	**14,30**
2nd batch	6,45	6,42	5,70	5,49	**6,01**	**7,10**		
3rd batch	5,62	6,25	6,39	6,07	**6,09**	**4,74**		

Table 7.5 (c)



Graph 7.5 (a)



Graph 7.5 (b)

7.6 Stress-strain measurements

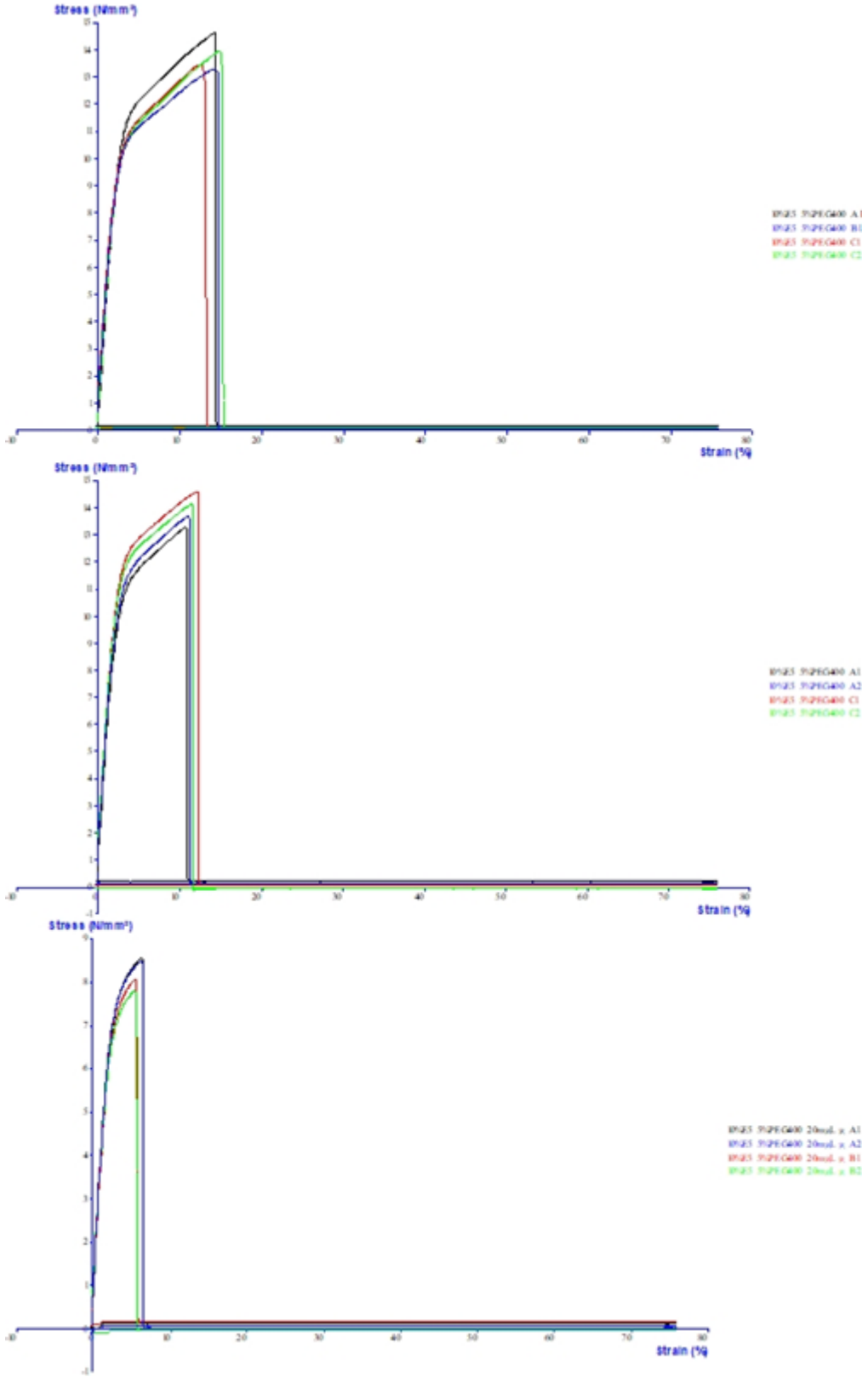


Fig. 7.6 (a) – A representative stress-strain curve of one batch from each formulation stage. Top to bottom: H2O (1st batch), PBS (2nd batch) and liposomal stage (2nd batch).

7.7 Residual moisture content

The evaluation delivered RMC values of 2,55% (RSD= 20,44%) in H2O stage, 3,41% (RSD= 9,98%) in PBS stage and 3,38% (RSD= 9,30%) in liposomal stage (*Graph 7.7 (a)*).

For batch variability data see *Graph 7.7 (b)*.

H2O	A1	A2	B1	B2	**Batch Average [%]**	**RSD [%]**	**Formulation Average [%]**	**RSD [%]**
1st batch	2,20	3,49	2,10	2,10	**2,47**	**23,82**	**2,55**	**20,44**
2nd batch	2,80	3,35	2,50	2,40	**2,76**	**13,38**		
3rd batch	2,10	3,30	2,00	2,30	**2,43**	**21,30**		

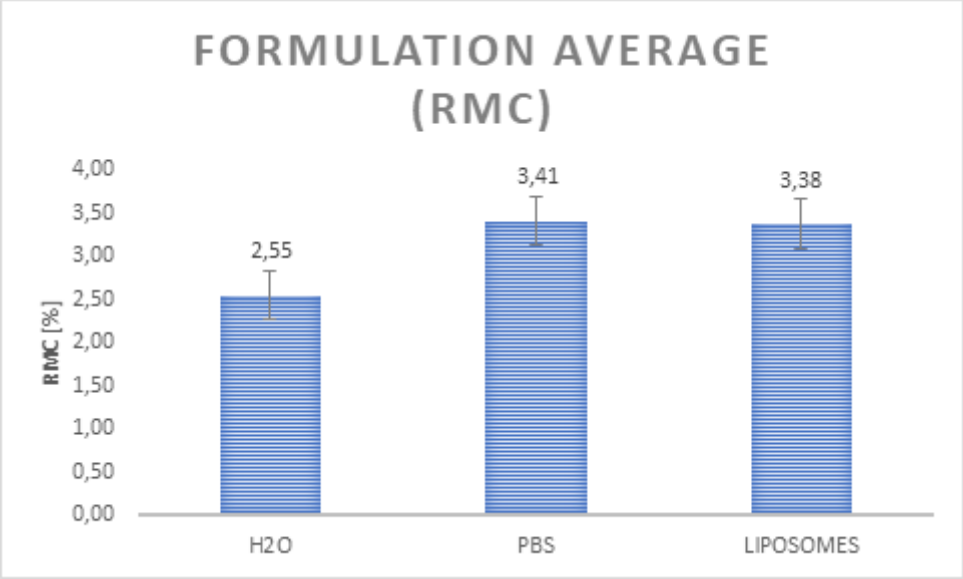
Table 7.7 (a)

PBS	A1	A2	B1	B2	**Batch Average [%]**	**RSD [%]**	**Formulation Average [%]**	**RSD [%]**
1st batch	3,70	3,80	2,89	2,90	**3,32**	**12,91**	**3,41**	**9,98**
2nd batch	3,50	3,60	3,00	3,00	**3,28**	**8,47**		
3rd batch	3,70	3,75	3,60	3,49	**3,64**	**2,74**		

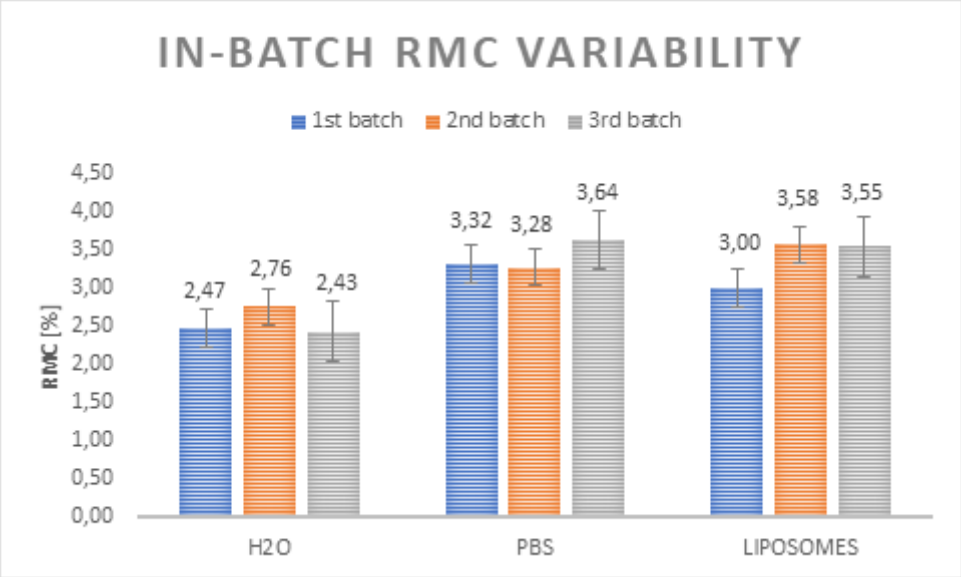
Table 7.7 (b)

LIPO-SOMES	A1	A2	B1	B2	**Batch Average [%]**	**RSD [%]**	**Formulation Average [%]**	**RSD [%]**
1st batch	2,70	3,10	3,00	3,20	**3,00**	**6,24**	**3,38**	**9,30**
2nd batch	3,70	3,70	3,50	3,40	**3,58**	**3,63**		
3rd batch	3,30	3,80	3,60	3,50	**3,55**	**5,08**		

Table 7.7 (c)



Graph 7.7 (a)



Graph 7.7 (b)

7.8 In vitro swelling

The film weight change after 5, 10 and 15 minutes (expressed percentwise) was as follows: 54,36% (RSD= 2,06 %); 51,46% (RSD= 0,93 %) and 28,79% (RSD= 62,08 %) in the first stage (H2O), 50,55% (RSD= 2,56 %); 47,46% (RSD= 6,06 %) and 28,42% (RSD= 2,14 %) in the second stage (PBS) and finally 42,42% (RSD= 2,80 %); 42,15% (RSD= 3,35 %) and 28,90% (RSD= 27,08 %) in the third stage (liposomes). *(Complete dissolution had been observed after approximately 90 minutes.)* For more detailed data see *Tables 7.8 (a–c)* and *Graph 7.8*.

H2O	Swelling batch averages [g]				Swelling batch averages [%]		
	m(0)	**m(5)**	**m(10)**	**m(15)**	**m(1)-m(0)**	**m(5)-m(0)**	**m(10)-m(0)**
1st batch	0,0481	0,1024	0,0998	0,0743	52,82	51,61	53,54
2nd batch	0,0483	0,1074	0,1011	0,0586	54,82	51,95	20,88
3rd batch	0,0446	0,0994	0,0904	0,0609	55,45	50,80	11,96
Formulation average	**0,0470**	**0,1031**	**0,0971**	**0,0646**	**54,36**	**51,46**	**28,79**
RSD [%]	**3,6149**	**3,2016**	**4,9096**	**10,7166**	**2,06**	**0,93**	**62,08**

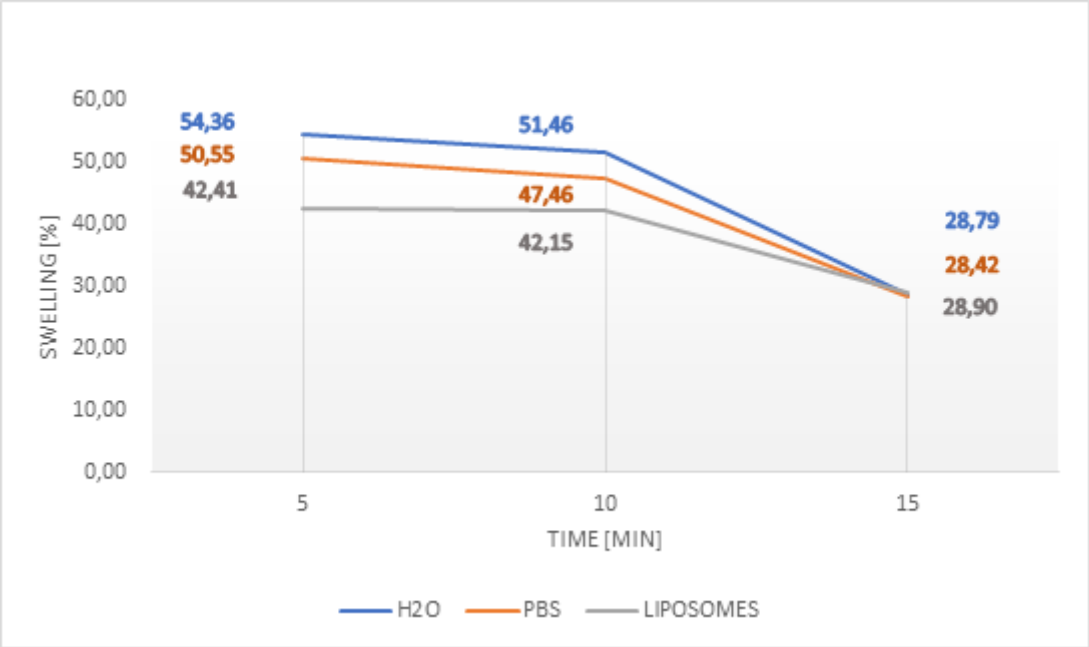
Table 7.8 (a)

PBS	Swelling batch averages [g]				Swelling batch averages [%]		
	m(0)	**m(5)**	**m(10)**	**m(15)**	**m(1)-m(0)**	**m(5)-m(0)**	**m(10)-m(0)**
1st batch	0,0572	0,1160	0,1164	0,0803	50,75	51,07	29,08
2nd batch	0,0602	0,1175	0,1139	0,0847	48,88	47,26	28,57
3rd batch	0,0493	0,1026	0,0879	0,0680	52,03	44,05	27,62
Formulation average	**0,0556**	**0,1120**	**0,1061**	**0,0777**	**50,55**	**47,46**	**28,42**
RSD [%]	**8,2736**	**5,9790**	**12,1492**	**9,0997**	**2,56**	**6,05**	**2,14**

Table 7.8 (b)

LIPOSOMES	Swelling batch averages [g]				Swelling batch averages [%]		
	m(0)	**m(5)**	**m(10)**	**m(15)**	**m(1)-m(0)**	**m(5)-m(0)**	**m(10)-m(0)**
1st batch	0,0604	0,1037	0,1027	0,0913	41,75	41,18	33,84
2nd batch	0,0596	0,1017	0,1067	0,0917	41,39	44,14	35,01
3rd batch	0,0543	0,0971	0,0922	0,0661	44,07	41,11	17,85
Formulation average	**0,0581**	**0,1008**	**0,1005**	**0,0830**	**42,41**	**42,15**	**28,90**
RSD [%]	**4,6588**	**2,7404**	**6,0822**	**14,4217**	**2,80**	**3,35**	**27,08**

Table 7.8 (c)



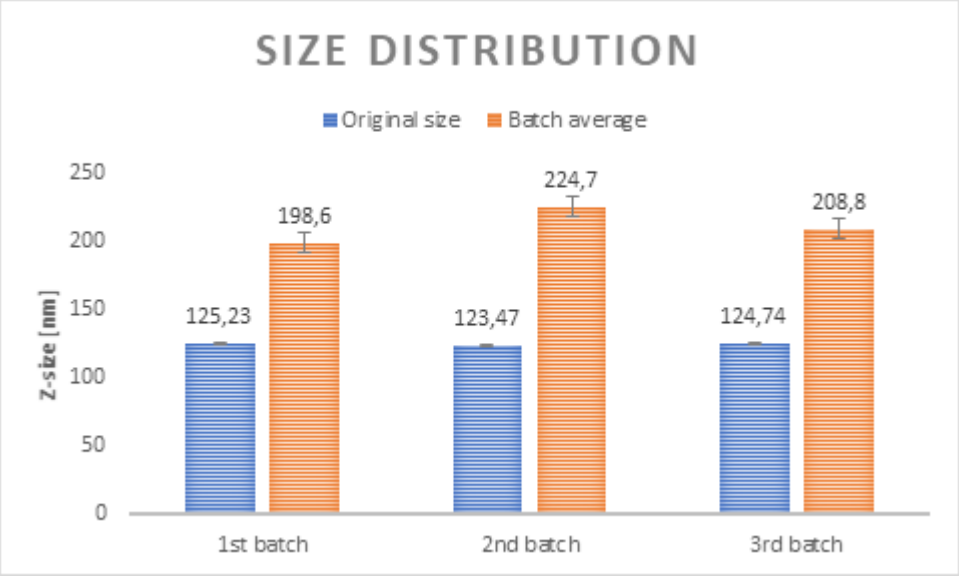
Graph 7.8

7.9 Liposome integrity after dissolution

The liposome size underwent change from 125,23 nm to 198,6 nm (RSD= 3,90 %) in the first batch, 123,47 nm to 224,7 (RSD= 2,59 %) nm in the second and 124,74 nm to 208,8 nm (RSD= 9,59 %) in third stage (mean change interval: 124,5 nm to 210,7 nm with RSD= 7,94 %).

SIZE [nm]	Original size	**Batch average**	**RSD [%]**	**Formulation average**	**RSD [%]**
1st batch	125,23	**198,6**	**3,90**	**210,7**	**7,94**
2nd batch	123,47	**224,7**	**2,59**		
3rd batch	124,74	**208,8**	**9,59**		

Table 7.9 (a)

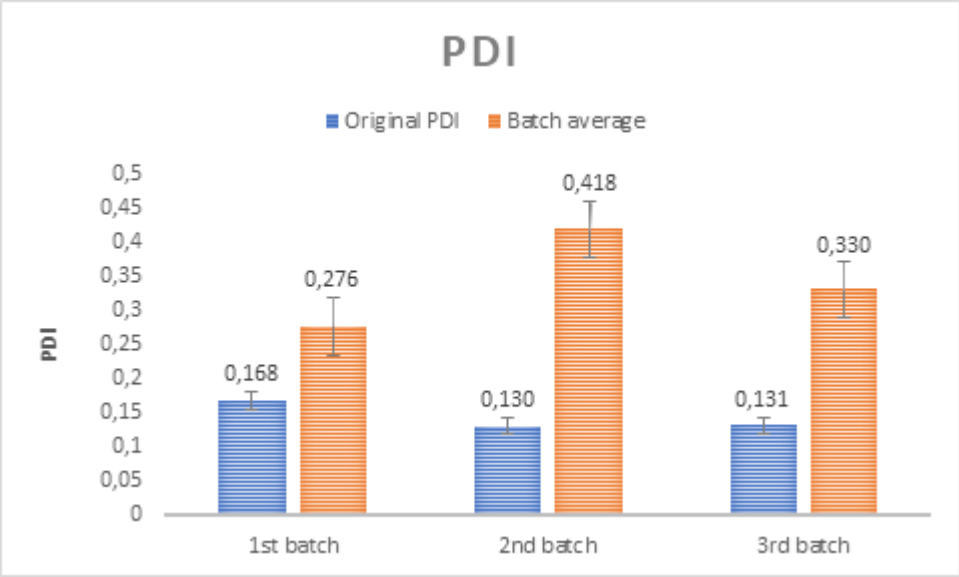


Graph 7.9 (a)

The PDI changed from 0,168 to 0,276 with RSD= 24,16 % (first batch), 0,130 to 0,418 with RSD= 13,35 % (second batch) and 0,131 to 0,330 with RSD= 12, 94 % (third batch).

PDI	Original PDI	**Batch average**	**RSD [%]**	**Formulation average**	**RSD [%]**
1st batch	0,168	0,276	10,01	0,3416	19,54
2nd batch	0,130	0,418	5,63		
3rd batch	0,131	0,330	12,95		

Table 7.9 (b)



Graph 7.9 (b)

8. Discussion

Method and formulation

Although initial stages incorporated work with both hypromellose E4M- and E5-type, only type E5 showed suitable handling properties and had therefore been chosen for further development. Solutions of E4M (3%, 4%, 5% and 7%) all possessed high viscosity detrimental to homogenity and handling ease.

Type E5 failed to produce films of sufficient rigidity in concentrations lower than 10%. Further increase had not been pursued as certain elasticity is crucial to film's resilience. Moreover, too dense polymer scaffolding could potentially result in liposome immobility causing the drug's entrapment.

The choice of plasticizer type and concentration had been based on a study by *S. Honary and H. Orafai, 2002* [87], which states clear benefits to using low molecular weight PEG of lower concentrations in hypromellose formulations. Contributing argument specific to this project was the expected plasticizing effect of liposomes. [88] Furthermore, as PEG is known for its hygroscopic properties, its high concentrations could have negative effect on residual moisture content and therefore strength of mucoadhesion. [12]

The drying temperature had been first set to 35°C and combined with a 29-hour drying period. These conditions were effective in the first two stages but caused characteristic phospholipid odor in Stage 3 formulation with liposomes. [88] In order to prevent presumed lipid degradation the temperature was lowered to 30°C, which resulted in 39- hour drying period.

Initial experiments also uncovered the dependence of batch homogeneity on number of films being dried in the same cycle. Original number of three delivered RMC and mechanical properties inconsistency mainly in the middle disc due to its central position and the chamber being heated from side panels. Two discs in one drying cycle exhibited acceptable uniformity and were therefore chosen as default option.

Thickness

The preparation method showed good reproducibility demonstrated on minimal differences among average batch thickness in all formulation stages. Slight RSD increase in PBS and liposomal stage was due to homogenization becoming more demanding with added components (*Graph 7.3 (b)*, *Tab. 7.3 (a–c)*). One of the possible explanations for the difference of 35,42 μm between H2O and PBS films could be the salting-out effect PBS ions have on HPMC polymer chains. PBS contains $(Cl)^-$ and $(H_2PO_4)^-$, both of which are considered to possess strong salting-out properties (according to the Hofmeister series [88]), plus Na^+ and K^+ cations with somewhat less pronounced salting-out capacity. These ions interact with water molecules in the dissociation process, which leads to decrease in water molecules free to form hydrogen bonds with HPMC hydroxyl groups thus causing its lower solubility at the same temperature. [89] As result more hydrophobic bonds within the polymer itself can form influencing polymer's spatial conformation and possibly contributing to thickness increase. However, further exploration requires SEM technique not available to the author at the time.

The 104,3 μm difference between H2O and liposomal stage was due to the addition of liposomal suspension.

To conclude, while the H2O and PBS film thickness lies below recommended value of 500 μm, the liposomal formulation intended for further development is within the acceptable range of 500–1000 μm and therefore suitable. [12]

Mechanical resilience

The larger strength needed for delivering breakage in PBS formulation compared to H2O and liposomal ones shows increase in formulation's resistance to mechanical stress upon pulling. The lower tensile strength needed in liposomal stage compared to that in H2O stage indicates liposomal interference with the polymer gel structure resulting in malleability increase.

Strain (*Fig. 7.4 (a)*) measurements showed decline in films' elasticity in H2O→ PBS→ liposome directional line supporting observations made in section *7.2 Appearance*.

Stress-strain measurements

Stress-strain curves depict the process of elongation when stress is applied. (*Fig. 7.4 (a)*) While stress-strain curves of all formulations show relatively small elastic region (the area under curve to the point of yield strength), none of them exhibited susceptibility to plastic deformation as ultimate strength was identical with yield strength (this classifies the films as brittle, as opposed to ductile [90]). The changes in film composition in different stages did, however, cause both the proportional and disproportional section of elastic region to become smaller with medium change from H2O to PBS and further with the addition of liposomes. (*Fig. 7.4 (c)*) This clearly demonstrates that both PBS and liposomal suspension lower the films' elasticity when the plasticizer concentration is kept the same (5% in all stages).

Residual moisture content

The aforementioned results demonstrate increase in RMC upon medium change with PBS causing the formulation in stage 2 and stage 3 to retain more moisture after being subjected to the drying period. The insignificant difference between stages 2 and 3 is due to PBS being the medium in both instances. (*Graph 7.5 (a)*)

RMC in all stages lies below the recommended 5%. [12] This parameter had been therefore declared acceptable for further development.

In vitro swelling

The decreasing swelling capacity implies water molecules penetrating polymer chains at a slower rate in PBS formulation, which becomes even more pronounced in liposomal stage. (*Graph 7.6*) [90]

Furthermore, the film disintegration follows a trendline of doubling in weight in the initial 5 minutes (54,36%; 50,55% and 42,41%) followed by relative plateau phase of 5 minutes (weight increase after 10 minutes) and concluded by the start of disintegration clearly visible after 15 minutes. (*Graph 7.6*)

Liposome integrity after dissolution

The average size after dissolution was 210,7 nm (*Table 7.9 (a)*), which is slightly above the acceptable size range of 200 nm. This value has shown correlation with longer circulation time of the liposomes. [91]

The change in liposome size indicates structural changes during drying and/or swelling period. (The influence of stirring and ultrasound period has been ruled out through separate measurements after the corresponding cycles.)

A possible explanation for the size change is liposome-polymer interaction during the swelling period which could result in formation of small liposome/polymer aggregates. ([92])

The PDI change corresponds with observed change in liposome size. The presumed cause of PDI fluctuations are liposome/polymer aggregates of uneven size and inhomogeneity through samples.

9. Outlook

To conclude, this thesis contains a comprehensive view of preliminary formulation stage of a new dosage form. The choice of backbone polymer had been focused on readily available substance with zero toxicity and sufficient mucoadhesive properties. Initial testing established the need for a plasticizer with minimum impact on film's mucoadhesion. The developed preparation method produced films of reasonable homogeneity while testing methods enabled conceptual changes throughout the development process. The third formulation of 10 % HPMC with 5 % PEG 400 and 20mg/ g liposomes presents a solid starting point for the advanced formulation stage.

Areas with room for improvement were identified and are outlined in the following lines.

The PBS as solvent and source of stability for future entrapment of model peptide has had negative effect on film's mucoadhesive strength thus establishing the need for additional components. The film preparation uncovered tricky steps creating an incentive for process modification. Lastly, considering liposome's key role in the dosage form's intended utilisation more extensive liposome stability tests are due to performed before moving on to working with model peptide.

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