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Faculty of Pharmacy in Hradec Králové

Department of Pharmaceutical Technology

Active Encapsulation of Imiquimod in Liposomes with Dendrimers

Diploma thesis

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Hradec Králové 2022

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In Hradec Králové 2022 Dina Manna

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Abstrakt

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Název diplomové práce: Aktivní enkapsulace imiquimodu do lipozomů s dendrimery

Jednou z hlavních překážek, které je třeba řešit při vývoji systémů pro dodávání léčiv jako jsou lipozomy, je účinná enkapsulace („drug loading“) adekvátní koncentrace účinné látky. Enkapsulaci léčiva do liposomů je možné provést dvěma způsoby: pasivním a aktivním. Při pasivní enkapsulaci se léčivo dostává do lipozomu během jeho přípravy. Při aktivní enkapsulaci však dochází k průchodu léčiva přes fosfolipidovou dvojvrstvu lipozomu poté, co je lipozom kompletně zformován. Bylo dokázáno, že tato metoda je účinná pro nízkomolekulární ionizovatelná léčiva a lze tedy předpokládat, že by mohlo jít o účinnou metodu enkapsulace léčiva imiquimodu (IMQ). IMQ je imidazochinolon známý pro svou extrémní hydrofobicitu a zásaditost., užívaný pro léčbu kožních onemocněních (např. karcinom kůže).

Příprava lipozomů v této práci probíhala pomocí klasické metody tenkého filmu, kdy byly lipidy hydratovány vodným roztokem v naší výzkumné skupině syntetizované první generace dendrimerů o dvou různých koncentracích (5 mM a 10 mM). Jakmile byly lipozomy připraveny, byl vytvořen pH gradient mezi jádrem a vnějším prostředím lipozomů, a to výměnou pufru (PBS, pH=7,4). Bylo testováno více inkubačních podmínek (včetně změn teploty, délky inkubace a využití mechanického míchání), aby se vyhodnotilo, zda je možné provést aktivní enkapsulaci IMQ do lipozomů. Naše výsledky prokázaly, že aktivní enkapsulace IMQ do lipozomů je závislá na délce inkubace a míchání při specifické inkubační teplotě. Nejvyšší koncentrace IMQ (1,24 mg/ml) byla pozorovaná u lipozomů, které byly vytvořeny z 10 mM roztoku dendrimerů a inkubovány po dobu 10 dnů při 60 °C bez míchání.

Abstract

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Title of the thesis: Active Encapsulation of Imiquimod in Liposomes with Dendrimers

One of the main obstacles to tackle when dealing with drug delivery systems such as liposomes is efficiently loading adequate concentration of active ingredient. Drug encapsulation in liposomes occurs in two possible methods: passive and active loading. Passive drug loading involves the capture of drugs into the liposome whilst it is being prepared. Active loading however is the passage of drug through liposome's phospholipid bilayer after the liposome is fully formed. This method has been shown to be effective for low molecular weight ionizable active components and it was hypothesized to be an effective method of encapsulation of imiquimod. IMQ is an imidazole quinolone drug known for its extreme hydrophobicity and basicity and have been used for skin diseases (e.g. skin cancer).

During our approach, liposomes were prepared using the classical thin layer method while the lipids were hydrated with aqueous solution of in-house synthesized first-generation dendrimer of two different concentrations (5 mM and 10 mM). Once the liposomes were prepared, a pH gradient was formed between the liposomes's core and the liposomes' exterior by buffer exchange (PBS, pH=7.4). Multiple incubation conditions were tested (including changes in temperature, incubation duration, and the incorporation of mechanical stirring) to evaluate if the active loading of IMQ to liposomes is possible. Our results proved that active loading of IMQ to liposomes is depended on duration and stirring at specific incubation temperature. The highest IMQ concentration (1.24 mg/ml) observed for the liposomes which were created with 10 mM of dendrimers and incubated for 10 days at 60 ^{o}C without stirring.

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1. List of abbreviations

AK	Actinic Keratosis
EPC	Egg Phosphatidylcholine
HPV	Human Papillomavirus
IMQ	Imiquimod
LUV	Large Unilamellar Vesicles
MLV	Multi-lamellar Vesicles
PBS	Phosphate Buffered Saline
PDI	Polydispersity index
SUV	Small Unilamellar Vesicles
Tc	Transition Temperature
TLR	Toll-like Receptor

2. Introduction and Aim of the work

Active loading has proven to be a formidable method of drug encapsulation for lipophilic drugs. The aim of this work was to successfully encapsulate a high concentration of the drug imiquimod into fully formed liposomes while using dendrimers as the driving force in various incubation conditions. The dendrimers, synthesized in our labs, contain a protonated amine group ending which is resulting acidic aqueous solutions after dissolving them in water. This acidity allows the formation of a pH gradient along the liposomal wall creating an acidic core. The gradient increases the driving force of the basic hydrophobic drug IMQ to move inside the liposomes which would get ionized and thus trapped. The tested samples were incubated in various incubation conditions which includes changed in temperature (Room temp, body temp, transition temp), duration (1,3,10 days), dendrimer concentration (5 mM, 10 mM) and the incorporation (or not) of mechanical stirring.

3. Theoretical part

3.1. Liposomes

3.1.1. Introduction

Liposomes are synthetic self-assembling spherical vesicles whose primary function is to deliver active ingredients (1). Due to their similarity to cellular membranes and small size, liposomes are considered to be one of the best methods of drug delivery (2). Similar to many biological membranes, liposomes are formed from phospholipid molecules. These molecules consist of a hydrophilic head and two hydrophobic tails (3). Due to their amphiphilic nature, they have the tendency to self-assembly (4). The formed vesicle is lined up in a bilayer where the hydrophilic head is found in the surface of the vesicle and the inner core while the hydrophobic tail is in between. A schematic representation of liposomes is given in Figure 1.

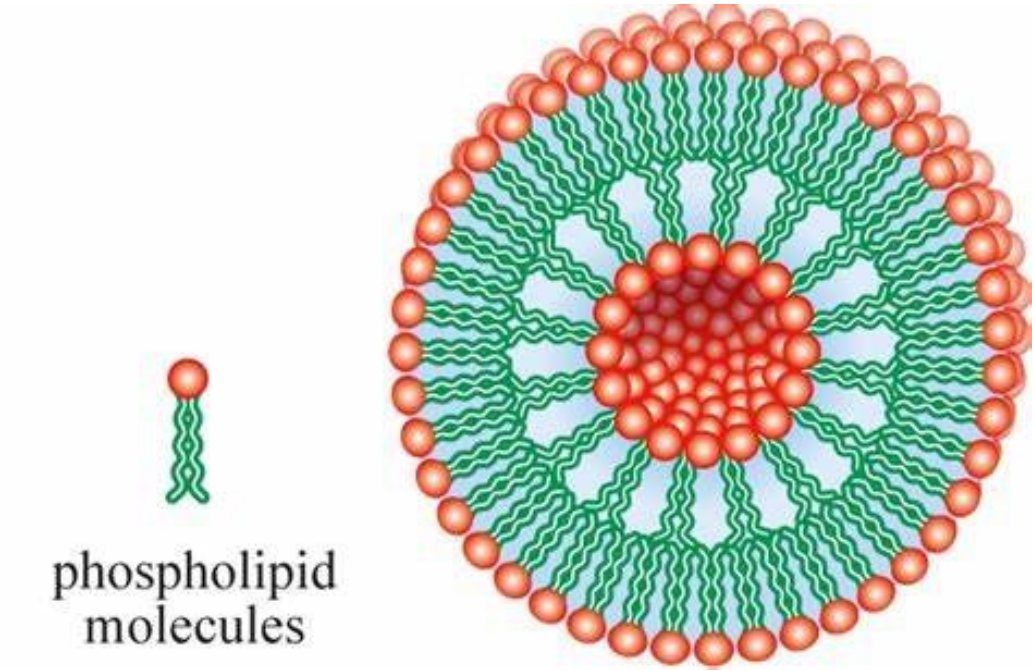

Figure 1: Liposome structure containing hydrophilic head and lipophilic tails

3.1.2. Characteristics

3.1.2.1. Size and Polydispersity Index

Characteristics such as size and polydispersity index (PDI) are critical to clarify the stability, encapsulation efficiency, cellular uptake as well as systemic drug release of the liposome (5). When discussing liposome size, it is said that liposomes larger than 600 nm cannot penetrate the skin layers whilst it's found that the range of 10-210 nm can in fact pass through the trans-follicular route (5) (6). This was proven when encapsulating amphotericin B,

it was found that liposomes smaller than 100nm were able to easily penetrate through pores since the diameter of the intercellular space between the corneocytes is 100nm (7).

PDI describes the size range of the liposome population, which is expressed quantitatively from the ranges of 0.0 (perfectly uniform) to 1.0 (multiple particle size distribution). PDI above 0.7 is considered unacceptable whilst if the measurement was shown to be less than or equal to 0.3, then it is considered monodisperse and homogenous (5). Measuring PDI is an important parameter to consider because it depicts the safety of liposome, a polydisperse population could induce a differential immune response (8) or even affect the accumulation tendency toward the target tissue (5).

Classification of liposomes can be considered depending on their size and number of bilayers formed (9);

Table 1: general summary of liposome classification

Type	Layers	Size	Preparation Method
SUV	Unilamellar	>100nm	Extrusion, High-energy sonic fragmentation, High-pressure homogenization, Solvent injection
LUV	Unilamellar	<100nm	Freeze-thaw cycling, De-/rehydration, Extrusion, Detergent dialysis, Reverse evaporation
MLV	Multilamellar	<100nm	Thin-film hydration (evaporation-dried, spray-dried or lyophilized lipid material)

It is important to understand that preparation generally starts with the creation of large liposomes which can be sized smaller according to needs (10). Typically, MLV or LUV liposomes are initially prepared followed by size reduction to become SUV (11).

3.1.2.2. Zeta potential

Colloidal nanoparticles such as liposomes can be examined by the electrical double layer theory which describes the relationship between the particle surface charge and the dispersed medium (12). Zeta potential measures the average potential of the moving liposome

in accordance with the electroneutral medium (13). A study analyzing the zeta potential of human Amelogenins verifies that zeta potential is influenced by pH changes. The study revealed that a positive zeta potential related to the increase in acidity of the media and vice versa applies (14). Figure 2 describes the results:

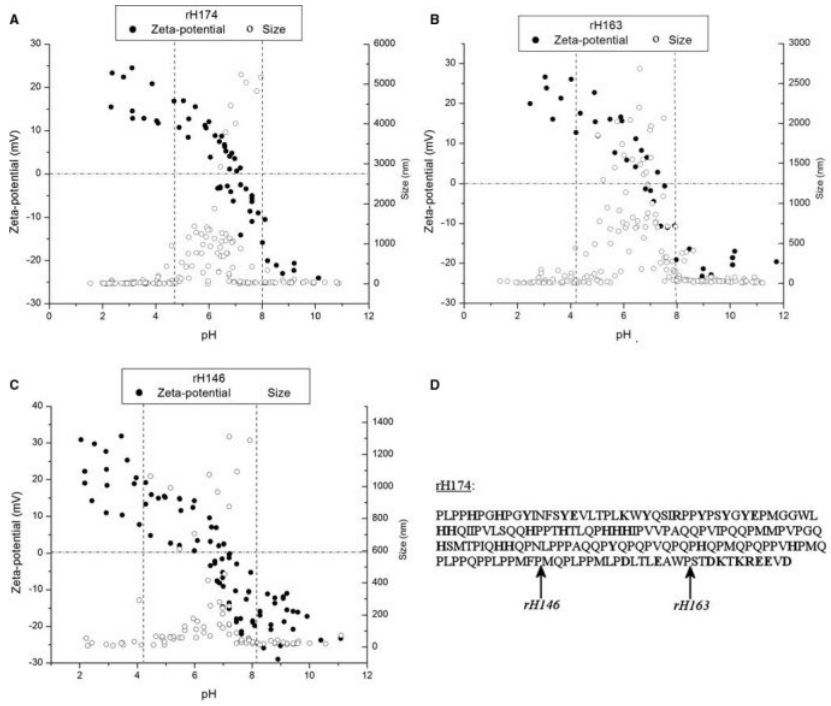

Figure 2 The graph shows both negative and positive zeta potential. In each sample, once the pH is near 7, the zeta potential is closer to zero. While it also indicates when pH drastically changes, zeta potential moves further away from zero

It is thought that the zeta potential can determine colloidal stability, and while this is true in general, the higher the zeta potential (in absolute value), the more stable the colloidal dispersion. Colloidal stability is a little more complicated than that; it is based on the sum of electrostatic repulsive forces and van der Waals attractive forces. The zeta potential assists in understanding repulsive forces but it does not describe the attractive forces (15). For example, the zeta potential of colloidal silica nanoparticles was measured to be low whilst also proven to be extremely stable (16).

3.1.2.3. Transition Temperature

Formation of liposomes is a multifactorial topic that revolves around electrostatic forces, changes in temperature, amphiphilicity, hydrogen bonds and much more. An important property of liposomes is the crystalline transition temperature, which is defined as the temperature where the lipid bilayer reaches a fluid gel like state and becomes less rigid (17) (18) . Liposome permeability is the cornerstone of drug encapsulation, this temperature constant

allows an easier movement of active ingredient when drug loading as well as increases the chances of a successful liposome preparation (19).

3.1.3. Applications

3.1.3.1. Dermal Application

Transdermal drug delivery is a broad topic within liposomes. When working with active ingredients, the usage of liposomes solely depends on the required effect. Some active substances have considerable side effects when applied orally; thus, liposomes could help with alternative drug delivery in this scenario (20). Transdermal liposome application can also be used to improve widely used medications in the market. For example, *in vivo* mice and human studies confirm a successful systemic delivery of insulin (21). Working on such a project will aid with developing a non-invasive drug administration method which will in turn improve the patients' quality of life and overall compliance (22).

Liposome applications on transdermal drug delivery is limitless, however general factors must be in mind when considering transdermal penetration. Factors such as liposomal size, formulation, composition, charge, and the presence of penetration enhancers (23).

3.1.3.2. Vaccine

Vaccines have gained great attention due to the pandemic; nanoparticles have been in the forefront of novel vaccines. Liposomes are used as both drug delivery system and adjuvants to stimulate an immune response (24) (25). As adjuvants, liposomes would decrease the excessive, unnecessary immune responses (26) thus making them more tolerable and safer. Nanoparticles also provide the necessary protection of genetic materials to arrive the antigen presenting cells (27).

3.1.3.3. Gene Therapy

Gene therapy uses nucleic acids to interfere with gene expression of certain genetic diseases (28). This method of therapy deals with many obstacles, such as poor stability and increased degradation of these nucleic acids, they also require active targeting (29). This is where the liposomes are beneficial since they can encapsulate the nucleic acids and protect them from degradation (30).

3.1.4. Preparation

Method of preparation dictates the size, encapsulation efficiency and even the structural integrity of the liposome (4). This indicates that the choice of liposome preparation method is

done depending on the physicochemical characteristics needed for the liposome as well as the drug being used (31). All preparation methods involve basic steps that are changed and curated according to the needs of the experiment (1). These steps are hydration, sizing, purification and drug loading.

3.1.4.1. Hydration

3.1.4.1.1. Mechanical Method

3.1.4.1.1.1. Thin Film Hydration

This method is known as the "conventional method" (4); it begins with dissolving the chosen mixture of lipids in an organic solvent such as chloroform or a chloroform-methanol mixture. It is then evaporated using a rotary evaporator at reduced pressure (9). After the lipid film is formed, the film is hydrated in an aqueous media of choice and maintained in Tc while actively shaken (32). This will allow the lipids to peel off the flask's walls and spontaneously form MLV liposomes with a polydisperse size and shape (33).

3.1.4.1.2. Solvent Dispersion Method

3.1.4.1.2.1. Ethanol Injection Method

This method of preparation makes relatively monodispersed SUV liposomes below 100nm (33) and it is simple to make liposomes in bulk. The major drawback for this method is that it is only effective using ethanol soluble drugs and produces a diluted amount of lipids (32). The method begins with lipids being dissolved in ethanol and then injected into the aqueous phase while agitated. The force of injection, injection rate, and the correct lipid concentration makes it possible to create SUV liposomes reliably (34). One point to keep in mind is that the ethanol should be removed from the liposome suspension by dialysis or rotary evaporation (32) (34). However, it could form an azeotrope which would make it very difficult to evaporate all the remnants (9).

3.1.4.1.2.2. Ether Injection Method

Another injection method, similar to ethanol injection technique, is when the lipids are dissolved in ether. The benefit of ether usage is that it is not miscible with water and its low boiling point will allow complete evaporation. Thus, the lipid concentration will not be diluted (9). The difference in the preparation method is that in this case ether and the aqueous medium must be at different temperatures and the injection rate must be slower (35). Unlike the ethanol injection method, this method does not produce SUV but mainly heterogenous LUV. This means that the formed particles need to go through a sizing technique to form small homogenous liposomes (1).

3.1.4.1.2.3. Reverse Phase Evaporation Method

Also known as RES method, it is based on the formation of w/o microemulsion (9). Like the injection methods, lipids are dissolved in an organic solution such as diethyl ether or methanol. Droplets of aqueous solution are added to the organic mixture causing an encapsulation by the lipids to form MLV liposomes (36) (32). These large liposomes are able to have a high encapsulation efficiency but also a wide size distribution dispersity (37) (9).

3.1.4.1.3. Size Transformation and Fusion Method

3.1.4.1.3.1. Freeze thaw

This method is attractive due to its ability to increase encapsulation efficiency of the liposomes (9). The liposomes are prepared using the thin film hydration method and then frozen, typically using liquid nitrogen at temperature of -196 ^{o}C (38). After freezing, they are thawed at a temperature above transition. This process is called a freeze-thaw cycle (39). Such a cycle is repeated multiple times to improve liposomes' homogeneity and size (9). Finally, MLV liposomes are formed, so they must go through a sizing method to be used according to the needs.

3.1.4.2. Sizing

3.1.4.2.1. Sonication

Sonication is a technique using sound waves to change prepared polydisperse MLV to uniformly sized SUV (9). There are two methods currently being used: probe sonication and bath sonication (1). Each with their own advantages and disadvantages; probe sonication is useful due to its ability to provide a stronger sonic energy thus a faster result. Unfortunately, with the higher sonic energy comes a higher risk of overheating the sample whilst also increasing the risk of titanium particles contaminating the sample (36) (40). This is the reason why bath sonication is much more preferred (9), it is a method capable of maintaining a constant temperature (due to the water bath) and considered safer overall (41).

3.1.4.2.2. Extrusion

Extrusion is a mechanical method used to reduce the size of the liposomes. The method includes the passage of the MLV lipids through polycarbonate membranes with specified pore sizes. Pore size options vary, and it is recommended to extrude the liposome starting with a larger pore size and working down to the preferred size (42). The main control of an accurate extrusion is presenting a constant pressure and flow rate through the pores, decreased flow rate

produces a much more uniform dispersion due to the increase shear rate (43). It is also important to maintain transition temperature of the vessels as it is much more fluid (41) (44).

3.1.4.3. Purification

3.1.4.3.1. Gel Permeation Chromatography

No matter the method of drug loading during the preparation process, liposomes must go through a purification process to be able to eliminate the free drug around the fully formed liposomes (45). This is done to accurately measure drug encapsulation.

Gel permeation column chromatography is a separation method which typically uses Sephadex or Sepharose bed to separate liposomes and free drugs using their size difference. To prevent the loss of loaded liposomes during their passage through the column, the gel is pre-saturated with empty liposomes and washed with a buffer before applying the actual samples (46). After pre-saturation, the liposomes are applied, and the separation process begins. Since the drug has a smaller size, they are more prone to be trapped in the gel pores whilst the much larger liposomes go through the column speedily. (47)

3.1.4.3.2. Mini Column Centrifugation

Like the column chromatography, this method took advantage of the clear difference in size between the liposomes and free unencapsulated drugs. Unlike the simple gel chromatography where gravity is the only force used to pass the mixture through the gel, this approach enforced the usage of centrifugal force combined with the concept of the gel chromatography (46). Mini syringes placed in test tubes went through several centrifugation rounds to allow the elutes to pass through with a fraction of the time compared to the traditional chromatography method (46) (48).

3.1.4.4. Drug Loading

Drug loading refers to the method of loading the active ingredient into the liposome. Typically, drug loading can be done either passively or actively. Passive loading corresponds to loading the drug whilst the liposome is in the process of being prepared (49). Active loading involves the encapsulation of drugs after the formation of the vesicle. In general, passive loading is effective for hydrophilic drugs while lipophilic drugs yield extremely poor encapsulation efficiency compared (9).

3.1.4.4.1. Passive Loading

Passive loading, as mentioned above, is done during the process of liposome preparation. Most methods of preparation conclude with the formation of MLV liposomes, such large liposomes are capable on higher encapsulation efficiency (50). However clinically, SUV are much more in use (45). Typically, during the preparation steps, hydrophilic drug is dissolved within the aqueous media while lipophilic drugs are dissolved in the organic solvent. For example, during the preparation using the ethanol injection method, the hydrophilic drug is present in the aqueous medium. When the ethanol is injected, the liposomes form spontaneously with the presence of the drug (51).

3.1.4.4.2. Active Loading

As mentioned, active loading of drug is the addition of active ingredient after the preparation of a fully formed empty liposome. Many factors influence the encapsulation efficiency, these factors include temperature of liposomes, duration of incubation period and formation of pH gradient (52) (53). Formation of a pH gradient along the liposomal membrane creates a strong driving force for weaky basic drugs to enter the acidic core to achieve equilibrium (54). Creation of the pH gradient can be obtained through multiple methods including the ammonium sulphate method, and ionophore method (55) (56).

3.1.4.4.2.1. Ammonium Sulphate Method

This process uses ammonium sulphate to create an elaborate pH gradient (57). Once the lipidic film is hydrated with the ammonium sulphate (58), the pH gradient occurs when the ammonia passes through the liposome causing the internal vesicle to be acidic due to the presence of the trapped sulphate (59). This overall gradient causes the drug to pass through the lipidic walls and get trapped inside the liposome due to the changes in charge (59). The key behind this method is the low permeability of sulphate salt inside the liposome as well as the increasing trapping efficiency due to the formation of precipitate with the drug (56) This method

is useful for amphipathic weak basic or acidic drugs such as doxorubicin and amphotericin B (60).

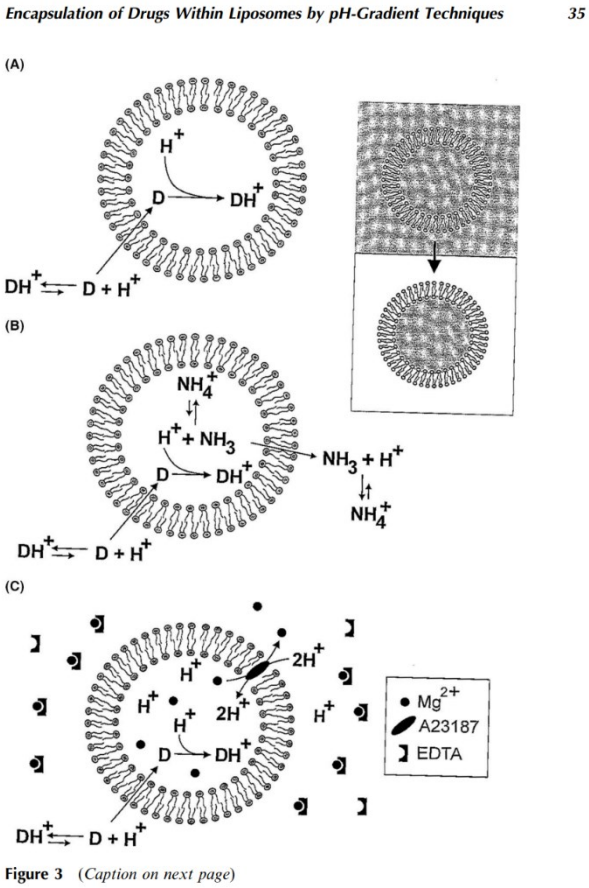

Figure 3 (Caption on next page)

Figure 3: (A): Citrate Method. (B): Ammonium sulphate method. (C): Ionophore Method

3.1.4.4.2.2. Ionophore Method

Building up on the previous mentioned methods, here ionophores are used to create a gradient. An ionophore is a transporter that can reversibly bind to ions and assist in the passage of cellular barriers (61). For this method, in addition to the ionophore, there must also be a transition metal to create an "ion gradient" (62). For example, in the encapsulation of Vincristine, one of the metals binds to the ionophore to be expelled from the liposome. This causes two protons to be encapsulated inside, causing the ultimate formation of an acidic internal vesicle thus a pH gradient and increased drug intake (59) (63). All the mentioned methods can be described in Figure 3 (59):

3.2. Dendrimers

3.2.1. Introduction

Dendrimers are tree like synthetic polymers that possess multiple functions. They are greatly beneficial in today's drug development era due to their predictable structural consistency (64). The dendrimer structure consists of a central core with branches which extend to functional groups, they can be classified into generations based on the number of repeating branches (65). This can be viewed in Figure 4.

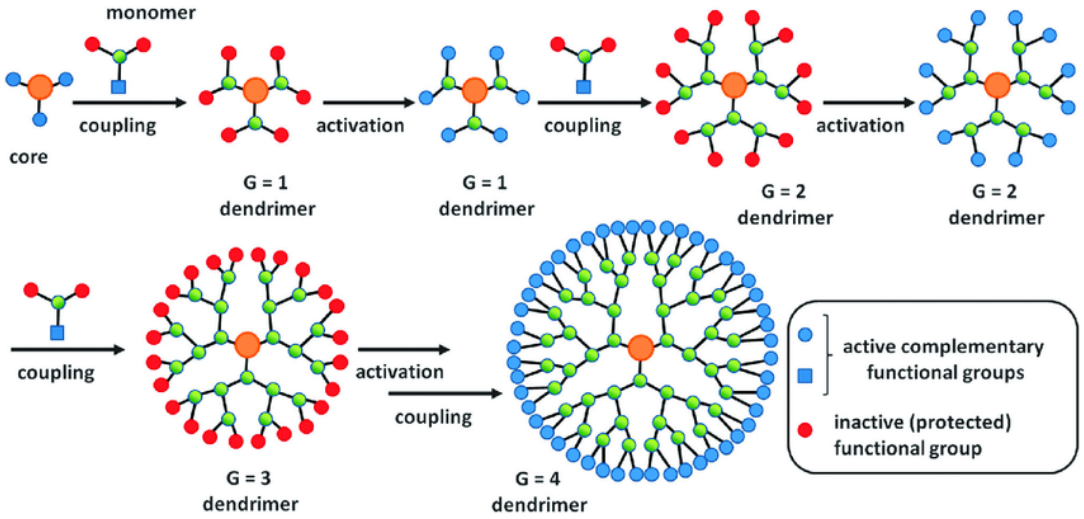

Figure 4: As increased branches are added, so are the increase in generation

3.2.2. Properties

3.2.2.1. Size

Dendrimer's size depends on the number of repeated branches, with such a unique consistent shape, as the generation increases so does their molecular weight and diameter. The significance of their size depends on the purpose of use. For example, if the dendrimer is used for targeted delivery of a drug with fast elimination, the larger sized dendrimer is more beneficial due to its impermeability at the renal glomerulus (66). On the other hand, when dealing with dendrimer delivery of DNA, the dendrimer must be much smaller to ensure its cellular permeability (67). As generations progress, the dendrimers start to have different structural characteristics. G 1,2,3 contain less branches, thus are much more open and linear. As the generations increase, they become more branched and start to partake a more spherical three-dimensional shape (68).

3.2.2.2. Functional end groups

Dendrimers' physical properties significantly depend on its functional group ending and it can also determine how it reacts with cell membranes (69). Dendrimer ending can be

positively, negatively charged, or neutral with several different origins, this is shown in the Figure 5 (70). Each type influences the application of the dendrimer. For example, cationic amine charge ending is substantially the most important due to the anionic nature of cell membranes thus allowing increased membrane permeability (71). The functional ending also helps with increasing drug solubility. This is shown in the increase of solubility of Paclitaxel due to dendrimers ending with ethylene glycol group (72).

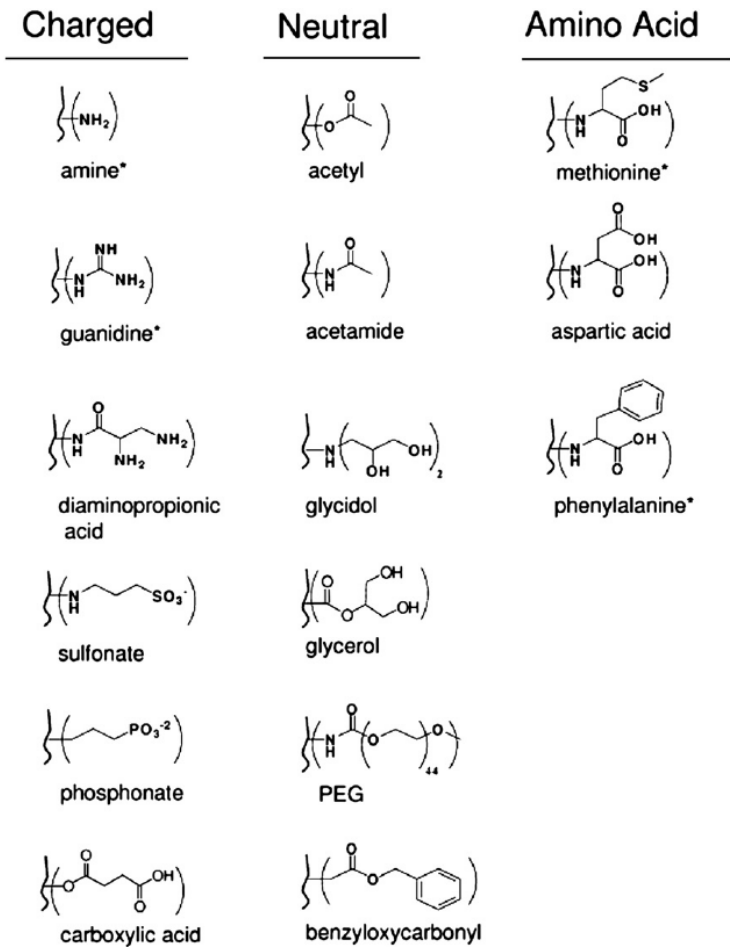

Figure 5: Shows several possibilities for dendrimer terminal functional groups

3.2.3. Synthesis

Dendrimers are "self-assembled" polymers with two main synthesis methods: divergent and convergent. The two methods differ in their branching techniques; divergent begins with the core and diverges outwards to make new branches whilst convergent is the opposite, starting with the synthesis of the dendrimer surface and branches whilst moving towards the core (73). These methods contain distinct pros and cons which depend on the type of dendrimer in need, divergent methods are good for higher generations and bulk synthesis however they must be created with utmost care thus to prevent creation of branches with uneven length (74).

Convergent method is restricted with the synthesis of lower generations of dendrimers, as well as preparing large amounts of dendrimer. Its greatest advantage is its ability to be purified relatively easily (75) (76).

3.2.4. Applications

3.2.4.1. Drug delivery

Dendrimers, like liposomes, have been considered to aid in drug delivery due to their structure, size, and mono-dispersity. Dendrimer, in theory, should operate as a carrier, assisting in boosting the therapeutic index of the medicine while decreasing adverse effects (77). This was shown when a research group examined the drug delivery effects of dendrimers with zidovudine, an anti-HIV drug. They concluded that the dendrimer in fact showed positive results in drug loading and sustained release whilst also providing a decrease in hemolytic toxicity (78).

3.2.4.2. As drugs

Dendrimers are differentiated depending on the number of branches (generations) as well as the functional ending groups, it has been found that some of these dendrimers hold significant pharmacological activity. This discovery dictates that certain dendrimers with anionic functional groups demonstrate antiviral activity (79) whilst dendrimers with cationic functional groups demonstrate antibacterial effect (80). Starting with the anti-viral dendrimers, it was found that dendrimers with 1-(carboxymethoxy)naphthalene-3,6-disulfinate surface group attached to generation 3 l-lysine possess a strong prophylaxis and treatment against HSV, HIV and hepatitis B infection (81) (82). It is marketed under the name “VivaGel” (81). Similar to the antiviral dendrimers, lysine type dendrimers were used during the research for possible antimicrobial properties (83). As there has been an increase in antibiotic resistance, this research could provide groundbreaking discoveries for future generations. It was found that cationic functional group endings could be attracted to the negatively charged bacterial membranes causing leakage and eventual apoptosis (84). Such research provided positive effect against both gram-negative (Escherichia coli) and gram-positive (Staphylococcus aureus) bacteria (85).

3.2.4.3. Solubility enhancement

Dendrimer structure resembles that of a static unimolecular micelle where the hydrophobic drug interacts with the dendrimer functional terminal group due to hydrogen bonding and electrostatic interactions (86). Thus, this aided scientists to experiment the possibility of dendrimers increasing drug solubility, therefore increasing drug bioavailability.

Many of the dendrimer properties must be considered before understanding the mechanism behind increasing drug solubility. Dendrimer generation size and concentration entails the ability to affect the hydrophilicity of active ingredients. It was found that the higher the generation concentration, the more soluble drugs become, the hypothesis behind such a claim indicates that with increased branches and amount, there would be increased drug entrapment in the dendrimer (87) (88). However, one important note must be mentioned, chosen dendrimers cannot be higher than generation 3 due to a possible immunologic reaction trigger (87). Such process is also heavily pH dependent; this is due to the dependency of solubility and possible electrostatic interaction between the dendrimer and drug molecule. Thus, the increase of solubility hangs on the ionized state of the drug (89).

One of the many real-life examples include amphotericin B solubility enhancement, it was experimented with multiple generations, concentrations, and pH variables to determine the most optimized effect. The results concluded that with increased dendrimer concentration, there was a linear increase in solubility. The pH dependence was measured using G3 and concluded as that the best pH was that of 7.4. This was because it presented the highest electrostatic interaction between the drug and dendrimer. pH 4 offered the worst solubility because it couldn't ionize the drug, so it heavily decreased the chances of interaction (90). This is properly explained in Figure 6.

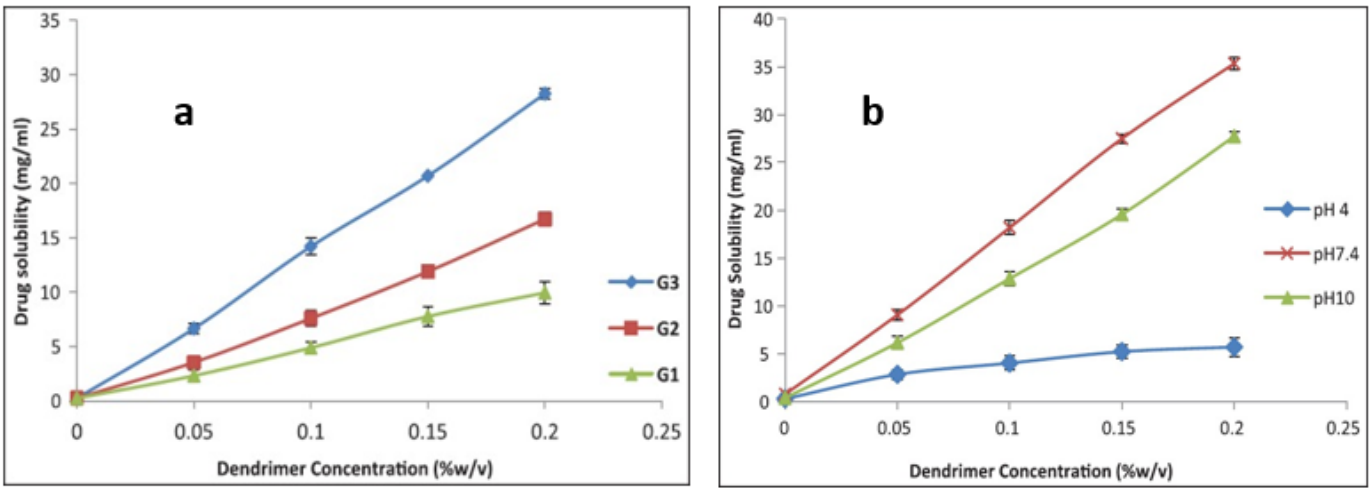

Figure 6: a; effect of dendrimer generation and concentration on the solubility of Amphotericin B. b; effect of G3 generation pH solution on the solubility of amphotericin B

3.2.4.4. Diagnostics

Surprisingly, dendrimers can be used as diagnostics, they hold the ability to carry ionic contrast agents for magnetic resonance imaging (69). Such an ability would allow safer

diagnostic materials as the dendrimer would be decreasing the possible toxic effects as well as aiding in the contrast distribution and clearance (91).

3.3. Imiquimod

3.3.1. Mechanism of Action

Imiquimod has shown both antitumor and antiviral activity, this is due to its imidazoquinoline structure which possesses agonistic action towards toll-like receptors 7 and 8 (TLR 7 and 8). TLR 7/8 were discovered to play a role in innate immunity and immune cell activation (92), this causes the activation of INF alpha, cytokines, and chemokines which would ultimately increase the cytotoxic T-helper cell immune reaction (93). This innate immune response is the key towards the antiviral and antitumor effect mentioned earlier (94). The Figure below describes the immune response (95)

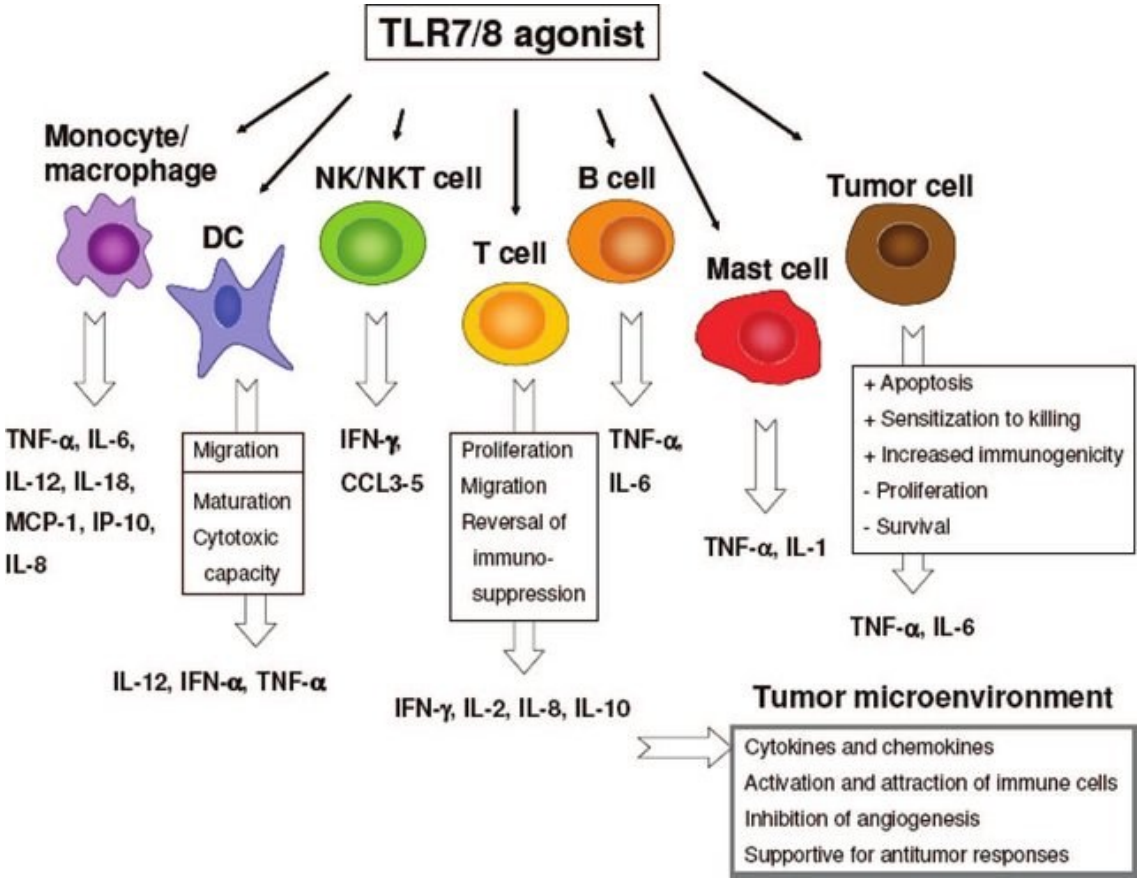

Figure 7: Immune response based on agonistic effect of TLR7/8

3.3.2. Properties

Structurally, IMQ is a low molecular weight guanosine derivative that is classified as an imidazoquinoline amine used to treat papilloma and skin cancers (96). It is a white crystalline powder which is lipophilic and contains a free amine group thus soluble in acidic pH. IMQ melting temperature is at 292-294 °C (97) (98). In current FDA use, this drug is only approved for topical use in maximum 5% concentration (99) (100)

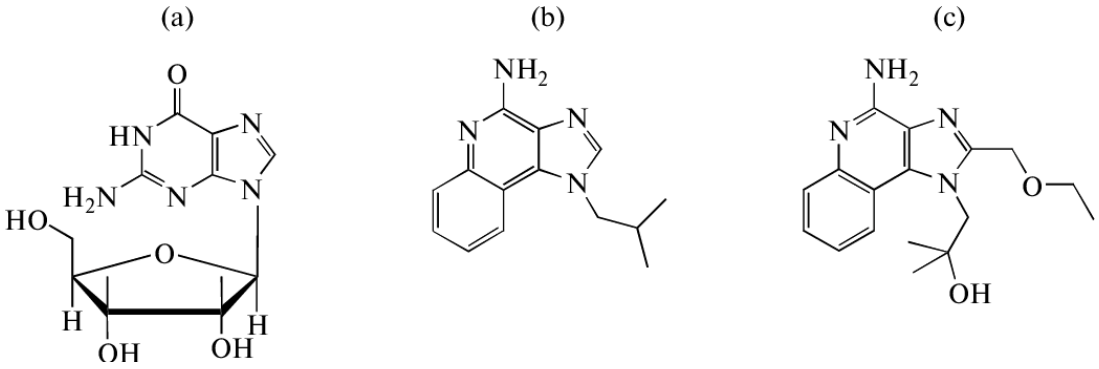

Figure 8: (a) guanosine structure. (b): Imiquimod structure. (c): Resiquimod structure

3.3.3. Adverse effects

Due to the physicochemical properties of IMQ, it is officially only used in 5% topical application (99). Due to that fact, most of the adverse effects reported were that of local nature. This included rashes, erythema, irritation, and ulcerations. It was also reported the presence of systemic effect, though it doesn't pass the stratum corneum, it was hypothesized that it occurred when spread over a large surface area and caused by immunomodulatory effect (100). Systemic effects include but not limited to fever, fatigue, pain, and dizziness (101) (102) (103).

3.3.4. Current Uses

3.3.4.1. Genital Warts

HPV 6 and 11 are the most common types of Human Papillomavirus (HPV) infections to cause the uncomfortable symptom of genital warts (104). In the UK, it was reported that genital warts are the most common sexually transmitted infections (105) and in the US, a report indicated that in the year 1996, more than 15 million people were infected (106). Such numbers improved due to the HPV vaccine (107), however there was still a great need in a topical treatment to aid infected individuals. IMQ is currently the first-choice drug that proved during clinical trials its effectiveness against warts and recurrence (108).

3.3.4.2. Actinic Keratosis

Actinic keratosis is an epidermal scaly dry lesion which occurs subsequently due to long UV exposure and is viewed as a progressor to possible squamous cell carcinoma (109). This

disease is cumulative where high prevalence is seen in men over 70 years old, typically bald with lighter skin color (110). `Previously treatment of AK involved dermabrasion, laser therapy and retinoid therapy (111), such treatments resulted in poor outcomes which could cost the patient scarring and changes in skin pigmentation, not to mention the painful procedures to endure (112).

Imiquimod was a topical drug used to treat genital warts, and multiple studies tested its efficacy against AK. One study assessed 2 phase 3 study where a total of 436 people participated, 215 were treated with 5% IMQ and 221 with a placebo vehicle cream. It concluded that the IMQ group achieved a clearance rate of 45.1% compared to its placebo counterpart of 3.2%, partial clearance also reported 59.1% for IMQ group while the vehicle group showed 11.8%. Groups being treated with IMQ reported adverse effects at target site such as itching (20.5%), burning (5.6%), bleeding (3.3%), and pain (2.3%). Other adverse effects included local skin reactions such as erythema (97.2%), scabbing (8.4%), and scaling (7.4%) (113).

4. Experimental part

4.1. Chemicals, materials and instruments

Water used throughout the experiments was of Millipore quality and the organic solvents (chloroform and methanol) were HPLC grade purchased from Sigma Aldrich, Germany. The dendrimer used in this study was an in-house synthesized dendrimer of first generation (Mw:1211 g/mol). For the liposome formation egg phosphatidylcholine (EPC) 60% pure was used and purchased from Sigma Aldrich, Germany. Imiquimod (Mw: 240.31 g/mol) was purchased from TCI Chemicals, Tokyo, Japan. Sephadex G-50 was purchased from Sigma Aldrich, Germany. Phosphate Buffered Saline (PBS) was previously prepared in the lab by combining $NaH_2PO_4.2\ H_2O$ (156 g/mol; 2 mM), $Na_2HPO_4.12\ H_2O$ (358 g/mol; 8 mM), NaCl (58,4 g/mol; 137 mM), and 0.201 g KCl (74.6 g/mol; 2,7 mM). All salts were purchased from Sigma Aldrich, Germany.

A rotary vacuum evaporator IKA RV 10 basic was used for the evaporation of organic solvents and gentle mixing of liposomes. Variable volume pippetes for liquid dispensing (Transferpette S Pipette), mechanical shaker (LT3) for long-term shaking and vortex machine (IKA vortex 3) for short-term shaking were all purchased from Sigma Aldrich, Germany. Sonicator used was the Kraintek 12 (Hradec Kralove, Czech Republic) and the Micro-pH meter was the Hanna precision pH meter model 210 (Sigma Aldrich, Germany). To decrease the size of liposomes, Avanti Lipids extrusion kit was used from Alabama, USA. The extruder was heated with a temperature-adjustable MR Hei-Standard magnetic stirrer, Heidolph. Liposome size, PDI and zeta potential were determined using Zetasizer Nano-ZS, Malvern including the DTS1060 cuvettes. MPW-260R from MPW (Warsaw, Poland) centrifuge were used to perform the column centrifugation. The heating of the samples during loading was achieved either in an Memmert Incubator I (Memmert, Germany) or with the use of a temperature-adjustable MR Hei-Standard magnetic stirrer, Heidolph while the samples were embedded in Lab Armor™ Bath Beads (FisherScientific). Drug concentration was determined using an Agilent 1200 series HPLC equipment (Agilent Technologies, Germany) with a G1379B degasser, G1310A isocratic pump, G1316A thermostatted column, G1329A autosampler, and G1321B fluorescence detector. The HPLC column was the HS Discovery C-18 1504.6 mm column 5 μm particles with a 100 Å porosity.

4.2. Specific procedures of experimental work

4.2.1. Preparation of aqueous dendrimers solutions

First and foremost, aqueous dendrimers solutions of the first (1st) generation (G1) were prepared with concentrations of 5 mM (G1-5) and 10 mM (G1-10) in Millipore water. Once the dendrimers were dissolved, the pH of the solution was measured.

4.2.2. Preparation of Liposomes

Liposomes were prepared by using EPC dissolved with 4:1 (v:v) ratio of chloroform to methanol. The typical ratio between lipids and organic solvent was 300 mg of lipids for 10 ml of organic solvent. Once fully dissolved, the solvent mixture was evaporated at rotary evaporator under reduced pressure and a thin lipidic film created at the bottom of the flask. This flask was placed in a high vacuum pump for one hour to eliminate any kind of moisture or organic solvents traces from the film. After one hour the film was hydrated with the previously prepared dendrimer solution (G1-5 or G1-10) to a final lipid concentration of 100 mg/ml. Blank/empty liposomes were also prepared by hydrating lipid film with PBS.

After the addition of aqueous solution, the lipid mixture was left stirring at 65ºC for 1 hour using a rotary evaporator without vacuum application. The obtained samples were further sonicated for 1 hour at 20ºC and were left overnight in the fridge.

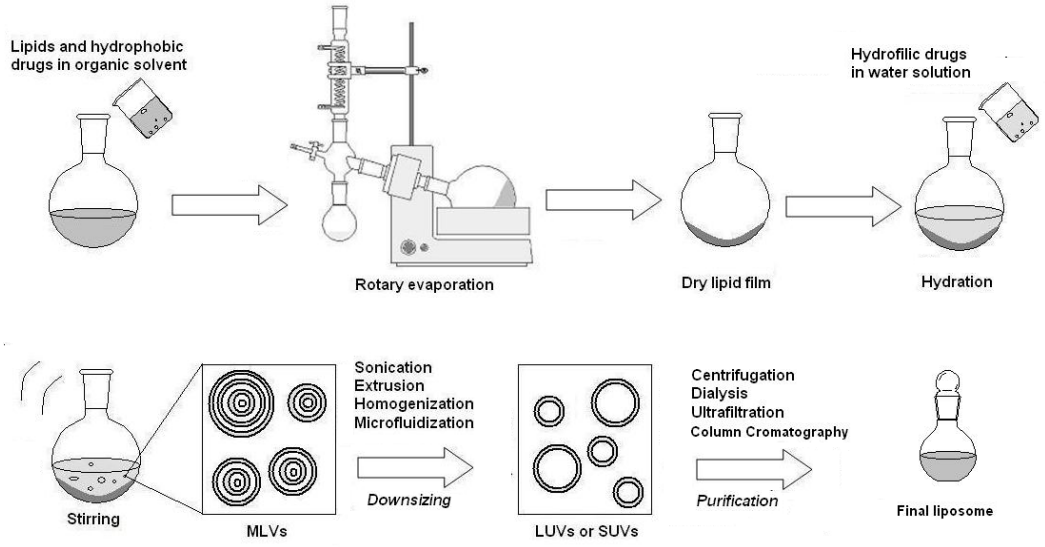

Figure 9: Schematic representation of the preparation of liposomes (129)

4.2.3. Extrusion of liposomes

The following day the samples were extruded through 400 nm and 100 nm polycarbonate membranes to ensure size reduction and uniformity. Each sample was extruded

11 times first through the 400 nm and then through the 100 nm membrane. Before every extrusion process, the membranes were hydrated by passing Millipore water though the extruder 11 times. The extruder was constantly placed on top of a hot plate stable between 55-70 °C (around transition temperature).

Figure 10: Extruder used during experiment. One of the syringes were filled with liposomal mixture and pushed through the membrane to pass to the second syringe.

4.2.4. Exchange of external media

The next day, the already extruded liposomes were eluted through a Sephadex column. Sephadex gel was prepared by using a 1:20 (w:v) ratio of Sephadex to PBS and left to swell overnight in the fridge prior to use. Plastic syringes of 3 ml equipped with a cotton stopper were filled with 3 ml of Sephadex gel, placed in test tubes and centrifuged for 3 minutes at 1500 rpm at 20 °C to remove excess of solution. Once completed, the syringe was pre-saturated with 132 µl of blank/empty liposomes and centrifuged under the same conditions. The column was washed and centrifuged 3 times using PBS to guarantee the complete passage of the blank liposomes. Once the process was complete, the eluates containing blank liposomes and PBS were discarded and the gel was ready for use. The prepped gel was saturated with the dendrimer filled liposomes and spun twice to ensure all the liposomes went through. The eluates were collected, and their pH was measured.

4.2.5. Liposomes' characterisation

After the media exchange, the liposomes were characterized at Zetasizer for their size and PDI. 0.1 ml of the sample was diluted with 0.6ml of PBS solution and placed in a disposable capillary cuvette to be analysed. The measurement ran using the manual conditions which included 10 number of runs for 10 mins with 0 delay and measured in triplicates.

4.2.6. Addition of IMQ

After the preparation of the dendrimer loaded liposomes, an excess of IMQ was added in each vial and the samples were incubated in various conditions described in Table 2. For the

stirring conditions, at room temperature the liposomes were stirred using magnetic stirrer however for 37 ºC and 60 ºC, heating beads were used to maintain a stable temperature and magnetic stirring was used.

Table 2: Samples are labelled according to letter (condition status) and dendrimer concentration (5 mM or 10 mM)

Sample	Temperature	Stirring	Duration
A-5	37°C	No	1 day
A-10	37°C	No	1 day
B-5	37°C	No	3 days
B-10	37°C	No	3 days
C-5	37°C	No	10 days
C-10	37°C	No	10 days
D-5	37°C	Yes	10 days
D-10	37°C	Yes	10 days
E-5	Room Temp	Yes	10 days
E-10	Room Temp	Yes	10 days
F-5	60°C	Yes	10 days
F-10	60°C	Yes	10 days
H-5	60°C	No	10 days
H-10	60°C	No	10 days

4.2.7. Purification

After the indicated incubation period, the samples were purified to remove the free IMQ that failed to be encapsulated into the liposome. The same “mini column” method used during the media exchange was utilized during this process.

4.2.8. HPLC analysis

The samples were diluted with MeOH (1000x dilution) before each analysis. IMQ concentration was determined using HPLC analysis at 25°C. The mobile phase was 185:275:540 v/v methanol/acetonitrile/acetate buffer (100 mM, pH = 4) at 1 mL/min flow rate with a 1 µL injection volume. The experiment lasted 5 minutes, and IMQ was discovered after 3.2 minutes using λexc = 240 nm and λem = 360 nm. Over a range of 0.02 – 10 µg/mL, the calibration curve was linear (R^2=0.999, p<0.001).

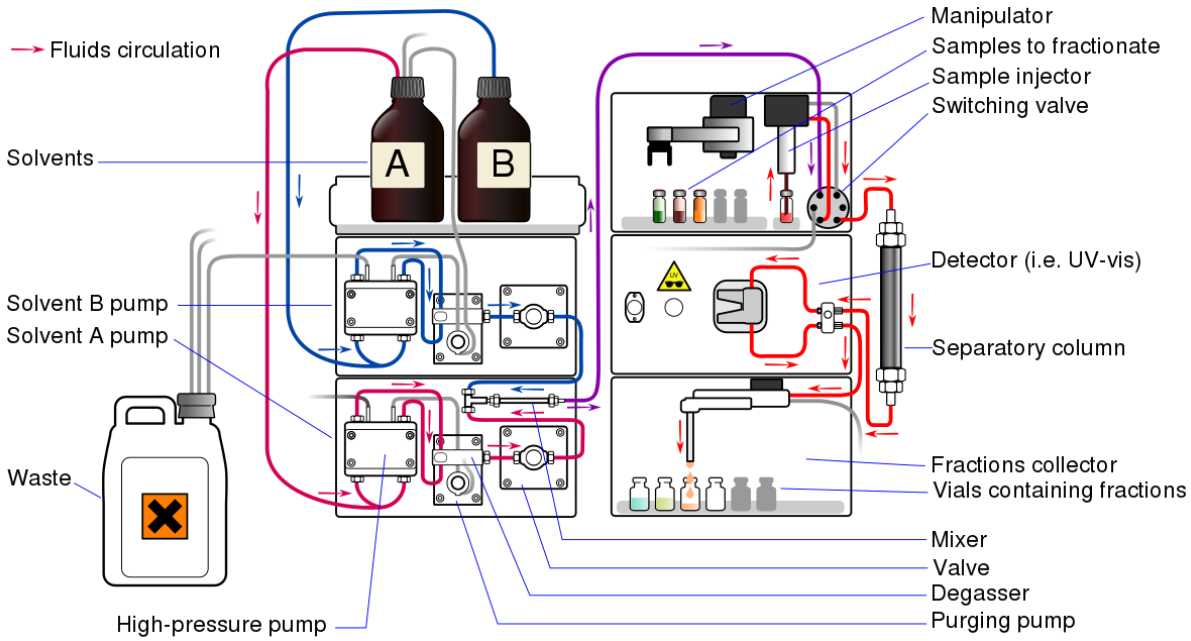

Figure 11: schematic overview of HPLC

5. Results and Discussion

5.1. Background

As previously explained, liposomes can be loaded with the active substance by two major methods: passive and active loading. Passive loading is realized when the active substance is incorporated in liposomes while they are formed, and active loading is the addition of active ingredient after the preparation of a fully formed empty liposome. Previous results of our research group have shown that hydration of the thin lipid film with aqueous medium which contains in-house synthesized dendrimers can promote the liposomes' loading with IMQ (114). This early study had proved that the encapsulation efficiency of IMQ is higher when the active substance is dissolved in the dendrimer solution rather than when it is incorporated in the lipid film. A more detailed study has revealed that the solubility of IMQ in aqueous media is highly depended on the dendrimer's generation and the dendrimer's concentration used (115). During that study it was proved that for each different dendrimer generation there is an optimum dendrimer concentration in which it is achieved the highest IMQ solubility. The optimum dendrimer concentrations for G0, G1, G2 and G3 was found to be 20, 10, 5 and 2.6 mM respectively, where the IMQ solubility was 7.34, 9.17, 9.24 and 8.45 mg/ml respectively. Recently, IMQ was successfully loaded to liposomes in the present of dendrimers using the passive loading (116). The data obtained from the latest study suggested that the first-generation dendrimer (G1) was the most effective molecule for IMQ loading since it produced the highest possible IMQ concentration in the liposomal formulation (1.98 mg/ml).

The dendrimers developed in our group are synthesized through repeated steps of deprotection and monomer conjugation to obtain the next generation molecule (117) (118)(Figure 12).

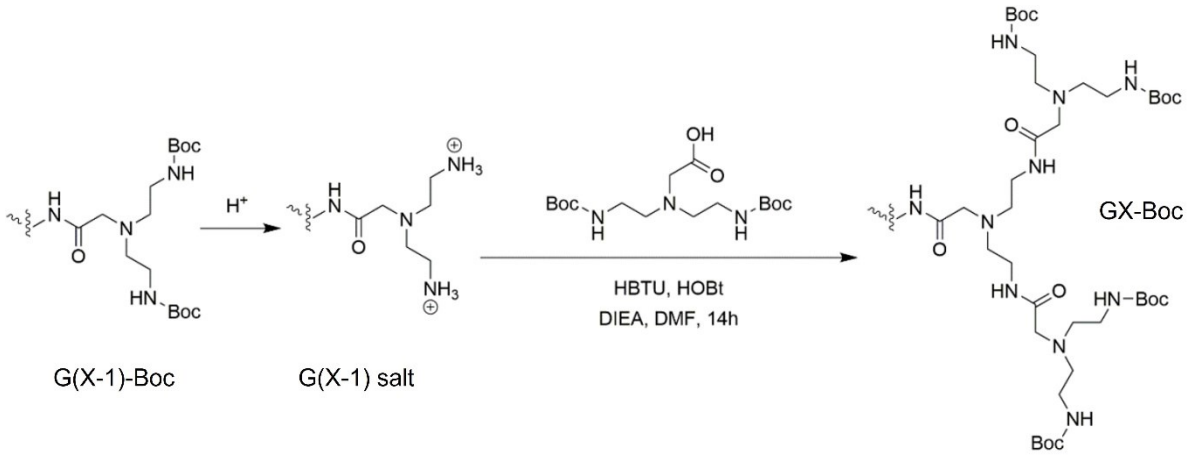

Figure 12: G-1 dendrimer synthesis cycle

The amine deprotection is achieved in acidic conditions and thus, a dendrimeric salt is isolated during every generation growth cycle (G(X-1)-salt). This salt, due to its nature, is soluble in water producing acidic aqueous solutions.

5.2. Method Discussion

In all our previous approaches, IMQ was loaded to liposomes by passive loading. During this study, the aim was to examine if dendrimers can promote active loading of IMQ to liposomes. More specifically, the idea was to initially create dendrimer loaded liposomes after hydration of the lipidic thin film with aqueous dendrimer solution. For the hydration of lipidic thin film, the salt of the first-generation (G1) dendrimer was used since it was the molecule with the most effective IMQ loading since (final IMQ concentration after purification was 1.98 mg/ml) during the passive loading.

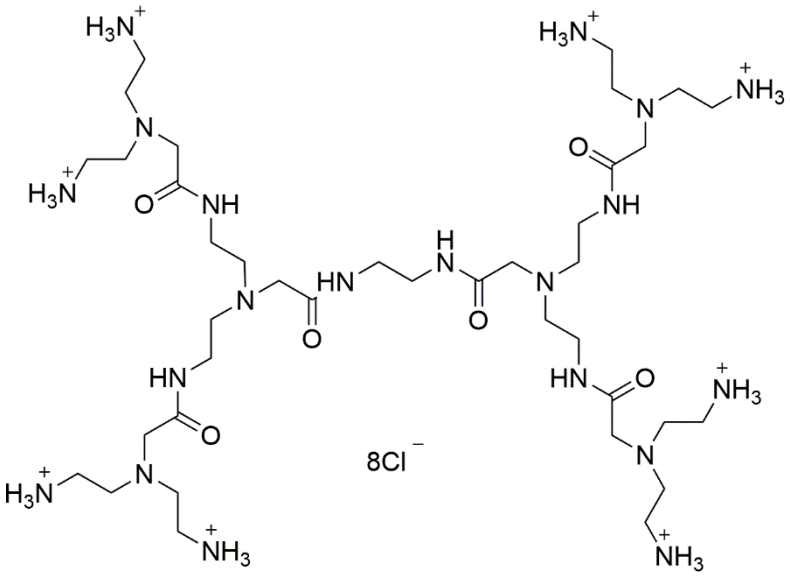

Figure 13: G-1 dendrimer with protonated amine group which is a salt acidic in nature

Two different dendrimer concentrations were tested to get an idea if the dendrimer concentration will have an impact in the active loading. Since 10 mM was the optimum G1 dendrimer concentration producing aqueous solutions of IMQ with concentration of 9.17 mg/ml, the concentrations selected were 5 and 10 mM.

After their creation, the dendrimer loaded liposomes were size reduced. The first size reduction was accomplished with a short sonication cycle (1 hour). Prolonged exposure of liposomes to ultrasound for size reduction is not recommended since it can lead to peroxidative lipid damage (119) (120). The main size reduction of liposomes was achieved with extrusion. Liposomes were passed through 400 nm and 100 nm extrusion membranes to get a gradual size reduction to the required size. The required size was set to 100 nm since previous studies have shown that this is the optimal size for liposomal formulations with when their target site is the lower layers of epidermis (5) (6). An extrusion temperature around the lipid transition

temperature was set to ensure higher flexibility of liposomes during size reduction and 11 passages through the membranes ensured the effective reduction and homogeneity of the resulting liposomal mixture (121). The odd number of passages through the membranes is to avoid potential contamination with larger vesicles which never got through the membrane.

After the size reduction, the liposomes were passed through Sephadex gel and washed with PBS to exchange the external medium with non-acidic PBS (pH=7.4). This procedure is helping to create the wanted pH gradient since the liposomes are loaded with an acidic dendrimer solution while they are surrounded with PBS (pH=7.4). Before the application of each liposomal formulation to the column, the column was pre-saturated using blank/empty liposomes to prevent liposomal breakage. This pre-treatment allows the "calibration" of the gel mixture and is preparing the column for the passage of the needed liposomes for the buffer exchange (122). To confirm the creation of the pH gradient, the pH of the formulations was measured before and after their passage through the Sephadex gel. The pH measurements before and after the buffer exchange are summarized in Table 3 for all the samples and the pH gradient formation was proved. The pH after the buffer exchange was in the range of 6.36-7.10.

Table 3: Initial and exchanged pH values

Sample	Initial pH	Post exchange pH
A-5	1.95	7.02
A-10	1.65	6.81
B-5	1.95	7.02
B-10	1.65	6.81
C-5	1.96	6.76
C-10	1.67	6.36
D-5	1.90	7.10
D-10	1.63	7.03
E-5	1.90	7.10
E-10	1.63	7.03
F-5	1.90	7.10
F-10	1.63	7.03
H-5	1.90	7.10
H-10	1.63	7.03

After confirming the creation of the pH gradient, the liposomes were characterized for their size and PDI. The expected size values are expected at the range of 100 nm since the 100 nm pore membrane was used last during the extrusion procedure. The PDI values can in general range from 0.0 to 1.0, where 0.0 means perfectly unisized liposomal mixture and 1.0 means populations containing particles with multiple particle sizes (highly polydisperse samples). For

liposomal formulations, a PDI value of 0.3 and bellow is considered to be acceptable indicating a population of homogenous lipid carriers (123) (124) (125).

Table 4: Sample was prepared in bulk then specific volume was separated and conditioned to specific incubation condition

Sample	Size (nm)	PDI
A-5	96.57 ±2.98	0.111 ±0.042
A-10	113.7 ±0.55	0.112 ±0.009
B-5	96.57 ±2.98	0.111 ±0.042
B-10	113.7 ±0.55	0.112 ±0.009
C-5	86.00 ±2.19	0.180 ±0.019
C-10	101.6 ±0.05	0.155 ±0.013
D-5	81.40 ±1.40	0.192 ±0.026
D-10	76.79 ±0.56	0.174 ±0.017
E-5	81.40 ±1.40	0.192 ±0.026
E-10	76.79 ±0.56	0.174 ±0.017
F-5	81.40 ±1.40	0.192 ±0.026
F-10	76.79 ±0.56	0.174 ±0.017
H-5	81.40 ±1.40	0.192 ±0.026
H-10	76.79 ±0.56	0.174 ±0.017

From the results presented in Table 4, the size reduction to the intended values was confirmed. All liposomes had an average size in the range of 76.79 ±0.56 to 113.7 ±0.55 nm. The PDI values confirmed the uniformity of the samples since they were in the range of 0.111 ±0.042 to 0.192 ±0.026.

After the buffer exchange and the characterization of each batch the liposomal formulations are ready to be loaded with IMQ by the active loading. IMQ was added and incubated in specific conditions mentioned in Table 2. After incubation period concluded, purification of the sample is necessary to confirm the IMQ loading and specify the final IMQ concentration of each formulation. Purification is the process of removing all undissolved IMQ and all traces of drug in the external media of the liposomes which is not capsulated. The liposomes were purified using the same centrifugation method as for media exchange. To calculate the amount of encapsulated IMQ, all samples were diluted with methanol (x1000). When methanol is applied, the liposomal walls are disrupted (126) and the HPLC measurements are representing the IMQ concentration inside the liposomes.

5.3. HPLC results

During this study, many different incubation conditions were tested. Each final outcome about the IMQ loading for every batch was a guide to set up new incubation conditions. Initially, the liposomal formulation was incubated at a set temperature of 37°C (body temperature), without mechanical stirring for 1, 3 and 10 days. This initial approach was performed to get an insight for the relevancy of time. Figure 14 describes the final IMQ concentration measured according to the incubation time.

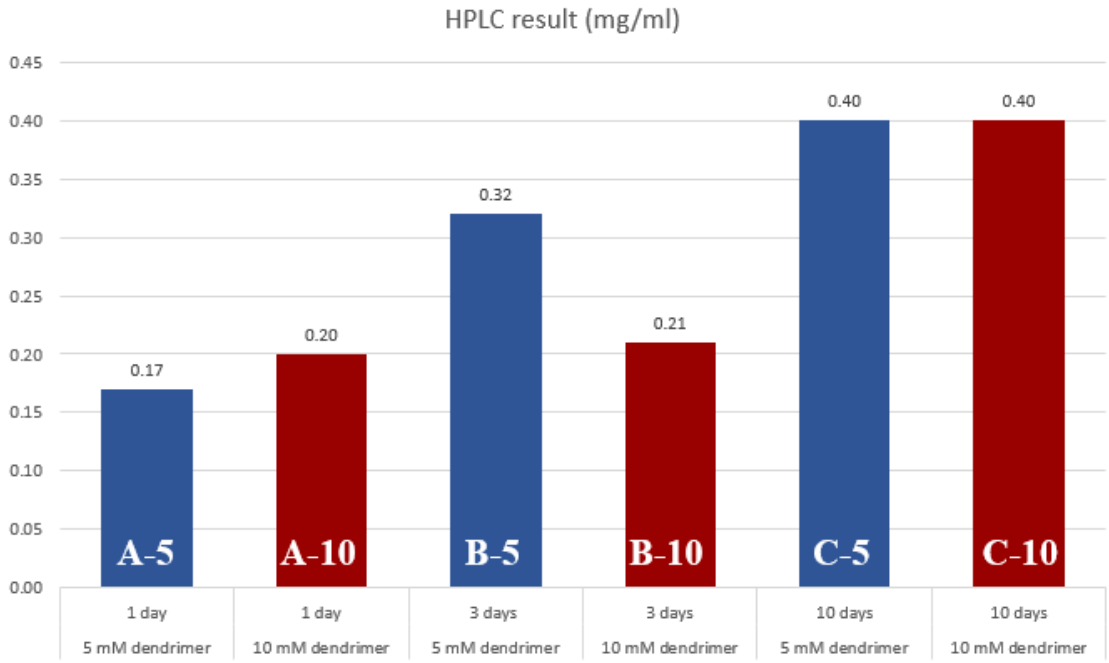

Figure 14: Relationship between time vs. IMQ concentration during incubation at 37 °C

In general, longer incubation time is producing liposomes with higher amount of encapsulated IMQ while the dendrimer concentration doesn't seem to directly affect the loading amount. The maximum IMQ concentration was noticed for the samples incubated for 10 days with 5 mM and 10 mM dendrimer concentration and it was 0.40 mg/ml.

In an additional effort to increase IMQ loading and examine the impact of different parameters, stirring and varying temperature was introduced by keeping the incubation time period at 10 days. As it is shown in Figure 15, the incorporation of stirring increased the drug encapsulation and proved to produced liposomes with higher IMQ concentration than the non-stirred samples.

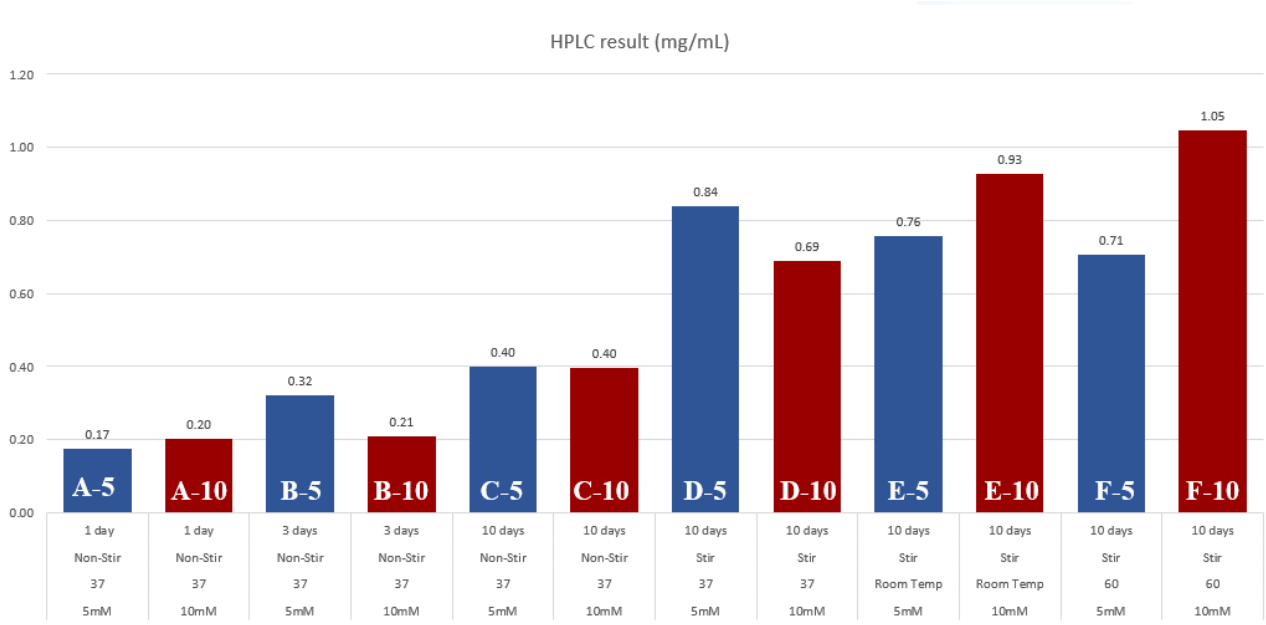

Figure 15: Comparison of drug concentrations between stirred and non-stirred samples

The IMQ concentration obtained to the stirred samples was in the range of 0.69 to 1.05 mg/ml. No direct corelation between the incubation temperature and IMQ concentration was observed. More specifically, for the liposomes created with 5 mM dendrimer concentration the IMQ loading increased in the order of temperature 60 ºC- room temperature- 37 ºC and for the liposomes created with 10 mM dendrimer concentration the IMQ loading increased in the order of temperature 37 ºC- room temperature- 60 ºC. The highest final IMQ concentration was noticed for the sample created with 10 mM dendrimer concentration after 10 days stirring at 60 ºC (1.05 mg/ml).

Finally, the last incubation condition included the 60°C temperature without stirring for 10 days. Figure 16 compares the IMQ concentrations for incubation at 60°C for 10 days without and with mechanical stirring of the liposomal mixture.

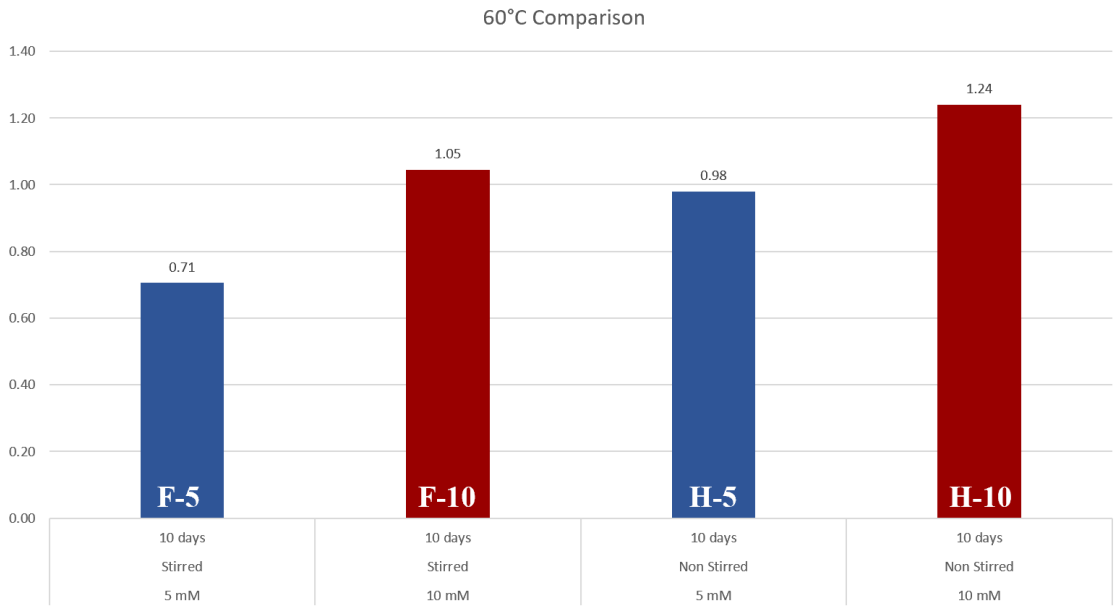

Figure 16: Comparison of IMQ concentration between stirred and non-stirred samples at 60°C

Interestingly, for both dendrimer concentrations (5 mM and 10 mM) the non-stirred samples were able to afford a higher concentration of IMQ than the stirred ones. More specifically, when the incubation temperature is 60 ºC, the liposomes created with 5 mM dendrimer concentration had a final IMQ loading of 0.70 mg/ml for the stirred samples and 0.98 mg/ml for the non-stirred samples. Similarly, when the incubation temperature is 60 ºC, the liposomes created with 10 mM dendrimer concentration had a final IMQ loading of 1.04 mg/ml for the stirred samples and 1.24 mg/ml for the non-stirred samples. A possible explanation of this phenomenon is that at transition temperature, the liposomes are much more fragile and likely to break. When stirring is added to the more fragile/non-sturdy liposomes, they begin to break and thus the amount of total drug in the liposomes is lower than non-stirred. This might mean, that the liposomes are able to encapsulate higher drug amount in higher temperature but the mechanical stirring at this temperature is disrupting the lipid bilayer and destroys the liposomes. To confirm this theory, further experiments should be performed.

The overall results of final IMQ concentration according to the incubation conditions is summarized in Figure 17.

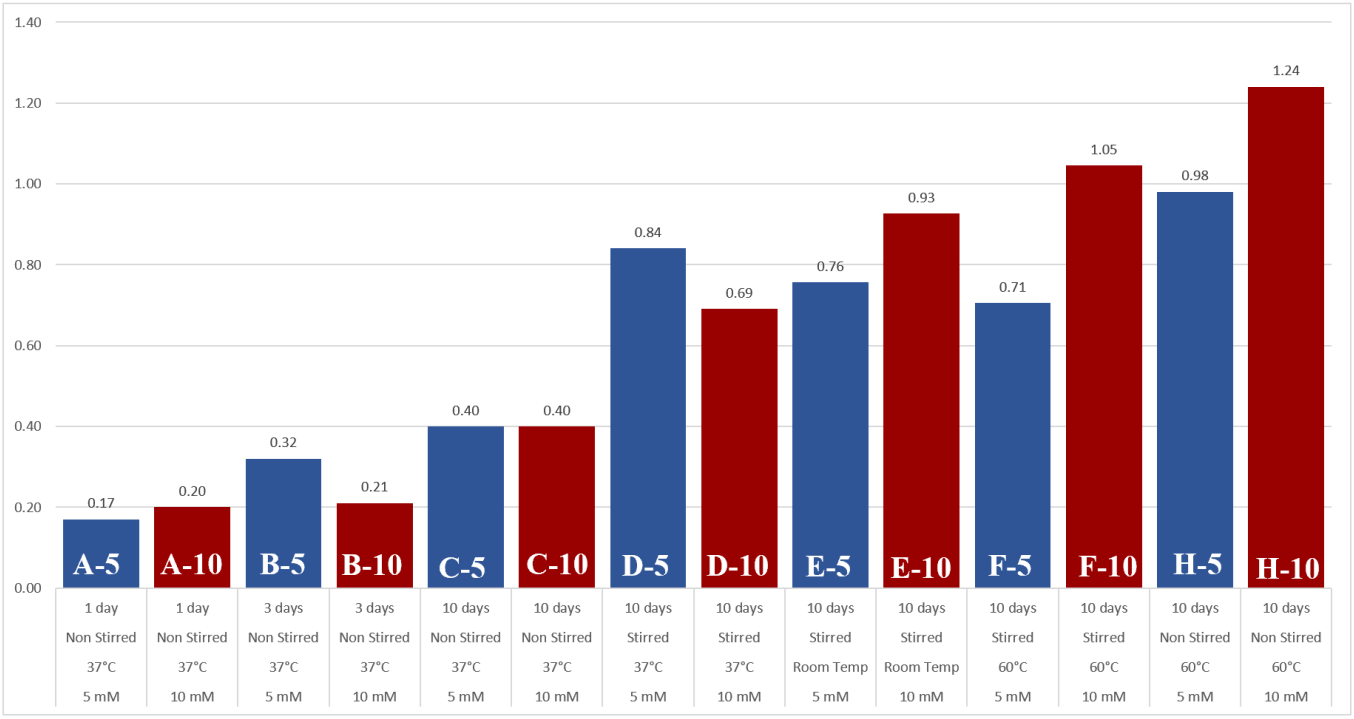

Figure 17: Final graph summary including all results obtained from the different incubation conditions

6. Conclusion

During this thesis, active loading of IMQ to liposomes was performed in different incubation conditions. The choice of dendrimers which are producing acidic aqueous solutions aided the weakly basic lipophilic drug to pass through the liposomal wall. Throughout this study, it was suggested that an increase in drug mobility while being incubated was the key to increase drug encapsulation. Mobility aided the drug to solubilize while simultaneously encouraged to follow the pH gradient inside the liposome. In a further extend, the incubation time is affecting the final concentration of the isolated liposomes. The more time spent under incubation, the more the drug had the possibility to be encapsulated. The difference between drug concentration between the 1-day incubation and 10-day incubation at 37 ºC was almost double. Finally, while temperature does play a role, the most significant temperature to use is the transition temperature. When liposomes are at "fluid" stage, they are more likely to allow the passage of drug. This is indicated by finding the two highest drug encapsulation concentration at 60°C.

In conclusion, the active encapsulation of IMQ to liposomes in the presence of dendrimers is possible, while the final concentration highly depends on the incubation conditions. The highest IMQ concentration noticed in this thesis was 1.24 mg/ml and observed for the liposomes which were created with 10 mM of first-generation dendrimers and incubated for 10 days at 60 ºC without stirring.

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