Development of long duration releasing liposomes for prolonged content release in dental care applications

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**DEVELOPMENT OF LONG DURATION RELEASING LIPOSOMES FOR
PROLONGED CONTENT RELEASE IN DENTAL CARE APPLICATIONS**

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2021

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A thesis submitted to the Nanyang Technological University

in partial fulfilment of the requirement for Master's Degree in Engineering (MAE)

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The contributions of the co-authors are as follows:

- A/Prof Hirotaka Sato provided the initial project direction and edited the manuscript drafts.
- Le Duc Long provided training and guidance for the synthesis of liposomes as well as advise on the experimental set up.
- Dr Sou Keitaro provided guidance on the project progress, advice on liposome characterization methodologies as well as assistance on liposome release data interpretation.
- I prepared the manuscript drafts. The manuscript was revised by Prof Sato and Dr. Sou.
- All synthesis, characterization, experimental data collection was done by me.

21/01/2021

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Abstract

In this study, we report the alkali-encapsulating thermosensitive liposomes (Tris-Lipo) using DPPC/SA/PEG-DSPE (9/1/0.06, molar ratio) to enable environment pH regulation by sustained continuous cargo release at endogenous temperatures of 36.5°C. Tris-Lipo successfully neutralized the environmental lactic acids from a starting 4.0 to 6.7 by releasing the alkalis for prolonged durations of up to 7 hrs and significantly prevented acid erosion of hydroxyapatite (HA) matrix. When compared with a control, Tris-Lipo was able to reduce acid erosion of HA by 94% ($p < 0.01$) and demineralization of the HA by 77% ($p < 0.05$), showing high efficacy at preventing dental caries formation in the oral cavity. The release characteristic of various other thermosensitive lipid compositions (DMPC, DSPC, DPPC) are also investigated to understand the potential of mixture design on achieving different release profiles. The sustained characteristics of thermosensitive liposomes showcased in this study can be extended for various novel therapeutic, dental, and drug delivery applications with further innovation with surface response methodology (SRM).

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Table of Contents

Abstract.....	5
Acknowledgements.....	6
List of figures.....	10
List of tables.....	13
Chapter 1: Introduction	14
1.1: Introduction to liposomes.....	14
1.1.2 Classification of liposomes	15
1.2: Motivation.....	16
1.2.1 Sustained release liposomes	16
1.2.2 Liposome applications in dental care	17
1.2.3 Mixture design of liposome to optimize release profile	17
1.3 Objective and scope	18
1.4: Organisation of the report	19
Chapter 2: Literature review	20
2.1: Liposomes	20
2.2 Synthesis of liposomes.....	22
2.3 Applications of liposomes.....	27
2.3.1 Applications of liposomes in therapeutics.....	28
2.3.2 Targeting of specific sites.....	28

2.4: Controlled release of liposomes	30
2.4.1 Stimuli responsive liposomes	30
2.4.2 pH triggered liposomes.....	31
2.4.3 Enzyme triggered release.....	33
2.4.4 Redox triggered release	34
2.4.5 Light triggered release	35
2.4.6 Ultrasound triggered release	36
2.4.7 Heat triggered liposomes	38
2.5: Prevalent issues faced in dental care	40
2.5.1 Enamel demineralisation by bacteria.....	41
2.5.2 Enamel demineralisation by dietary acids	44
2.5.3 Opportunity for long sustaining liposomes in dental care	46
2.6: Enamel remineralisation in literature	50
2.7: Calcium and phosphate loaded liposomes in enamel remineralisation.....	52
Chapter 3: Liposomal release.....	56
3.1: Experimental procedures	56
3.1.1: Synthesis and characterization of Tris-encapsulating liposomes	56
3.1.2 Characterization of liposome size by Dynamic Light Scattering (DLS) and Transmission Electron Microscopy (TEM).....	57
3.1.3: Liposome Encapsulation efficiency of Tris solution.....	57
3.1.4: Release of payload from Tris-Lipo.....	59
3.1.5: Preparation of hydroxyapatite samples	60

3.1.6: <i>in vitro</i> Tris-Lipo release in lactic acid environment	60
3.1.7: Characterization of corrosion and demineralisation volume on hydroxyapatite samples	62
3.1.8: Statistical analysis	62
3.1.9: Synthesis of mixed lipid liposomes for release optimization	62
3.1.10: In-situ release characterization with UV-Vis spectroscopy	63
3.2: Results and discussion	65
3.2.1: Effects of temperature on payload release rate of Tris-Lipo and pH change	65
3.2.2: Effect of pH on payload release rate of Tris-Lipo and pH change	69
3.2.3: Quantitative analysis of acid erosion in hydroxyapatite substrate (erosion and demineralisation)	72
3.2.4: Release profiles of copper-containing mixture-designed liposomes	77
Chapter 4: Conclusion and future work	86
4.1: Contributions	86
4.2: Limitations	88
4.3: Future work direction	89
Publications	91
References	92

List of figures

Figure 1: Schematic representation of the different classifications of liposomes and their activation mechanisms [7].	15
Figure 2: A liposome is a very versatile structure capable of various methods of drug encapsulation and surface and composition modifications that make it a very attractive vesicle structure for pharmaceutical research. [23].	21
Figure 3: Schematic representation of SUVs, LUVs, and MLVs. [28]	22
Figure 4: Advantages of liposomes in medicine [36].	28
Figure 5: Schematic showing the different types of stimuli triggered liposome release mechanisms [43]	31
Figure 6: TEM image of Doxatoxel-loaded liposomes exhibiting a high degree of instability at low pH, resulting in the breakage of the liposomal structure and release of encapsulated Doxatoxel, effectively showing the pH-dependent release of drugs. [48].	33
Figure 7: Schematic reactions of (a) photo-induced cis-trans conformational changes, (b) photocleavage of lipids upon exposure to light-mediated formation of reactive species, and (c) photopolymerization of lipids containing double or triple bonds [57].	36
Figure 8: Phase transition of thermosensitive lipids such as DPPC from crystalline phase to a gel-like liquid phase when exothermia is exerted to increase the temperature of the lipid past its phase transition temperature (T_c) [7, 61].	38
Figure 9: Erosion depth of enamel by soft drinks over time. The pH values of the soft drinks were A) 2.42, B) 3.33, C) 3.46, D) 3.03, respectively [78].	46
Figure 10: pH vs. time of liposome suspensions isothermally held at 30, 34, and 37 °C. The decrease in pH indicated the release of calcium and phosphate contents from the liposomes and formation of calcium phosphate mineral [88].	52

Figure 11: A) Enamel etched with 37% phosphoric acid only; b) etched enamel surface treated with liposomes suspension; c) etched enamel surface treated with liposome and polyanion peptide. Figure C shows highly crystalline and oriented apatite growth when calcium and phosphates are release for long durations (2 weeks) with the incorporation of peptide seeds. This is a stark improvement over the calcium phosphate precipitation by Murphy et al which was formed over a short duration in figure 12.	53
Figure 12: Acid etched human dentin surfaces following treatment with liposome suspension for 20mins at 37 °C.	54
Figure 13: The size distribution of prepared Tris-Lipo measured by dynamic light scattering (DLS).	65
Figure 14: TEM image of Tris-Lipo negatively stained by phosphotungstic acid.	66
Figure 15: Profiles of (A) payload release and (B) pH change during incubation of Tris-Lipo dispersion at 36.5 °C or 25 °C through a time period of 7 h. At an average temperature of 36.4 ± 0.08 °C, the pH increases from 4 to 5.53 ± 0.27 in 1.5 h. While an average temperature of 25 ± 0.09 °C increases the pH from 4 to 5 ± 0.07 in 6 h.	69
Figure 16: Profiles of (A) payload release and (B) pH change during incubation of Tris-Lipo dispersion at 36.5 °C. Lactic acid was added to the dispersion immediately after sampling at 3.5 h.	71
Figure 17: In vitro model to evaluate erosion of hydroxyapatite. (A) The hydroxyapatite samples are masked with a 5 mm tape and acid resistant nail varnish is applied at the sides to protect the reference area from corrosion. (B) Digital photograph of the masked hydroxyapatite substrate with 5mm exposed erosion window on the left, and the hydroxyapatite substrate as-in on the right.	73
Figure 18: Environment pH with and without Tris-Lipo introduction (n = 5, N = 10). With treatment of Tris-encapsulated liposome, the pH of the acid environment increases above 5.5	

within 1.5 h while the pH of the sample without treatment remained below 4.5 throughout the test.....	74
Figure 19: In vitro erosion test using hydroxyapatite substrate.....	76
Figure 20: Average eroded area calculated from the profilometry analysis data of the confocal scan (n=5) and average demineralised volume calculated from the profilometry analysis data of the confocal scan (n=5).....	77
Figure 21: Mixture design methodology implemented to obtain 10 lipid compositions from 3 different lipids. The 10 mixtures can then be analysed and optimized through surface response methodology to obtain an ideal lipid composition.....	78
Figure 22: Release profiles of the 10 liposome compositions formulated with DPPC (16 PC), DSPC (18 PC) and DMPC (14 PC) through mixture design methodology.....	79
Figure 23: Release profiles of the liposomes with the 3 main lipids as well as an equal part composition liposome consisting of the 3 lipids.....	80
Figure 24: Release profiles of liposomes with DSPC (18PC) as either a main or secondary constituent.....	81
Figure 25: Release profiles of liposomes with DPPC (16PC) as either a main or secondary constituent.....	82
Figure 26: Release profiles of liposomes with DMPC (14PC) as either a main or secondary constituent.....	83
Figure 27: Surface response of liposome size variations as a function of lipid compositions, as a optimization method demonstrated by Behnouch et al[100].....	84

List of tables

Table 1: Distribution of Lactobacillo, Streptococci, Prevotellae and Fusabacteria across different pH ranges, showing the predominance of lactobacilli and streptococci at lower pH ranges [70].	43
Table 2: pH lowering potential of mutans streptococci on enamel after glucose stimulation after 10mins and 60mins [72]	44
Table 3: Lipid compositions for mixture design optimization.....	63
Table 4: Erosion data of Hydroxyapatite samples after 5 days of lactic acid immersion in-vitro	75

Chapter 1: Introduction

1.1: Introduction to liposomes

Liposomes are lipid capsules composed of lipid bilayer membrane which can encapsulate water-soluble molecules in their aqueous core[1]. By dispersing amphiphilic lipids in a solvent, the lipids self-assemble into a capsule which are termed as liposomes, encapsulating the solvent in the core. And depending on the solvent in which the lipids are dispersed in, liposomes are able to encapsulate both hydrophilic and hydrophobic solutions. Due to this unique nature, as well as the high biocompatibility of the lipids that make up the structural shell of the liposome, it is highly suitable as a nano drug carrier. Gregory Gregoriadis was the first to demonstrate the encapsulation of drugs using liposome shells, and since then their use in drug delivery has been extensively explored[2].

Since then, many kinds of functionalized liposomes have been extensively studied as a drug carrier which show relative high biocompatibility [3, 4]. Liposome drug delivery systems are mostly researched as selective cell targeting systems for advanced biomedical applications such as treatment of cancer cells [5, 6]. Currently, the active target of cancer cells and burst release of drugs by using stimuli-responsive liposomes are technical matter for advanced drug delivery system [7-9]. In these systems, the liposomes are the drug vessel that protects the payload/drug from bodily fluids during delivery to the target site, before activation and bursting of the liposome to deliver a large dosage of drugs through applying specific exogenous stimulations such as heat, light, magnetic fields, lasers, and many other novel methods.

1.1.2 Classification of liposomes

These numerous methods in liposome delivery control and release activation resulted in liposomal drug delivery to be classified into 2 different groups in terms of targeting and activation. Liposomes are that passive targeting do not home in onto the target cells but are rather just injected near the target site and left to circulate passively. Activate targeting liposomes, however, are functionalized with moieties [10] that have chemical affinity with the targeted cells or areas, allowing the liposomes to deliberately bind onto the target cells. Research has also been done on novel targeting systems such as magnetically controlled liposomes that are embedded with paramagnetic metal nanoparticles [8].

Figure 1: Schematic representation of the different classifications of liposomes and their activation mechanisms [7].

Liposomes are also classified according to their release profile. Triggered release liposomes rely on exogenous stimuli such as ultrasound, heat and light to activate the release of drugs or payload inside the liposomes, while conventional release liposomes release their drugs through leakage of the drugs over time, without any exogenous stimuli.

While there are many researches and innovations on externally triggered liposomes, conventional release liposome development is scarce. Despite the numerous studies on liposomes as drug carriers for intravenous applications in which stealth liposomes stably maintain the encapsulation of payload at body temperature [11, 12], the studies on design of liposomes for slow sustained release at the endogenous heat is still limited.

1.2: Motivation

1.2.1 Sustained release liposomes

While there are already many novel and successful research on liposome activation in vivo to good effect of drug release, these methods require highly specialised equipment in controlled environments, and thus are not widely and readily available as treatment options. There is much opportunity for research on a conventional-releasing drug liposome that is passively activated by solely using natural endogenous body heat instead of the stimuli-triggered burst-release liposome drug delivery methods to achieve a slow and sustained release profile.

This is important to provide constant and sustained drug delivery to target sites without the need for sophisticated medical equipment for activation of the drug release, that are inaccessible outside of advanced medical compounds. Extended and sustained drug release from the liposome also increases the effective treatment duration of the drug on the targeted cells for better medical results [13, 14]. Without the need for external stimuli, such liposomes

can be used more readily and be accessible to a wider population. The sustained release model also poses a future potential for specially functionalized liposomes to act as an embedded passive defensive therapeutic, activated by intravenous signals of infection or mutation [15].

1.2.2 Liposome applications in dental care

Dental care is an area of research where liposomes as a therapeutic vehicle has not been widely researched but has great potential in. With dental related conditions such as the formation of dental caries being prevalent on a vast majority of the human population as compared to relatively rare diseases such as cancer, improvement in dental care using liposomes can be far wider reaching than the current trends of liposomal research as drug therapeutics.

Continuous release of chemicals in the oral cavity can present opportunities for regulation of pH, which is one of the leading contributors of dental caries formation [16, 17], as well as provide mineral saturation for the reparation [18] and growth of hydroxyapatite crystals to strengthen and eventual complete repair of enamel surfaces on dentine.

1.2.3 Mixture design of liposome to optimize release profile

Thermosensitive liposomes in our research are able to release drugs for a prolonged period. However, the use of one lipid type (DPPC) limits the duration and release profile of encapsulated solutions without the capability to tailor for different release rates and exposure durations. By investigating the release profiles of a mixture of thermosensitive lipids, we are able to tailor the critical temperature to trigger self-sustained release, as well as the release rate, all under endogenous temperature. This presents an opportunity to set a standardised liposome design for specific durations and profiles, to suit different pharmaceutical needs.

1.3 Objective and scope

The objective of this research is to investigate the viability of using thermosensitive liposomes to provide continuous and gradual release using endogenous body heat. This method would be less invasive and be more accessible as it does not require hyperthermia in excess of the body's nature temperature which could be damaging to body tissues at temperatures above 42.5 °C [19, 20]. A sustained release targeted drug liposomal carrier could be used for daily application without the need for sophisticated medical equipment for the activation of the liposome release.

For this purpose, we will be studying the release characteristic of liposomes mainly composed of thermosensitive lipids such as DPPC, DMPC and DSPC with various phase transition temperature at between 25 - 55 °C [21] to explore the applications of the liposomes in an oral environment with a thermal characteristic similar to endogenous body temperature. By encapsulating alkali buffers into our liposomes, we will investigate whether sustained release of drug contents with thermosensitive liposomes is viable, and whether it has tangible benefits in prevention of oral caries in an acidic environment.

1.4: Organisation of the report

This confirmation report is organised into 4 chapters. Each chapter begins with a short note reflecting the main contents of that chapter.

Chapter 1 is an introductory chapter and it gives an overview about the research opportunity, aims, objectives, scope, and organization of this report.

Chapter 2 contains a comprehensive literature review that has been carried out for this project. An overview of the theory, principle and process of liposome formation, design and applications is covered.

Chapter 3 outlines the work done so far related to the experimental designs, procedures, results, and discussion for the sustained release of thermosensitive liposomes.

Chapter 4 is the last chapter of this research thesis. It gives the overall summary of the benefits of this project. Suggestions for future work are also described here.

Chapter 2: Literature review

2.1: Liposomes

A liposome is a term given to a spherical nanostructure that is formed by a lipid bilayer. The hydrophilic tail mainly consisted of phosphoric acid bonded to a water-soluble molecule, while the hydrophobic tail contained fatty acid chains with carbon chains ranging from 10-24. Due to the lipids having a hydrophobic and hydrophilic end, they form bilayer lamellar sheets when disperse in an aqueous solution, and spontaneously align into a spherical form to achieve thermodynamic stability [22].

Liposomes in literature are commonly composed of phospholipids, with phosphatidylcholine in particular being a commonly used class of lipids. Other common lipids and components include phosphatidylethanolamine, lysolipids, phosphatidylglycerol and phosphatidylserine. These components exhibit the lipid bilayer structures in solvents and are biocompatible, and as such are used as the base components for liposomes. These liposomes can also be extensively modified on the surface for various functions such as

1. targeting of liposome to site or away from site
2. protection and/or enhancement of liposome stealth from immune system
3. modification of chemical characteristics such as phase transition temperature or surface charge
4. activation of liposome content release

Figure 2: A liposome is a very versatile structure capable of various methods of drug encapsulation and surface and composition modifications that make it a very attractive vesicle structure for pharmaceutical research. [23].

Liposomes were first described by Alec D Bangham [24, 25] in a 1964 publication describing the bilayer lipid structure of dry phospholipids modified by negative staining under an electron microscope. In a subsequent paper, Bangham, Standish and Weissmann also demonstrated the capability of liposomes on releasing their enclosed contents after treatment with detergent, thus kicking off the potential of liposomes being a viable novel drug delivery system [26].

Through observation via negative stain electron microscopy performed by Yashroy et al, the microstructure of liposomes could also be distinctively distinguished from micelles and hexagonal lipid structures [27].

One important method of classifying liposomes is to group them according to the size and quantity of bilayers within a liposome [28]. Based on common convention of naming, they can mainly be grouped into

- Unilamellar vesicles (SUV): Smallest of the 3 classifications, a SUV is a liposome with diameter ranging between 10nm – 100nm, made from a single bilayer shell structure.
- These liposomes are thermodynamically unstable and can hold the least encapsulated contents due to the small volume to surface ratio.
- Large unilamellar vesicles (LUV): A liposome with sizes ranging from 100nm – 3000nm, it is a larger version of an SUV. With a more favorable size for increased thermodynamic stability due to lesser angles of curvature, as well as a more economical volume to surface ratio, this type of liposome is widely studied in research.
- Multilamellar vesicles (MLV): The largest of the 3 classifications, it is classified as a liposome of diameter ranging from 0.5 μ m to 10 μ m, consisting of a large vesicle encapsulating several smaller vesicles of various sizes with. These smaller internal vesicles may consist of LUVs and SUVs with unilamellar or multilamellar structures.

Figure 3: Schematic representation of SUVs, LUVs, and MLVs. [28]

2.2 Synthesis of liposomes

Liposomes can also be classified by the method in which they are synthesised. The general procedure for liposome synthesis can be generalised into 4 procedures:

- 1) Drying or evaporation of organic solvent in which the lipid composition/mixture is
- 2) dissolved.
- 3) Dispersion of dry lipid into aqueous media to be encapsulated and formation of liposome from dispersed lipid through injection of energy into the system
- 4) Separation and purification of the resultant liposomes.

Drying or evaporation of organic solvent

Drying or evaporation of organic solvent of lipid mixtures are commonly done through freeze drying [29]. Dissolving mixtures of different lipid components are necessary for the formation of a homogenous lipid composition comprising of different lipid types and phases. This is usually done by dissolving the lipid mixture in an organic solvent such as tert-butyl alcohol/water cosolvent systems [30]. After a homogenous lipid composition is achieved, it is separated from the tert-butyl alcohol/water cosolvent system by a freeze-drying process. By freezing the lipid and cosolvent mixture at a temperature below the triple point of the cosolvent, which crystalized the cosolvent, the pressure of the environment is lowered, while heat is added to the system simultaneously to cause the cosolvent to sublime and evaporate, leaving the dry homogenous lipid mixture behind.

Dispersion of dry lipid in aqueous media and formation of liposomes

For the formation and loading of liposomes, there are two main classification of methods, namely by mechanical dispersion and solvent dispersion.

Some of the common methodology used for the mechanical dispersion of lipids in aqueous media include sonication, extrusion, freeze-thaw, emulsification and lipid film hydration.

Solvent dispersion methods include ether injection, ethanol injection and reverse phase evaporation.

Mechanical dispersion: Sonication

One of the most common methods for synthesis of liposomes is the use of sonication [31]. By using sonication, MLVs that naturally form during the dispersion of freeze-dried lipid films into aqueous media are broken down by destructive energy produced by mechanic cavitation of the solvent by sonication. The broken fragments of the MLVs thus become the lipid films that are the basis of the formation of SUVs subsequently as thermodynamics drives the film edges to conjugate and enclose into a sphere to reduce the free surface energy present within the film-solvent boundaries. The disadvantages of using this methodology for liposome synthesis is that due to the miniscule size of cavitation caused by sonication, the resultant sizes of liposomes are almost always in the SUV range, while having no real defined control over the resultant sizes. In addition, due to the high angles of surface curvature, the resultant SUVs are also less stable than MLVs and LUVs and are not suitable for use and storage over long periods.

There are 2 methods of sonication, which is to use either a probe sonicator or a bath sonication. For probe sonication, a sonicator probe is immersed directly into the lipid dispersion. This allows for excellent transference of energy, resulting in a highly energetic sonication and dispersion of lipids into smaller fragments. Such a method is excellent for synthesis of SUVs in the range of less than 10nm. However, a characteristic disadvantage of probe sonication is that the localised heating at the probe tip caused by the high energy transfer and vibration causes degradation of the lipids, as well as potential contamination of the solvent and liposomes by sloughing of the probe tip material.

For bath sonication, the lipid dispersion is immersed into a bath sonicator while contained in a vessel, without direct contact with the bath. This method offers excellent temperature control of the lipid dispersion, in contrast to using probe sonication, as well as reducing contamination as the lipid mixture is in a closed vessel. The size and mechanism of liposome formation are similar to that in probe sonication.

Mechanical dispersion: Extrusion

The extrusion method to synthesise liposomes involve using high pressures to force lipid films, or naturally occurring MLVs, through a small orifice. In membrane extrusion, a filter membrane with defined pore sizes is used to control the sizes of the liposomes synthesized. As a result, this method has several advantages over sonication, being able to control the liposome size, as well as the resultant liposomes being larger than those synthesized by liposomes, generally being in the LUV size range. This inherently larger liposome batch size results in better encapsulation efficacies as well as increased storage and activation stability due to the larger LUVs having lower surface tension. However, due to the high pressures involved in extrusion, the synthesis volumes are generally limited and smaller than in sonication processes.

Mechanical dispersion: Freeze thawing

Freeze-thawing synthesises LUV liposomes by the fusion and aggregation of multiple SUVs. By rapidly freezing and slowly thawing SUVs synthesised from sonication methods, the collapsed and fragmented SUV lipid bilayers fuse together to reduce free surface energy by adopting a lower surface curvature and forming LUVs as a result. This method is able to achieve high levels of encapsulation efficacies [32].

Solvent dispersion: Organic solvent injection

Ether injection involves dissolution of lipids in ethers, and subsequently injecting this ether to an aqueous solution of encapsulated materials. By precipitation of the lipid films out from this ether-aquo cosolvent at increased temperatures above the phase transition temperature of the lipids, liposomes are synthesized, and ether can subsequently be removed by vacuo. This method results in a non-homogenous size range of liposomes which could be a disadvantage depending on the application. The aqueous compound that is encapsulated are also exposed to high temperatures that could result in degradation [33].

Ethanol injection involves a similar methodology where ethanol is used as the organic solvent instead of an ether. After injection into a strong buffer, clusters of MLVs are precipitated immediately. The method shares the same disadvantage of non-homogenous liposome sizes as ether injection, as well as an additional disadvantage of being difficult to purify due to ethanol forming azeotropes with water in the aqueous cosolvent [34].

Solvent dispersion: Reverse phase evaporation

Reverse-phase evaporation is based on an aqueous cosolvent mixture of water and organic phase solvent in which the amphiphilic lipids are solubilized. In this cosolvent, the lipids form an inverted micelle where the micellar structure collapses and form a gel upon evaporation and removal of the organic phase under vacuum. The collapsed gel then breaks up into bilayers and encapsulates smaller micelles in the mixture and forms MLVs. The encapsulation efficacy using this method can be very high, and can be controlled by the ionic strength of the buffer used in the water-organic cosolvent[35].

2.3 Applications of liposomes

Due to the unique properties and capabilities of liposomes, researchers have found widespread usage for them in a wide range of applications, ranging from highly specific in-vivo site targeted therapeutics, to ex-vivo aesthetic cosmetic applications.

Many drugs used for cancer therapy have a very small window between therapeutic efficacy and toxic dosage. However, with a nano drug carrier like a liposome, the toxicity in-vivo can be reduced as a result of reducing the leakage and spatial distribution of the drug throughout the body, as well as increased therapeutic efficacy by site targeting and controlled release of the drug. The interaction between the liposomal carrier and the body's natural immune and biological responses are critical to the administering of drugs in-vivo and have thus been the common focus in most liposome drug delivery research.

In vitro and in vivo studies of liposome-cell interactions show that the predominant interaction of liposomes with cells is either endocytosis or adsorption, where the liposome and its contents are adsorbed into the cell membrane, therefore delivering the drug directly. Lipid exchange as a result of similarity between liposome lipid bilayers with cell membrane phospholipids is also possible. The result is in an exchange of bilayer constituents, such as cholesterol, lipids, membrane bound oleates and peptides with components of cell membranes.

Figure 4: Advantages of liposomes in medicine [36].

2.3.1 Applications of liposomes in therapeutics

Liposomal drug delivery systems can offer advantages over traditional forms of drug, especially for parenteral, topical, and pulmonary methods of delivery. There are many ways that distribution of the drug molecules can be altered by the usage of liposomes, and in many cases, even yield benefits such as the improved therapeutic efficacy of encapsulated drugs. Some of these benefits include:

2.3.2 Targeting of specific sites

By modification of the liposome surface through peptides and moieties, delivery of the liposomes to the desired diseased or infected site is made possible, reducing the drug's overall exposure and toxicity to normal tissues during its journey through the physiological path, while increasing the drug load to the targeted site. Long circulating liposomes that are undetectable by the body's natural immunocytes have also been a subject of research, being able to bind to

the targeted cells through several passes of circulation throughout the body without releasing the contents in the liposomes during its circulation [37].

Site-avoidance delivery

Just as the targeting of sites can increase the therapeutic efficacy of the drugs, site-avoidance delivery can reduce the cytotoxicity of drugs towards healthy cells in the subject's physiology during the liposome's passage towards the site [38]. Anti-cancer drugs such as doxorubicin are well documented to have a high toxicity to healthy cells and cancer cells alike, and has a narrow therapeutic index, where a slight increase in concentration can result in therapeutic effects to toxic effects such as acute cardiac toxicity. Encapsulation of such drugs in liposomes can reduce the downsides of drug exposure to healthy tissues as compared to direct methods of drug delivery such as intravenous or site injections.

Improved solubility of lipophilic and amphiphilic drugs

Some cases of cancer drugs such as Ancrylovir or Doxorubicin can be encapsulated in concentrations higher than their usual saturation point in aqueous solutions when encapsulated in liposomes[39]. This is due to the formation of a concentrated gel or precipitation state due to their hydrophilic nature of the compounds. Some other examples include drugs like Porphyrins, Amphotericin B and Minoxidil. This allowed for larger drug doses to be delivered to the targeted sites without the drawback of increasing toxicity towards healthy tissues during the drug's delivery to the site through the physiobiological system as well as increasing the treatment efficacy.

Selective activation of drug release

Many novel methods of liposome activation and release have been researched, resulting in a wide range of liposomes that activate only upon very specific stimuli or triggers not naturally occurring in human physiology, thus making the liposomes highly effective at site treatment, while remaining dormant in the rest of the body. Such examples include photon triggered release [40], x-ray stimuli [41], and spot targeted ultrasonic waves [42].

2.4: Controlled release of liposomes

2.4.1 Stimuli responsive liposomes

Stimuli-responsive liposomes have been widely researched and emerged as a promising method of liposome drug delivery to specific sites. Many novel liposome creations incorporate stimuli-selective properties by means of imbuing various surface modifications such as lipids, polymers and nanoparticles to react with several biological environment factors to trigger the release of the liposome contents.

Certain specific features in the physiological environment such as environmental pH, presence of infection/disease specific bioindicators such as enzymes, and redox potential, can serve as a local endogenous stimulus. Other forms of stimuli are remote or exogenous triggers such as heat, ultrasound and light to initiate destabilization of the liposomes through photochemical reactions, physical breakage or disruption of phase transition of the liposome lipid structures to release the encapsulated drugs.

Figure 5: Schematic showing the different types of stimuli triggered liposome release mechanisms [43]

Some of the literature featuring liposome delivery strategies involving pH triggers, enzymic environmental triggers, photochemical reactions caused by light, and redox reactions will be discusses, as well as their benefits and problems faced. Despite the promising academic results from these stimuli-responsive liposomes, and an extensive amount of work onto these researches, not many have had successful applications in clinical practice.

2.4.2 pH triggered liposomes

Diseased pathological targets are widely reported in literature to have different pH ranges as compared to healthy tissues in the biological system. Cancer cells are reported to often be more acidic than healthy tissues of similar species, ranging from 6.8 – 7.0, and some reports of pH below 6.0 [44, 45] These changes in the physiological environment as the liposomes passes from healthy sites to pathologically diseased sites of interest can trigger changes in the

permeability of the bilayer membranes by deprotonation or protonation of functional groups in the modified lipid bilayer and induce morphological changes.

One such example is the polymorphic phase transformation of 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) which has an inverted hexagonal phase II (HII phase) at low pH and a bilayer structure ($L\alpha$ phase) at neutral pH [46]. This change in the structural outer shell of the liposome modified by DOPE will promote the destabilization of the shell and result in the release of the encapsulated contents when passing through sites with low pH (Figure 6).

Amine group functionalization have also been utilized to trigger pH-dependent drug releases in liposomes by exploiting the deprotonation/protonation effect. By using pH-sensitive copolymers, composed of poly(2-(diisopropylamino) ethylmethacrylate) (PDPA) and mPEG segments⁵⁰ which are amphiphilic and assemble to form normal liposomes in physiological environments but become hydrophilic upon protonation of di-isopropyl tertiary amines under acidic conditions to initiate drug release, the PDPA liposomes were able to release 80% of its encapsulated contents at pH 6.0, while only 20% were released at pH 7.4 [47].

Figure 6: TEM image of Doxorubicin-loaded liposomes exhibiting a high degree of instability at low pH, resulting in the breakage of the liposomal structure and release of encapsulated Doxorubicin, effectively showing the pH-dependent release of drugs. [48]

2.4.3 Enzyme triggered release

Enzyme-sensitive release systems most often employ elevated enzyme expression levels to drive biochemical transformations that can activate drug release at specific tumor microenvironments. In general, enzyme-responsive carrier systems most often rely on the cleavage of esters or amides in short peptide sequences by esterases or proteases, respectively, to release the therapeutic agent selectively.

There are extensive research on the range of enzymes that can be used to activate the many liposomes of the studies, including Human sPLA2-IIA [49], phospholipase C (PLC) [50] and even glucose-triggered liposomes that incorporated poly(N-isopropylacrylamide-co-

methacrylic acid-co-octadecylacrylate) (P(NIPAM-co-MAA-co-ODA)) [51]. However, much of enzyme related liposome release mechanisms are not feasible for dental car studies as these enzymes will be extremely unlikely to be present in oral environments during non-exceptional circumstances.

2.4.4 Redox triggered release

Redox-responsive liposomes have been reported that use electron-transfer reactions to trigger drug delivery. Vesicles of this type have been destabilized by changes in charge and/or hydrophilicity of the amphiphile, chemical reducing agents, or removal of cross-links to drive phase transitions of the lipid system. The high redox potential differences (100- to 1000-fold) existing between the reducing environment of intracellular space and the more oxidative extracellular environment can serve as an activating stimulus for both tumor targeting and intracellular drug delivery[52].

One of the most commonly utilised redox activation systems uses disulfide linkages within amphiphiles which are disrupted using thiolytic reducing agents, such as dithiothreitol (DTT). Upon reaction with the agent, the critical micelle concentration of the cleaved amphiphile by-product are typically increased. A similar strategy involves reducing or fragmenting the functional headgroups in fusogenic liposomes, leading to the activation which fuses and aggregates the liposomes, resulting in subsequent drug release[53].

Changes in charge can also induce liposome release by using amphiphiles that include surfactants based on ferrocene and metal cation-ligand complexes, with the redox moiety attached to either aliphatic chains or cholesterol. The cationic versions of these amphiphiles

existed as vesicles that, upon chemical reduction to less-charged species, were destroyed, resulting in content release [54].

2.4.5 Light triggered release

Photon from light sources can also be used to activate the release of contents encapsulated in liposomes. To use the light to trigger release, photons from light in 600-900nm wavelengths are able to transmit deep into the biological system to activate photosensitizing agents that are modified onto the liposome surface [55]. Photodynamic therapy utilizes photosensitized liposomes to activate on-demand or selective release of drug contents using light of different wavelengths, intensities and pulse durations to achieve direct eradication of diseased cells, and is a therapeutic modality for cancer treatment [56].

The photosensitizer absorbs energy from photons and transfer the energy molecular oxygen, leading to the formation of reactive species that can subsequently react with the liposomes to induce contents release. Since most photosensitizers are hydrophobic, nano drug capsules such as liposomes and micelles are widely used to enhance the stabilization of drugs during delivery to site as well as site targeting using moieties. These light-induced reactions can involve either the irreversible modification (photocleavage) of a reactive amphiphilic molecule or reversible conformational changes in the lipids to trigger release via a localized increase in membrane permeability [57].

Figure 7: Schematic reactions of (a) photo-induced *cis-trans* conformational changes, (b) photocleavage of lipids upon exposure to light-mediated formation of reactive species, and (c) photopolymerization of lipids containing double or triple bonds [57].

Photo-polymerizable phospholipids such as DC8,9PC (1,2-bis(tricoso-10,12-diynoyl)-sn-glycero-3-phosphocholine) can be used to modify and prepare light-trigger liposomes. UV light irradiation initiates the polymerization reaction, causing the covalently linked lipids to polymerize within the bilayer outer shell, creating defects in the structure and resulting in breakage and leakage of the liposome contents. Studies demonstrated that these liposomes were able to release large percentages of liposome contents within a short amount of time upon UV stimulation, able to release 100% of its contents within 15 – 40 mins [58].

2.4.6 Ultrasound triggered release

Liposomes modified to increase their acoustic sensitivity are sometimes also referred to as echogenic or Acoustically Activated Liposomes. These liposomes make use of ultrasound, consists of sound waves (pressure waves) with a frequency higher than 20 kHz. The sound wave is a physical wave that needs a medium to travel through, unlike light or electromagnetic waves. Sound waves propagate by means of energy transfer between the molecules of the medium, which occur as the pressure changes from compression (high pressure) to refraction

(low pressure). There are different methods in which ultrasound can be used to trigger release in echogenic liposomes, which are through hyperthermia, cavitation and collision.

Lipid membranes have an acoustic impedance, similar to muscle tissues, causing it to absorb and dissipate energy when an acoustic wave is absorbed. This acoustic energy absorbed is converted to heat energy, and when in abundance, the heat energy added to the liposome raises its temperature above its phase transition temperature, causing the liposome to undergo phase transition from a crystalline structure to a gel-like structure, which causes its contents to leak or release. However, such a method of raising the liposome's temperature also results in the drawback of causing damage to the healthy tissue being irradiated. At high high frequency at high intensities required to penetrate deep into the tissues [59] to reach the targeted site, the outer layers of the body will be subjected to extremely elevated temperatures which causes unnecessary damage to otherwise normal tissues.

Cavitation is a natural phenomenon that occurs when a wave is incident on a spherical capsule with liquid phase having the same resonance frequency as that of the incident wave. When the resonance frequency matches, the sphere will oscillate along with the wave, and in the case of liposome bursting, result in transient cavitation. Cavitation-induced release from the liposomes is triggered due to either the shear wave produced from the collapse, directed or not, and/or due to the elevated temperatures that are generated in the process [60]. At high shear forces, the vibrations experienced by the liposomes can sometimes result in bursting through collision with neighbouring liposomes at high speeds, and not necessarily due to the breakage of the lipid structure during to transient cavitation.

2.4.7 Heat triggered liposomes

Heat triggered liposomes utilize thermosensitive phospholipids as the main composition, where the lipids have a defined phase transition temperature (T_c) depending on the length of the carbon chain tail. A phospholipid with a higher number of carbon chains on its hydrophilic tail will have a higher phase transition temperature. When the phospholipid is at a temperature below its T_c , it has a crystalline state that is highly stable; but when heated to near or beyond its T_c , it transforms from the stable crystalline state to a more amorphous gel-like liquid state that is more permeable and unstable, thus making thermosensitive liposomes made from such thermosensitive lipids able to be activated by heat for content release.

When the temperature of a thermosensitive liposome is increased, the mobility of the polar starts to increase, along with the transition of carbon chain tails from the crystalline to the gel-like liquid phase as the temperature increases further towards the T_c of the liposome. The interface of both solid and liquid lipid phase at or near the T_c results in leaky release of the contents due to the formation of such grain boundary defects.

Figure 8: Phase transition of thermosensitive lipids such as DPPC from crystalline phase to a gel-like liquid phase when exothermia is exerted to increase the temperature of the lipid past its phase transition temperature (T_c) [7, 61].

When the phase transition temperature of the liposome is exceeded, the entire liposome outer shell is transformed into the gel-like state, which results in drastically increased permeability which significantly increases content release, or in conventional liposomes without surface ligands to keep the liposomes in a colloidal state, induce fusion and aggregation of the liposomes and result in a 'burst' release of the contents. Such models of 'burst' release of thermosensitive liposomes are common in methods of heat or exothermia induced release using heat sources such as high intensity focused ultrasound, infrared spot heating or advanced magnetic resonance-guided high intensity focused ultrasound to achieve non-invasive heating.

Research on thermosensitive liposomes typically revolve around modification of the T_c of the liposome through lipid composition [62], lysolipid modification [61], and thermosensitive polymer [63] surfactant modification. However, the common theme behind these studies of thermosensitive liposome compositions is the ability to optimize the release of large percentages of contents within a short frame of time (60mins>) to reduce the need for hyperthermia as well as to reduce the drawbacks of damage to tissue from prolonged exposure to high temperatures which will damage healthy cells [19, 20].

These liposomes are also mostly designed to activate under excess exothermia generated through external means, or excess exothermia as a result of internal irregularities such as diseased sites or biological responses towards inflammation and infection, but are generally stable in normal physiological conditions and will not release their contents in any significant amount otherwise [64].

2.5: Prevalent issues faced in dental care

The formation of dental caries is a medical condition where the enamel of the dentine is eroded away and is an unavoidable condition that the vast majority of humanity will experience at some point in their lives. As such, prevention of dental caries formation has always been an area of continual research and innovation in the field of dentistry.

Despite the natural protective qualities of the enamel, there are several factors that can cause the demineralisation of tooth enamel minerals and dental tissue that are widely common. These include acids generated by bacteria in plaque and the oral environment following metabolism of fermentable carbohydrates derived from the diet, foreign sources of acids such as dietary acids from consumable drinks and food, and physical wear and tear from natural abrasion and attrition from using teeth for normal consumption of foods. The result of enamel demineralisation or enamel loss can be the softening or degradation of the enamel and the subsurface enamel which can lead to changes in tooth hardness, shape, function, aesthetic qualities, increased sensitivity and even ultimately to complete tooth loss[65].

Due to physical wear and tear being a mostly unavoidable phenomenon, the target of most dental caries preventable research is on the reduction of enamel erosion and demineralisation by acids. The composition of the tooth is made out of 85% hydroxyapatite (HA), 12% water, and 3% of organics of proteins and lipids, with a non-homogenous composition throughout the teeth, mostly due to specific tooth morphology required at different areas. At the working surfaces of the enamel, the composition is made out of hydroxyapatite to give it a high surface hardness and toughness, although highly susceptible to erosion by acids.

The erosion rate of hydroxyapatite by acids increases with decreasing pH and is also a function of the concentrations of Ca^{2+} , PO_4^{3-} as shown by this simplified equilibrium equation:

The influence of calcium and phosphate ions as a function of the chemical reaction also meant that demineralisation will not occur when there is saturation of calcium and phosphate ions in the system as they cannot be precipitated out from the HA in the enamel. Thus, there exists a critical pH where the system becomes undersaturated and enamel demineralisation is possible. This critical pH value is widely quoted to be 5.5 in earlier research[66, 67], but also believed to be in the range of 4.5 – 5.5 [68] in recent reviews.

2.5.1 Enamel demineralisation by bacteria

The demineralisation of dentine enamel by bacteria can occur when dietary carbohydrates such as glucose, sucrose and fructose are consumed and metabolised by bacteria in plaque such as streptococci, lactobacilli, prevotellae, and fusobacteria to produce organic acids which causes the pH of the localised area around the plaque to decrease. Examples of such organic acids produced by the bacteria are lactic acid, acetic acid/acetate and propionic acids[69]. Lactic acid is present in the most significant quantities and is the main driver of decreasing the oral pH below critical levels for HA demineralisation. Dental caries produced by using lactic acid in-vitro have also been indistinguishable from acids produced naturally by plaque bacteria, and have been accepted as a method of artificially produced caries lesions [17].

pH of the plaque also influences the bacteria population and distribution within the plaque itself. Amongst the 4 families of bacteria that are the most predominant and widely discussed in dentistry, lactobacilli and streptococci are the high acidogenic family of bacteria, producing mainly lactic acids which are the main driver of enamel demineralisation, while prevotellae and fusobacteria are less acidogenic, producing weaker acetic and propionic acids. A study into the distribution of bacteria in dental plaque has shown that prevatellae and fusabacteria are more predominant in higher pH ranges of 5.5 – 6.0, while lactobacilli and streptococci thrive at lower pH ranges of 4.5 – 5.0 [70] and also produce more lactic acid at this range than at higher pH ranges.

This creates a vicious cycle where the lower the pH of the oral system, the more acidogenic bacteria can thrive due to their acid tolerance [71] and more lactic acid will be produced. However, it also presents a window of opportunity where if the system pH is restricted from dropping below the critical pH for lactobacilli and streptococci to thrive in the first place, dental caries prevention caused by plaque bacteria could be achieved.

	pH range	Percentile concentration (cell/mg)					
		P ₅	P ₁₀	P ₂₅	P ₅₀	P ₇₅	P ₉₅
Lactobacilli	4.5–5	37,734	37,986	48,773	144,437	427,739	1,570,823
	5.0–5.5	64	184	3,132	17,073	76,295	137,577
	5.5–6.0	7	23	1,010	6,209	79,560	859,200
	6.0–6.5	1	46	143	1,443	4,109	43,098
	>6.5	3	22	125	556	1,622	858,798
Streptococci	4.5–5	18	28	82	1,380	26,971	48,401
	5.0–5.5	5	9	25	205	7,046	21,114
	5.5–6.0	1	159	1,188	1,761	12,435	85,956
	6.0–6.5	2	17	55	532	4,246	42,171
	>6.5	6	7	36	186	3,004	306,770
Prevotellae	4.5–5	8	8	14	101	1,554	5,054
	5.0–5.5	0	1	6	88	1,063	15,926
	5.5–6.0	1	17	499	2,402	16,317	60,926
	6.0–6.5	0	1	2	225	2,746	21,288
	>6.5	1	2	6	355	2,011	28,576
Fusobacteria	4.5–5	2	2	3	43	598	1,616
	5.0–5.5	0	0	1	2	8	1,013
	5.5–6.0	0	1	3	170	983	5,887
	6.0–6.5	0	0	0	17	481	5,283
	>6.5	0	1	2	17	546	5,656
Total load of bacteria	4.5–5	435,153	496,692	884,003	1,651,535	5,876,933	18,043,634
	5.0–5.5	50,513	80,862	264,972	598,880	780,630	2,837,841
	5.5–6.0	10,985	30,779	112,218	1,292,971	4,522,828	16,140,983
	6.0–6.5	1,434	16,203	43,337	161,326	429,581	2,285,414
	>6.5	2,033	4,752	17,766	48,980	344,247	7,354,811

Table 1: Distribution of Lactobacillo, Streptococci, Prevotellae and Fusabacteria across different pH ranges, showing the predominance of lactobacilli and steptococci at lower pH ranges [70].

The final pH reached by streptococci bacteria in plaque after stimulation by glucose has also been studied [72], showing that the increase in acid production by streptococci is able to decrease the pH levels from neutral 7.0 at regular saliva buffered conditions to pH as low as 4.0 just after 60mins. Thereafter, the pH levels remained consistent and does not drop drastically and could possibly rise back to buffered levels due to production and refreshed saliva introduced in the oral environments. However, this increase in acidity due to glucose stimulation is a cause of gradual enamel degradation due to fermentable carbohydrates being a part of modern diets, as well as decreased saliva production levels during periods of rest and sleep [73].

Subject	Lower Anterior Area					Upper Posterior Area				
	pH*			% of Total Plaque Flora (Mean)		pH*			% of Total Plaque Flora (Mean)	
	Δ pH					Δ pH				
	0-10 Min	(0-10 Min)	Final	MS++	TS++	0-10 Min	(0-10 Min)	Final	MS	TS
1	6.87-5.48	1.39	4.89	0.01	10.9	6.50-4.61	1.89	4.36	2.30	45.1
2	6.10-5.38	0.72	4.71	0.30	27.3	6.58-4.94	1.64	4.30	5.80	13.2
3	6.88-4.92	1.96	4.35	0.02	73.8	6.23-4.34	1.89	4.00	11.80	50.0
4	6.93-5.60	1.33	4.96	0.003	33.3	6.47-4.44	2.03	4.18	0.05	26.7
5	7.03-6.10	0.93	4.96	0.009	50.0	7.19-4.90	2.29	4.37	0.24	16.7
6	6.19-5.00	1.19	4.31	0.01	63.8	6.38-4.40	1.98	4.06	0.90	68.8
7	6.84-4.92	1.92	4.69	0.002	21.9	6.02-4.65	1.37	4.26	0.90	74.0
8	6.67-5.20	1.47	4.61	0.003	40.0	6.10-4.44	1.66	4.16	0.40	31.4
9+	7.06-6.36	0.70	5.01	ND	83.3	6.92-5.67	1.25	4.83	ND	61.1
10+	6.87-4.98	1.89	4.61	0.0001	16.7	6.74-5.07	1.67	4.48	0.03	21.9
11+	7.02-6.11	0.91	5.20	ND	34.1	6.42-4.74	1.68	4.28	0.003	44.2

Table 2: pH lowering potential of mutans streptococci on enamel after glucose stimulation after 10mins and 60mins [72]

2.5.2 Enamel demineralisation by dietary acids

Another major factor of HA demineralisation in dentine enamel is through the acids in the dietary foods that people consume. In a review study to investigate the correlation between sugar consumption and development of dental caries, the arrived conclusion was that sugars in foods is an important factor in determining its cariogenicity, and both the quantity and frequency of consumption of sugars is strongly related to caries in humans [74].

One source of concern is sugary beverages that are widely consumed by the developed world in alarming quantities. These drinks are extremely high in sugar and carbohydrate content, as well as being highly acidic due to the presence of citric, phosphoric, malic and lactic acids as flavouring or stabilizers in carbonated drinks [75]. These drinks have pH values well below the

accepted 'critical pH' in which HA demineralisation from the precipitation of calcium and phosphate out from the HA structure in a desaturated state will start to occur.

While the exposure duration of such sugary and acidic beverages should not be significant as compared to exposure to plaque and the acid generated by bacteria, the enamel erosion studies done on acidic beverages shows that they are still a significant cause of erosion due to their lower pH, with certain popular drinks reaching 2.50 pH [76], and also due to the glucose stimulating plaque bacteria activity.

The same study also showed that short exposure periods to such acidic sugary beverages around roughly 15 mins per day could result in 20um of erosion in enamel depth in as little as a week, or 40um after a month. For people with existing conditions of hyposalivation, the lack of saliva production in the oral means the lack of a buffer system to regulate the oral pH and neutralize the excessive acids left onto the dentine by consumption of such drinks [16, 77]. The constant exposure to such low pH without an inherent buffer from saliva could erode as much as 10um in as short as 1 hr.

Figure 9: Erosion depth of enamel by soft drinks over time. The pH values of the soft drinks were A) 2.42, B) 3.33, C) 3.46, D) 3.03, respectively [78].

2.5.3 Opportunity for long sustaining liposomes in dental care

With the discussion of issues present in dental care in the formation of dental caries through erosion and demineralisation of HA by plaque bacteria and sugary acidic beverages and foods in the modern human diet, it presents an opportunity for liposomes to come in as an innovation and improve dental care standards and potentially protect and prevent the erosion of HA on enamel through the mentioned causes.

With pH being the main indicator of demineralisation in dental HA, an alkali or basic substance that is encapsulated in liposome can act as a pH regulator in oral environment to further augment the buffering capabilities of natural saliva. Such an alkali should be able to increase the pH of the oral system while not bringing the pH into the alkali range in case of excessive content release from the liposomes. As such, strong alkalis, while able to mitigate the pH drops

causes by acids easily, could potentially set the oral pH into the alkali range and result in discomfort which would offset the benefits that such an application would potentially have due to a bad user experience. Buffering alkalis and bases would be a more suitable class of chemicals to use due to the ability to neutralize acids while keeping the pH within a stable range even if release in excess.

Despite the wide range of liposomes that can be selectively activated by stimulation, the liposomes triggered by light, ultrasound, enzymes, and redox reactions are not feasible for use in the oral environment. The exogenous triggers of light and ultrasound are not feasible due to not being readily available as well as simply being expensive and impractical equipment for daily use by the common human. Enzyme and redox triggered liposomes would also not be suitable due to the lack of bioavailability of the enzymes in the oral system, or the redox reactions covered by literature. The reasonable consideration for use would be pH activated and heat activated thermosensitive liposomes.

Both heat and pH triggered liposome designs would make great candidates for dental care applications regarding pH control and regulation to prevent HA demineralisation in enamel. The potential advantages that a pH triggered liposome mechanism will have over a heat triggered liposome would be:

- Capability to design the liposome to activate its release at specific pH thresholds. Modification using different pH-sensitive copolymers allow for liposomes to be designed to be released when the pH of the oral system or localized site (such as at plaque sites) drops below the critical pH of 5.5 for HA demineralization.

- Higher efficiency for content release. Since the liposomes are only triggered when the critical pH is breached, the contents encapsulated inside the liposomes are conserved and not wasted during times when buffering is not required.

While some disadvantages that a pH triggered liposome would have:

- Narrow scope of application in dental care apart of pH regulation and buffering. Due to the pH sensitivity of the liposome, it would be difficult to employ for uses of drug delivery for oral care in the absence of a spike of pH levels. Examples of such applications would be enamel remineralization or treatment of gum infections.
- Most pH sensitive liposome in literature become unstable near the critical designed pH, which leads to the release of encapsulated contents. At pH significantly lower than the trigger pH, pH sensitive liposomes tend to undergo total collapse of the capsule spherical structure, leading to a ‘burst’ release of contents. Such a collapse would result in a one-off activation and drastic regulation of the pH in the oral system simply due to consumption of a highly acidic beverage and render the pH regulation to be non-existent consequently.

Heat triggered liposomes used to encapsulate buffering contents for dental care is a unique situation where the conventional methods of exothermia to trigger the release of the liposomes render the application to extreme ineffectiveness but will be strongly considered for this study. The stimuli methods for exothermia covered in the previous sections of this literature review involves unfeasible methods previously mentioned such as ultrasounds, infrared and UV light sources. The ‘burst’ release triggered by these methods will also result in the same disadvantages faced by pH sensitive liposomes where they are rendered ineffective in regulating oral pH after one short bout of buffering.

Instead of relying on excess methods of exothermia to activate the release of liposome contents through heating beyond normal human body temperatures as per ‘conventional’ heat triggered liposomes, an innovative approach would be to use endogenous heat to activate the release of liposome contents at a very slow release rate relative to ‘burst’ release models. Such a slow-releasing heat trigger liposome is actually reminiscing of earlier conventional liposomes where the outer shell was less stable and susceptible to leakage in the physiological system as well as endocytosis into various tissues where drug release was not intended. Such conventional liposomes eventually were improved with new methods of modification of polyethylene glycol ligands to create stealth liposomes as well as improved stability for long circulation within the human biology to accumulate at the desired sites.

The difference of exploiting leakage of thermosensitive liposomes at endogenous heat to trigger slow-releasing buffers in the oral environment versus the mentioned earlier forms of conventional liposomes is the encapsulated contents as well as the physiological environment that it is released in. Conventional liposomes were first explored as drug delivery systems to delivery drugs in-vivo that were highly toxic to healthy cells, and leakage is undesired.

However, in the case of a long releasing liposome in the oral system, a buffer can be non-toxic, and leakage is intended and desired, especially so if the total leakage period is prolonged as it will increase the pH regulatory buffer period that the liposome provides. A lack of an exothermic source also means that the leakage is generated through a small percentage of the lipids phase transition to gel-like state from endogenous heat, causing mild disruption of the liposome shell structure. This leakage mechanism and profile can be consistently reproduced across the nominal human genus, and further improvements to the liposome design and

controlled and sustained release of other substances for various applications at human body temperature.

2.6: Enamel remineralisation in literature

Just as how hydroxyapatite in enamel can be demineralized in an environment of low pH (<5.5) and desaturation of calcium and phosphate ions, remineralization of hydroxyapatite on enamel is also possible in the reverse scenarios of moderate physiological pH environment with an abundance of calcium and phosphate ions. In a natural setting without interjections from technology and healthcare, saliva serves as evolution's protection against dental caries by buffering the oral pH and providing a high concentration of calcium and phosphates for the precipitation of hydroxyapatite onto enamel.

With the advancement of dental research and healthcare, decades have been spent studying and researching this process of tooth remineralisation, leading to the development of technologies that can promote enamel remineralisation, augmenting our own biological repair systems.

One of the most common and dated methods of dental care is the use of fluoride through fluoride containing toothpastes. Fluoride is able to protect the enamel through remineralising hydroxyapatite in the enamel by inducing the formation of fluorapatite or fluoridated hydroxyapatite through substitution reaction with HAP directly or promote the transformation of other calcium phosphate phases to fluorapatite [79]. This fluorapatite formation reduces the acid solubility of hydroxyapatite, reducing the demineralization of enamel by acids, as well as removal of acid producing plaque by abrasive polishing of the enamel surface. However, fluoridated remineralization systems face negative side effects or drawbacks such as the

formation of fluoride induced occult caries [80], diminished remineralization effects and potential chemical neuroticization at higher fluoride concentrations [81].

Calcium and phosphate systems are also used in a similar manner to fluoride systems, to provide mineral reservoirs to promote natural remineralization of mineral apatite through systemic saturation of calcium and phosphate ions. Increased calcium and phosphate concentration in saliva and the oral cavity has been widely reported to reduce the solubility of enamel and also increase the driving forces for remineralisation providing strong evidence that caries incidence is inversely correlated to the concentration of these minerals [82-84]. Along with the absence of side effects seen in higher fluoride concentrations, calcium and phosphate are commonly used as building blocks in many other remineralization literatures.

One other area of such research involving calcium and phosphates as building blocks are biomimetic systems. Using bioactive materials that simulates a natural physiological response to initiate formation of hydroxyapatite [85] from calcium and phosphate similar to natural healing and grafting of bone tissues, artificial remineralization of hydroxyapatite in the oral system can also be stimulated in a similar fashion. Bioactive materials such as phosphoprotein peptides[86] and amelogenin peptides[18, 87] are able to capture calcium, phosphate or even fluoride ions to form new mineral deposits on demineralized enamel. With the peptides as a seed attached to demineralized enamel sites, precipitates of small apatite form in an abundance of mineral ions to achieve remineralization of apatites with improved properties such as increased toughness, abrasion resistance, and reduced porosity and roughness.

2.7: Calcium and phosphate loaded liposomes in enamel remineralisation

There are some studies of calcium phosphate saturation in such remineralization studies utilizing thermosensitive liposomes as a vesicle to provide reservoirs of calcium and phosphate ions. In 2000, Murphy et al [88] used thermosensitive liposomes that comprised of 9:1 DPPC/DMPC to load calcium and phosphate ions separately. These calcium and phosphate containing vesicles were heated to 37 °C to induce a burst release of contents and a saturation of calcium and phosphates caused calcium phosphate to precipitate as marked by a decrease in the system pH as a result of the base formation. This formation of calcium phosphate was further experimented on heated dentine and enamel slabs, which successfully repeated the process of forming calcium phosphate minerals from the vesicle released ions. The experiment process was conducted over 20mins, but the release of contents from the liposomes were concluded in approximately 5 mins as no further pH drop from the mineralization process was observed by the researchers.

Figure 10: pH vs. time of liposome suspensions isothermally held at 30, 34, and 37 °C. The decrease in pH indicated the release of calcium and phosphate contents from the liposomes and formation of calcium phosphate mineral [88].

In another similar research published in 2012, Luo et al improved on the research by using an amelogenin-inspired peptide in addition to calcium and phosphate loaded thermosensitive liposomes to seed and control the precipitation of calcium phosphate to grow oriented and evenly distributed enamel-like hydroxyapatite onto enamel surface[18]. In the release and precipitation experiments, the mixture of peptide and liposomes solution was exposed to enamel surface at 37 °C in oven for two weeks. The end result of apatite precipitates was observed by SEM to be highly oriented and crystalline, and much more similar to enamel than the previous research by Murphy et al.

Figure 11: A) Enamel etched with 37% phosphoric acid only; b) etched enamel surface treated with liposomes suspension; c) etched enamel surface treated with liposome and polyanion peptide. Figure C shows highly crystalline and oriented apatite growth when calcium and phosphates are release for long durations (2 weeks) with the incorporation of peptide seeds. This is a stark improvement over

the calcium phosphate precipitation by Murphy et al which was formed over a short duration in figure 12.

Figure 12: Acid etched human dentin surfaces following treatment with liposome suspension for 20mins at 37 °C.

However, both researches did not characterise and analyse the relationship between the release rate of the calcium and phosphate ions from the liposomes and the growth rate of the apatite crystals. While the research by Luo et al yielded promising results, the release period of 2 weeks is not practical in situ as salivation will readily remove the state of saturation provided by the liposome release and does not provide any insight as to what was the effective period that the liposome release resulted in hydroxyapatite formation.

To further improve the research on thermosensitive liposomes on the remineralization of hydroxyapatite on enamel and bone structures, the understanding of a few additional critical aspects is required:

- 1) The growth rate of hydroxyapatite and its correlation to the concentration of calcium and phosphate ions in the surroundings.
- 2) Whether a constant supply of mineral ions at an optimal concentration, or a saturated but finite reservoir of mineral ions is more conducive for the growth of hydroxyapatite.
- 3) Optimization of liposome lipid composition to optimize and maximize the release efficiency of mineral ions from the thermosensitive liposomes.

Chapter 3: Liposomal release

3.1: Experimental procedures

3.1.1: Synthesis and characterization of Tris-encapsulating liposomes

DPPC/SA/PEG-DSPE (9/1/0.06, molar ratio) were dissolved in *t*-butyl alcohol (5g lipid/100 mL alcohol) to prepare the liposomes. This solution was then freeze-dried to obtain a lipid powder mixture. The lipid mixture was hydrated in 4.1 M Tris deionized (DI) water solution (200 mg lipid in 10 mL Tris solution). Tris was used as the cargo due to its properties to act as both an acid neutralizer and buffering agent to prevent excessive pH increases in the alkaline region.

The mixture was dispersed using a vortex mixer machine (Genie 2, Vortex Mixer, Scientific Industries), and then extruded through an extruder (LIPEX extruder 10mL, Evonik, Canada) heated at 55 °C. The mixture was extruded 4 times through membrane filters (Durapore, Millipore Corp) fitted in the extruder using high pressure nitrogen gas (first with 0.45 µm pore size, and subsequently 3 times with 0.22 µm pore size, with a membrane filter change between each pass).

Non-encapsulated Tris was removed by ultracentrifuge at 100k RPM for 1hr (Optima MAX-XP Ultracentrifuge, Beckman Coulter) of the mixture, and removing the supernatant, and this process is repeated 3 times.

3.1.2 Characterization of liposome size by Dynamic Light Scattering (DLS) and Transmission Electron Microscopy (TEM)

The final Tris-encapsulating liposomes (Tris-Lipo) were re-dispersed in DI water and the size distribution of the liposomes were analysed by dynamic light scattering (Zeta-Sizer Nano ZS, Malvern Instruments). For transmission electron microscopy (TEM), one drop of the liposome dispersion was dropped onto a carbon-coated copper grid, then the excess solution was removed using filter paper. One drop of an aqueous solution of sodium phosphotungstate (2 wt%, pH 7.0) was then dropped on the grid and the excess solution was removed using filter paper. The grid was observed using TEM (JEM 2010, JOEL)

3.1.3: Liposome Encapsulation efficiency of Tris solution

The theoretical entrapment capacity of Tris-Lipo was estimated based on geometrical calculations, as described below.

The encapsulation efficiency is defined as

$$E_{efficiency} = \frac{Total\ encapsulated\ volume}{Total\ volume}$$

The inner aqueous volume (V) of the Tris-Lipo (outer diameter, $Z_{avg.d}$ nm; bilayer thickness, r nm) was calculated as

$$V_{Lipo} = \frac{4}{3} \pi \left(\frac{Z_{avg.d}}{2} - r \right)^3$$

The number of the Tris-Lipo (N) in the extruded solution was calculated as lipids in the bilayer membrane (N) was calculated as

$$N = \frac{A_{lipids}}{A_{Lipo}}$$

where A_{Lipo} is surface area of Tris-Lipo calculated as

$$A_{Lipo} = 4\pi\left(\frac{Z_{avg.d}}{2}\right)^2 + 4\pi\left(\frac{Z_{avg.d}}{2} - r\right)^2$$

and A_{lipids} is the summation of total average area of membrane where A is Avogadro's number and M_w is the average molecular weight of membrane lipids.

The calculation was performed for vesicles of DPPC/SA/PEG-DSPE (9/1/0.06, molar ratio, average $M_w = 689$) with 193.8 nm (based on DLS analysis of Tris-Calcein lipo synthesised for the measurement of encapsulation efficiency test) for the outer diameter. The bilayer thickness was estimated as 5 nm ($r = 5$) based on photographic analysis of the TEM imaging and average area of DPPC membrane lipid is 0.4 nm^2 [89]. The distance between bilayers was assumed as 0 in this calculation.

In the sample calculation below for the encapsulation efficiency test in the manuscript, 100 mg of DPPC/SA/PEG-DSPE lipid was solubilized in 5 ml of Tris-Calcein.

1. $A_{lipids} = \frac{100mg}{689gmol^{-1}} \times A \times 0.4nm^2 = 3.495 * 10^{19} nm^2$
2. $A_{Lipo} = 4\pi\left(\frac{193.8nm}{2}\right)^2 + 4\pi\left(\frac{193.8nm}{2} - 5nm\right)^2 = 2.241 * 10^5 nm^2$
3. $N = \frac{A_{lipids}}{A_{Lipo}} = 1.56 * 10^{14}$
4. $V_{Lipo} = \frac{4}{3} \pi\left(\frac{193.8nm}{2} - 5\right)^3 = 3.251 * 10^6 nm^3$
5. $Total \text{ encapsulated volume} = N * V_{Lipo} = 5.071 * 10^{20} nm^3$
6. $E_{efficiency} = \frac{5.071*10^{20} nm^3}{5 cm^3} * 100\% = 10.14\%$

The actual encapsulation efficiency of Tris solution by liposomes is measured using the equation:

$$\frac{C_{wash}}{C_{extrude}} \times 100\%$$

Where C_{wash} is the concentration of calcein in the final washed state of Tris-Lipo and $C_{extrude}$ is the concentration of calcein in the final extruded state of Tris-Lipo. The volume of the final washed Tris-Lipo is identical to the volume of the final extruded volume of Tris-Lipo.

3.1.4: Release of payload from Tris-Lipo

The release of payload from the Tris-Lipo was characterized with calcein as a fluorescent tracer. The lipid mixture was hydrated in 4.1 M Tris solution containing 1.75 mM calcein. The mixture was dispersed using the vortex mixer, extruded through the extruder, and then washed with ultracentrifuge under the same conditions as described in preparation of Tris-Lipo. The obtained liposome dispersion was incubated at 25 °C or 36.5 °C to test the release characteristic of the liposomes. At specific sampling times 30 min apart, samples of the experiment solution were drawn out and ultracentrifuged (100k rpm, 1 h) to separate the supernatant and liposomes. The supernatant was added to pH 7.4 PBS in a 1:1 volume ratio. The release profile of the liposomes was characterized by measuring the fluorescence intensity of the calcein in the supernatant using a multi-mode microplate reader (Spark 10M, Tecan). The release profile was modelled using the equation:

$$\frac{(C_x - C_i)}{(C_f - C_i)} \times 100\%$$

Where C_i is the concentration of calcein in the supernatant before the commencement of incubation, C_f is the final concentration of calcein in the supernatant after solubilizing the liposomes with the detergent (octyl β -D-glucopyranoside), and C_x is the sample taken at specific sampling times.

3.1.5: Preparation of hydroxyapatite samples

Hydroxyapatite (HA) discs were used instead of extracted dentine due to the consistency of a manufactured HA structure and close approximation to actual enamel composition and formation path of dental caries [90, 91]. The HA discs were sonicated in DI water for 10 min in a bath-type sonicator (E70H Elmasonic, Elma) to remove dirt or loose HA structure. The treated HA discs were then air blown and dried in an oven at 50 °C for 1 h. A tape with 5 mm width was applied onto the HA discs as a mask, and acid resistant nail varnish was applied over the unmasked areas of the discs. After 24 h, the tape was removed, leaving a 5 mm width window as the test area.

3.1.6: *in vitro* Tris-Lipo release in lactic acid environment

DI water (50 mL) was prepared in a glass beaker and left in the 36.5 °C thermostatic water bath to simulate an artificial mouth system [92, 93]. A pH electrode and temperature probe from a data-logging pH meter (PHH-SD1, Omega) was inserted into the beaker to monitor and log temperature and pH changes. Parafilm was used to cover the top of the beaker. When the temperature of the solution has reached 36.5 °C in equilibrium with the environment, the pH of the environment is adjusted to 4.0 with 1 M lactic acid solution [70].

HA samples were added into the lactic acid environment and data logging commenced. The solution was gently agitated with a dropper pipette every 1 h to remove air bubbles on the HA samples, and the experiment was conducted for 7 h. At the end of the 7 h, the HA samples were rinsed in DI water and stored in pH 7.4 PBS until subsequent use. Similar procedures were used to prepare the lactic acid environment with Tris-Lipo. DI water (48 mL) was prepared in a glass beaker and left in the thermostatic water bath to reach 36.5 °C. Tris-Lipo dispersion (2 mL) was added to make up 50 mL of the lactic acid/ Tris-Lipo environment.

Final pH adjustments were made to adjust the solution pH to 4.0 with 1 M lactic acid solution before the HA samples are added into the test solution. The experiment was then conducted similarly to the lactic acid environment variation. Both experiments were repeated 5 times to obtain 5 data sets, with the results averaged and shown with SD (standard deviation).

This *in vitro* experimental setup was chosen as it is more suitable to reflect the static state of inactivity, such as periods of sleep, during which majority of dental caries form due to the lack of buffering from natural saliva [73]. The static model of Tris-Lipo release over 7 hours to mimic the period of sleep is thus more suitable to accurately study and document the release characteristics of Tris-Lipo in reaction to differences in environmental temperature and pH [94].

3.1.7: Characterization of corrosion and demineralisation volume on hydroxyapatite samples

After initial masking of the HA samples, the varnish was removed by ethanol and soft tissue, and the 5 mm test window was first characterised by 3D volumetric profilometry [95] with a confocal laser microscope (Keyence, VK-X1000, Japan). A reference plane was selected and initial profilometry was done to obtain the reference volumetric data. Volume analysis was done 2 more times, once after the HA samples were re-masked and experimented in the *in vitro* lactic acid solution to obtain the volumetric data for corrosion; and once more after the demineralised HA was removed from the samples by sonication in DI water for 1 min to analyse demineralisation.

3.1.8: Statistical analysis

The differences in erosion and demineralised volume of the HA samples in the acid-only tests and Tris-Lipo treated tests were analysed using the Welch's unequal variances t-test with the significance level was set at $\alpha = 0.05$. All statistical analyses were conducted using Excel (Office365, Microsoft).

3.1.9: Synthesis of mixed lipid liposomes for release optimization

Phospholipids DPPC, DSPC, and DMPC were mixed at various percentages to form 10 lipid compositions in Table 3 using mixture design principals for the purpose of optimizing release durations at a constant endogenous temperature. The lipids were mixed in the molar ratios at the mentioned percentages to form 90 mg of lipid mixtures. The lipid mixtures were added in 1ml of t-butyl alcohol (90 mg/1 ml) and heated to 60 °C to dissolve the lipids to obtain a clear homogenous solution. The lipid-t-butyl alcohol solutions were subsequently freeze dried at -90 °C using a freeze dryer (Alpha 2-4 LSC, Martin Christ, Germany) for 8 hrs to obtain the

final dry-phase lipids. The lipids were mixed with 4.5 ml of 16 mMol Copper Sulfate pentahydrate (Sigma Aldrich) and synthesized with the usual extrusion method as previously mentioned in (3.1.1).

Lipid	Composition (total 100%)									
	1	2	3	4	5	6	7	8	9	10
DPPC	100	-	-	33	33	33	66	66	-	-
DMPC	-	100	-	33	66	-	33	-	33	66
DSPC	-	-	100	33	-	66	-	33	66	33

Table 3: Lipid compositions for mixture design optimization

3.1.10: In-situ release characterization with UV-Vis spectroscopy

Using a UV-Vis thermoelectrically Temperature-Controlled Cell Positioner (CPS-100, Shimadzu, Tokyo Japan), characterization of the liposomes' release profile is analysed *in-situ*. 0.5g/L polyethyleneimine (800 Mw, Sigma Aldrich, USA) is prepared by dissolving polyethyleneimine (PEI) in DI water, and 3ml of the PEI solution is heated to 37 °C and added to 10 mm path length synthetic quartz glass cuvettes. The glass cuvettes are inserted into the thermal controlled cell positioner that is calibrated and pre-set at 37 °C. 300 µl of liposome solution is added to the cuvette right before the commencement of the release experiment, and the cuvette is capped and covered with a foam insulator.

The UV-Vis spectroscopy is then programmed to measure the absorbance of the PEI-copper sulfate liposome solution every 15 mins for a total duration of 7 hrs. The measured spectrum is from 250 nm to 350 nm, and the values at 285 nm are taken to characterize the formation of Cu-PEI complex which results from the complexation of released copper ions from the copper sulfate liposomes by the surrounding PEI.

3.2: Results and discussion

3.2.1: Effects of temperature on payload release rate of Tris-Lipo and pH change

The average diameter of the prepared Tris-Lipo was determined to be 193.8 nm with a polydispersity index of 0.096 by DLS (Figure 9). Such a low PDI indicates that the liposomes were predominantly homogenous in size around 190 – 200 nm in diameter each without significant variation.

Figure 13: The size distribution of prepared Tris-Lipo measured by dynamic light scattering (DLS).

Observation of the phosphotungstic acid stained liposomes under TEM also supported the formation of liposomes with 200 nm in diameter (Figure 10)

Figure 14: TEM image of Tris-Lipo negatively stained by phosphotungstic acid.

The encapsulation efficiency of Tris in Tris-Lipo is measured to be 8.9%, in close approximation to the theoretical calculated encapsulated efficiency of 10.14% in the methodology section. Previous studies have shown that a larger osmolality gap between inside and outside of the liposomes causes a minor release of cargo below the phase transition temperature of the liposomes (40 °C) [21].

The Tris-Lipo were prepared in 4.1 M Tris solution which has significantly higher osmolality than that of normal human saliva (less than 150 mOsm/L). Therefore they were expected to have a slow and sustained release of Tris in oral cavity even at temperatures lower than endogenous heat. The Tris-Lipo were incubated in thermostatically controlled water baths set at 36.5 °C and 25 °C for 7 hrs to investigate the release profile of the Tris-Lipo and the its effect on an acid environment of pH 4.

In numerous studies of causes of dentine caries formation as discussed in the literature review, it has been found that an acid environment below a critical pH threshold of 5.5 effectively triggers the demineralisation and erosion of the dentine and the hydroxyapatite structure [66, 67, 78].

At 36.5 °C, the Tris-Lipo released its contents at a higher percentage and higher initial release rate than the Tris-Lipo incubated at 25 °C (Figure 11A). Cargo released peaked at 18 % when the Tris-lipo was incubated at 36.5 °C compared to 12% under 25 °C incubation. The differences of 6% resulted in significant effect on pH regulation capability as demonstrated in Figure 11B. Within 1.5 h of incubation at 36.5 °C, Tris-Lipo brought up the environment pH above the dentine erosion critical pH of 5.5 and proved its effectiveness in counteracting the acid erosion by lactobacilli in human oral environments. It has been discussed and shown that acidification of teeth by lactobacilli under glucose stimulation brings pH down to around 4.5 in 1 h. Tris-Lipo is shown to be able to counteract this acidification within a similar timeframe [72]. This will be further investigated in profilometry analysis of the HA samples before and after the acidification experiments.

The Tris-Lipo incubated at 36.5 °C or 25 °C both showed similar release profiles at first glance. However, the Tris-Lipo release in 36.5 °C environment peaks at 2 h of incubation while the cargo release from Tris-Lipo at 25 °C only reached its peak release at 3.5 h. This is likely due to the decreased permeability at a lower temperature. This suggests that the temperature of the environment is not the only factor in triggering the release rate of the contents in Tris-Lipo. The difference in pH gradient between the inner and outer wall of the lipid bilayer membrane could be a contributing factor as well. The decreasing rate of pH increase in 36.5 °C supports that the cessation of content release of Tris-

Lipo after 2 h of incubations, suggesting an osmotic equilibrium could be reached with a decrease in pH gradient across the lipid bilayer membrane.

The continued but gradual increase of pH despite the lack of Tris buffer release by the liposome suggests that a reverse osmotic flow of acids into the concentrated alkali buffer content of the liposome is possible. This mechanism could potentially explain why the pH of the acid environment *in vitro* is increasing even though there is no alkali buffer release. It could also explain why the gradient of pH increase in 36.5 °C, where the initial 2.5 h release phase of DPPC brought the environment pH above 5.5 is less steep than in 25 °C, where the pH of environment is still under 5.5 after the initial release phase and relatively more acidic. This effect of pH would be touched upon in w subsequent experiment with reacidification in the middle of the 7hr run.

Figure 15: Profiles of (A) payload release and (B) pH change during incubation of Tris-Lipo dispersion at 36.5 °C or 25 °C through a time period of 7 h. At an average temperature of 36.4 ± 0.08 °C, the pH increases from 4 to 5.53 ± 0.27 in 1.5 h. While an average temperature of 25 ± 0.09 °C increases the pH from 4 to 5 ± 0.07 in 6 h.

3.2.2: Effect of pH on payload release rate of Tris-Lipo and pH change

To evaluate the hypothesis that the cargo release of Tris-lipo is dependent on the pH gradient difference across the lipid bilayer membrane, a reacidification experiment was conducted where the acid environment was refreshed with lactic acid halfway (3.5 h) through the experiment.

DI water (48 mL) was prepared in a glass beaker and left in the 36.5°C thermostatic water bath. A pH electrode and temperature probe from a data-logging pH meter was inserted into the

beaker to monitor and log temperature and pH changes. Parafilm was used to cover the top of the beaker. When the temperature of the solution has reached 36.5°C in equilibrium with the environment, the pH of the environment is adjusted to 4.0 with 1 M lactic acid solution. Tris-Lipo dispersion (2 mL) was added to make up 50 mL of the lactic acid/ Tris-Lipo environment. Final pH adjustments were made to adjust the solution pH to 4.0 with 1 M lactic acid solution. No HA samples were added to the solution, and samplings were taken at 30min intervals as per the cargo release characterization procedures to determine the cargo release.

After the sampling at 3.5 h, 20 μ L of 1M lactic acid is added to the solution and lightly stirred to bring the pH down to 4.3. Afterwards, the experiment is conducted as per usual procedures for the subsequent 2 h and terminated at 5.5 h.

Figure 16: Profiles of (A) payload release and (B) pH change during incubation of Tris-Lipo dispersion at 36.5 °C. Lactic acid was added to the dispersion immediately after sampling at 3.5 h.

In the reacidification experiment, the cargo release rate from Tris-Lipo was hovering at 11%, and environment pH has increased to above 6.0 after 1 h of sustained release. These results were consistent and characteristic with the findings from the pH and Tris-lipo release data from the cargo release characterization and *in vitro* acid erosion experiments up until the 3.5 h point, when 20 μ L of 1M lactic acid was added into the *in vitro* environment.

The reintroduction of acid reduces the environment pH to 4.3, back in the dentine erosion range. The cargo release sampling reveals that the flat trend of 11% release is disrupted by this reacidification as significant amount of cargo is released over the next 2 h to 15.5%. There is also a corresponding increase in pH caused by the Tris cargo release from 4.3 to 4.9.

The result in the reacidification experiment verified that the Tris-Lipo is not only reactive to the balance of osmotic pressure caused by the differences in pKa strength between cargo and environment. It is also strong evidence to show that sustained release over a longer period than 2.5 h is possible. This observation of pH-triggered cargo release in the thermosensitive Tris-Lipo suggests that the initial cargo release of 7% during the first 30 min of acid erosion experiments in 25 °C environment is due to the initial acid stimulus rather than to the effects of temperature and osmotic pressure. This result supports the hypothesis that a sustained release liposomal carrier can be tailored to react to environmental triggers caused by a target phenomenon like pH changes due to infection or cellular mutation [15].

3.2.3: Quantitative analysis of acid erosion in hydroxyapatite substrate (erosion and demineralisation)

To investigate the *in vitro* effects of Tris-Lipo on environment pH and subsequently, the erosion and demineralisation on the HA substrate, 3D profilometry will be to characterise 2 groups of 5 HA samples before and after immersion in the acid. After masking and preparation of the HA samples as per Figures 13A and 13B, the control group will be immersed into an acid environment of pH 4 without the addition of Tris-Lipo. This is to determine the effects of the Tris-Lipo on the demineralisation and erosion, while the test group is immersed in an acid environment of pH 4 with the introduction of Tris-Lipo to regulate the acidity of the environment. The resulting pH data collected was presented in Figure 14.

The results were similar to the pH profile from the analysis of Tris-Lipo release rates, showing that the behaviour of Tris-Lipo in a fixed environment is consistent and have little variance.

Figure 17: *In vitro* model to evaluate erosion of hydroxyapatite. (A) The hydroxyapatite samples are masked with a 5 mm tape and acid resistant nail varnish is applied at the sides to protect the reference area from corrosion. (B) Digital photograph of the masked hydroxyapatite substrate with 5mm exposed erosion window on the left, and the hydroxyapatite substrate as-in on the right.

The *in vitro* model to evaluate erosion of hydroxyapatite have samples are with a 5 mm tape and acid resistant nail varnish is applied at the sides to protect the reference area from corrosion. The 5 mm tape is then removed to expose a 5mm window on the substrate for the experiment. In the subsequent 3D volumetric analyse by confocal microscopy, the varnished area is used as reference diatom to analyse the corroded volume in the 5 mm exposed window.

Figure 18: Environment pH with and without Tris-Lipo introduction ($n = 5$, $N = 10$). With treatment of Tris-encapsulated liposome, the pH of the acid environment increases above 5.5 within 1.5 h while the pH of the sample without treatment remained below 4.5 throughout the test.

The physical characterization of the eroded exposed window of the HA samples by 3D confocal profilometry is done with a reference plane shown in Figure 13A. The 3D profilometry results show a significant reduction in both eroded volume (Figure 15A and 15B) and demineralization volume (Table 3) with the application of Tris-Lipo in the acid environment.

The average eroded volume of HA samples in the Tris-Lipo tests were significantly reduced compared to the untreated acid-only tests, with the eroded volume being only 6.1% ($p < 0.01$). In addition, by using ultrasonication to remove demineralised HA by micro-cavitation of water above the substrate surface and characterising the volumetric before and after sonication, we quantified the averaged demineralized volume of HA samples. The demineralized volume of Tris-Lipo tests was significantly lower, at 22.7% on average when compared to that of the HA samples in the acid-only tests ($p < 0.05$).

Sample	Reference volume (mm ³)	Volume after erosion (mm ³)	Eroded volume (mm ³)	Average eroded volume (mm ³)	Volume after sonication (mm ³)	Demineralised volume (mm ³)	Average demineralised volume (mm ³)
A1	0.179	0.319	0.14	0.187 ± 0.05	0.348	0.029	0.078 ± 0.04
A2	0.408	0.668	0.26		0.706	0.038	
A3	0.801	0.944	0.143		1.055	0.111	
A4	0.242	0.422	0.18		0.540	0.118	
A5	0.397	0.611	0.214		0.703	0.092	
L1	0.047	0.053	0.006	0.011 ± 0.01	0.059	0.006	0.018 ± 0.01
L2	0.045	0.048	0.003		0.058	0.01	
L3	0.496	0.506	0.01		0.543	0.037	
L4	0.089	0.09	0.001		0.098	0.008	
L5	0.132	0.169	0.037		0.196	0.027	

Table 4: Erosion data of Hydroxyapatite samples after 5 days of lactic acid immersion in-vitro

An interesting point to note is that the demineralised volume of the Tris-Lipo-treated HA is higher than its eroded volume, in contrast to the acid-only HA, where the eroded volume is more than twice as high as the demineralised volume. This observation supports the study by Eisenburger *et al* that demineralisation of the dentine is a precursor to erosion [96]. This suggests that the effects of demineralisation negation by Tris-Lipo lead to the reduction of acid erosion Tris-Lipo treated HA samples.

With the findings in this study, it is clear that Tris-Lipo is able to reduce demineralisation and acid erosion of HA structures through prolonged alkali release to neutralize an acidic environment. When the surface of liposome is functionalized with targeting moieties such as tetracycline and alendronate [97-99], Tris-Lipo has a high potential to be applied *in vivo* as an effective liposomal nanotherapeutic for the prevention of dental caries formation. More importantly, the sustained release model of Tris-Lipo under endogenous body heat showcases that a slow, stable, and prolonged release of drug cargo is possible, with the possibility of regulatory reactive release towards triggers such as environmental pH.

Figure 19: *In vitro* erosion test using hydroxyapatite substrate.

(A) Confocal laser images of the surface morphology of hydroxyapatite sample in lactic acid-only environment without Tris-Lipo treatment. Significant difference in step height between the masked and exposed area was the result of acidic erosion.

(B) Confocal laser images of the surface morphology of hydroxyapatite sample in lactic acid environment treated by Tris-Lipo. Minor difference in step height between the boundary of the masked and exposed area suggested acidic erosion was prevented.

Figure 20: Average eroded area calculated from the profilometry analysis data of the confocal scan ($n=5$) and average demineralised volume calculated from the profilometry analysis data of the confocal scan ($n=5$).

* Statistical significance ($p < 0.05$),

** Statistical significance ($p < 0.01$).

All statistical analyses were performed using Welch's unequal variances *t*-test.

3.2.4: Release profiles of copper-containing mixture-designed liposomes.

To further improve the constant release characteristics of thermosensitive liposomes for a prolonged period, as well as study of its release characteristics without the influence and variability of intravesical and extravascular osmotic balance, an optimization of thermosensitive lipids and their release characteristics is performed.

By employing mixture design optimization theory, 3 phospholipids 1,2-dihexadecanoyl-sn-glycero-3-phosphocholine (DPPC, 16 PC), 1,2-dimyristoyl-sn-glycero-3-phosphocholine (DMPC, 14 PC), and 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC, 18PC) are mixed. The steps to synthesize it into a single phase, dual phase or triple phase lipid composition (Figure 21) through the freeze-drying method described in section 3.1.19.

Figure 21: Mixture design methodology implemented to obtain 10 lipid compositions from 3 different lipids. The 10 mixtures can then be analysed and optimized through surface response methodology to obtain an ideal lipid composition.

The in-situ release profiles of the liposome compositions are characterized by UV-Vis with a constant temperature control spectrophotometer accessory to keep the samples at a constant temperature of 37.0 °C. The resulting release profiles are shown on Figures 22. By focusing initially on the liposomes consisting purely of the 3 main lipids DPPC, DSPC and DMPC, the resulting release profiles are expected and characteristic of their physical properties and permeability under endogenous heat. 100% 14 PC liposome, with its relatively low phase transition of 24 °C, is in a liquid-like gel state at the pre-set environment of 37°C. Therefore it has the fastest release rate of all the compositions, releasing 100% of its contents within 120 mins. 100% 18 PC however, is on the other end of the spectrum with its higher phase transition temperature at 55.5 °C. Due to the T_c being significantly higher than the environment temperature of 37 °C, it remains in an ordered crystalline state and does not allow for inter vesicle permeation of payload towards the environment. The analysis shows 0% release rate over the entire 7 hrs of in-situ characterization.

100% 16 PC, which has a T_c of 41 °C, is phased into a liquid-like gel state to a certain extent and the copper ion payload is able to permeate outward. However, an important observation is that the release rate and profile is starkly different from 100% 14 PC liposome which has phased completely at 37 °C. 100% 16 PC only has a total release rate of 39% over 7 hrs compared to 100% 14 PC liposome's 100% release over 2 hrs, with a much lower release gradient comparatively.

The fact that 100% 16 PC lipid liposome is able to release its contents is indicative that the lipids have undergone phase transition, but to a much lesser extent than the total phase transition evident in 14 PC liposomes. This partial phase transition, if employed effectively, can be used to optimise liposome compositions for fixed temperatures to design release profiles.

Figure 22: Release profiles of the 10 liposome compositions formulated with DPPC (16 PC), DSPC (18 PC) and DMPC (14 PC) through mixture design methodology.

Figure 23: Release profiles of the liposomes with the 3 main lipids as well as an equal part composition liposome consisting of the 3 lipids.

By analysing the release profiles of the mixed liposomes, we are able to observe that the mixtures of different lipids with different T_c is able to alter the release profile to obtain a mixture of characteristics from each basic lipid. In Figure 24, we are able to analyse the release profile for liposomes with 18 PC as a component. With any modification of 100% 18 PC with a 14 PC or 16 PC lipid, the mixed liposome is able to release its contents through permeation through the bilayers of the lipid with lower T_c . While 18 PC acts as a structural core supporting the shorter chained phased lipids as it remains crystalline.

The difference in degree of permeability between 14 PC and 16 PC at endogenous temperature is once again apparent in the 14:18 and 16:18 mixture liposomes. 14:18 liposomes of either composition have significantly higher initial rate of release as compared to the 16:18 liposomes. They exceed 50% release in around 30 mins due to the total phase transition undergone by the 14 PC lipids, as compared to a graduated release of less than 10% in 30 mins due to the partial phase transition of the 16 PC lipids.

Figure 24: Release profiles of liposomes with DSPC (18PC) as either a main or secondary constituent.

In the analysis of 16 PC liposomes however, a result that is unexpected of the current findings can be observed. With the understanding that 16 PC lipids will be partially phase transitioned at endogenous temperature, while 18 PC lipids will remain crystalline, the presumption would be for 14:16/33:66 liposome to have a higher release rate and total release than 14:16:18/33:33:33 liposome. Which is due to the higher percentage of partially phased 16 PC lipids in the bilayer compared to the 33% crystalline 18 PC in 14:16:18/33:33:33 liposome. The rate of release for both liposomes are similar after the 45 min mark, but before which, 14:16:18/33:33:33 liposome released 5% more of its contents during the 15 to 45 mins mark.

The release profile of both liposomes from the 45 min mark to the end of the release experiment could draw the conclusion that the release mechanisms between the 2 liposomes are largely similar and likely to be significantly contributed by the permeability of 14 PC, and 16 PC to a

smaller extent. The additional 5% content release from 14:16:18/33:33:33 liposome could be due to the bigger difference in hydrocarbon chain lengths in the tail portion of the 14 PC and 18 PC lipids. The difference in molecular size of the lipid tails could have resulted in a disruption in the geometrical stability of the spherical liposome structure and resulted in additional leakage through the grain boundaries or faults between the 14 PC and 18 PC lipids. It would also explain why the increased content release is only evident in the beginning of the release profile experiments where the liposomes undergo their respective phase changes and not after the system is in a relative state of equilibrium after the first hour.

Figure 25: Release profiles of liposomes with DPPC (16PC) as either a main or secondary constituent.

Figure 26: Release profiles of liposomes with DMPC (14PC) as either a main or secondary constituent.

This phenomenon is even more apparent in Figure 26, where 14 PC liposomes are analysed. In the 14:16/66:33 and 14:18/66:33 liposomes, the release profile and total release percentage are comparable and similar. This can be largely attributed to 14 PC making up as the majority of the liposome composition and thus releasing its contents through a primarily total permeation route. 14:18/33:66 liposome, however, has significantly higher total content release and a higher initial release rate than 14:16/33:66 and 14:16:18/33:33:33 liposomes. The comparison with 14:16/33:66 as a direct counterpart can draw strong conclusions about the hypothesis that leakage can be achieved through the disrupted grain boundaries forms between 2 lipids with highly different molecular chain lengths. The amount of content release difference between the 2 liposomes also sheds some insight into the degree of leakage that is possible such an incorporation of leakage design. 14:18/33:66 liposome releases almost twice as much contents as 14:16/33:66 liposome (52% vs 95%). This effect is apparent and would warrant more in-depth investigation in the field of liposome design.

From the study of the release profiles of these 10 mixture liposomes, we are able to observe 3 main types of release mechanisms, namely:

- 1) Permeation through total phase transition, such as in the case of 14 PC in endogenous temperatures.
- 2) Permeation through partial phase transition, such as in the case of 16 PC in endogenous temperatures, that does not reach or exceed the lipid's critical phase transition temperature.
- 3) Leakage through disruptions between grain boundaries of lipids with significantly different carbon chain lengths. In the case of this experiment, the difference is of at least 4 or more carbon chains between 14 PC and 18 PC.

Figure 27: Surface response of liposome size variations as a function of lipid compositions, as a optimization method demonstrated by Behnoush et al[100]

Through optimization by surface response methodology[100], these 3 release mechanisms that are uniquely possible from the combination of 2 or more lipid compositions can be designed to have the ideal release profiles for use in various endogenous applications. By evaluating the different parameters of each lipid composition to be optimized, a surface response plot can be obtained, with an example in Figure 27.

By analysing parameters such as

- a) total release as a percentage of encapsulated volume,
- b) total effective release duration under endogenous heat,
- c) release rate,
- d) rate of change in release rate,

mixture design methodology in combination with surface response analysis would be able to effectively optimize a lipid composition for the total release and release duration of the liposome. It would also be possible to achieve a release profile that is as consistent as possible for the mixture of lipids used.

Such an optimized lipid composition can be further augmented and modified with other moieties such as thermosensitive polymers and lysolipids[101] to further improve on its release characteristics with another stage of optimization if necessary. The finished liposome composition would potentially be a great resource within therapeutics and drug delivery systems, with many potential widespreading applications.

Chapter 4: Conclusion and future work

4.1: Contributions

In our findings, the alkali encapsulated liposomes were able to sustain releases of its contents up to 3 hours and is able to prevent the acid erosion of hydroxyapatite. When compared to a control, Tris-Lipo was able to reduce acid erosion by 94% ($p < 0.01$) and demineralization by 77% ($p < 0.05$). This finding indicates that the liposomes with sustained release of alkali is highly effective to neutralize the acidification of the dentine by lactobacilli after glucose stimulation and to combat the dentine erosion and demineralisation in oral cavity.

This indicates that Tris-Lipo could have wide spreading applications when functionalized with hydroxyapatite binders such as tetracycline and alendronate, ranging from daily oral washes to improve oral health in veterinary care, to specialized medical oral treatment for hyposalivation in humans.

After the sustained release of contents for 3 h, the continued release is subdued due to the presence of osmotic equilibrium and is not activated again until triggered by a disruption to the equilibrium in the case of reacidification in our study. After acid was added to the buffered environment, release of the alkali buffer initiated again for 2 hrs of further release. With the investigation into release profiles of different lipid compositions, it is proven that Tris-Lipo has the potential to release its content for up to 7 hrs.

This characteristic provides the possibility that liposomal drug delivery systems may be incorporated as a passive medical treatment, embedded in the biological system to be reactive as a regulatory front-line defence in the presence of hostile triggers, or as a sensor designed with controlled leakage of indicators.

Furthermore, the present liposomes would be effective for drug delivery system with sustained payload release characteristics by endogenous heat of body without applying a stimulation of exogenous heat.

By employing mixture design methodology in liposome compositions using phospholipids of different phase transition temperatures, we studied and analysed the release mechanisms and release profiles of the lipids DMPC (14 PC), DPPC (16 PC), and DSPC (18 PC) as well as their composite liposomes. By determining that three possible routes of content release or leakage is possible through partial phase transition and permeation, total phase transition, and leakage through grain boundary defects of lipids with significantly different chain lengths, we determine that it is possible to optimize lipid compositions to design a liposome for constant and long duration release in endogenous heat using different lipids and surface response analysis.

Controlling the release profile of thermosensitive liposomes for prolonged periods using liposome composition design might enable a future where targeted drug delivery systems could be facilitated orally without invasive methods of drug activation to provide improved medical treatment and reduced side effects for treatments of cancers and should be explored by future nanosized liposomal therapeutic research.

4.2: Limitations

The relationship between environment acidity and the release profile of thermosensitive liposomes observed in this research has also not been studied extensively. When further explored, the causal relationship may provide an opportunity to develop advanced thermosensitive and pH sensitive liposome drug systems to activate upon triggers caused by differing acidity levels in bodily fluids.

An interesting aspect that is not considered in the application of Tris-Lipo in the oral cavity is the effect of lipid build-up on the enamel and how it would affect bacteria and plaque accumulation. When used in excess, the HA-binders binds liposomes onto areas of dentine that are not accessible by mechanical brushing. When allowed to accumulate, the liposomes could potentially become lipid piles, devoid of the alkali buffer that reduces environmental acidity, and serve as a food reservoir for bacteria to flourish.

Other limitations presented by this research lies in the vast differences and specificity of the environments in which the vesicle can potentially be used in. As a drug vesicle for *in-vivo* therapeutics, more studies will need to be done regarding the release characteristics, immune-response towards the drug vesicle, vesicle stability and removal of the vesicle from the physiological systems. Each potential application of such passive and roaming liposomes would require extensive studies on each of the aspects mentioned to begin assessing its viability for clinical trials to be considered.

4.3: Future work direction

While Tris-Lipo has been effective in buffering and regulating the pH in a simulated oral environment in-vitro and has a proven efficacy in reducing the demineralisation of HA by acids, and we have a hypothesis on how to improve the liposome composition through a combination of mixture design using different phospholipids and surface response methodology, there are still studies that could be done to gain more insight into liposomal designs for dental and therapeutic applications.

1. Optimization of liposome composition using response surface methodology (RSM) to optimize the phospholipid composition [100] such that the release profile is optimized for maximum release duration, maximum accumulated release, and lowest rate of change (delta) in rate of release (which results in a near constant release rate). This can be done in 3 stages: by firstly analyzing the surface response plot for the 3 main lipids in terms of each parameter, namely release duration, accumulated release, and rate of change in rate of release. The 3 surface response plots can then be optimized using a mixture design software such as Design Expert.
2. Incorporation of lysolipids and thermosensitive polymer moieties into the optimized liposome composition may improve the release profile of the liposomes by providing additional mechanisms of content release. The thermosensitive moieties can be further optimized using the same mixture design and surface response methodology to work on the release parameter that is still lacking after the liposome composition optimization.

3. Calcium and phosphate ions can replace copper ions as the encapsulated and released drug in future studies of liposomal release onto amelogenin functionalized apatite substrates to stimulate remineralization of hydroxyapatite on dentine. This would be able to provide a second account of the optimized liposomes having a consistent release profile across different encapsulates and provides research insight into the efficacy of remineralization between constant mineral ion supplies and bulk supply methods in other literature, as well as any difference in crystallinity and other physical properties. It also further demonstrates a practical application of a constant and prolonged releasing liposome in dental applications.

Publications

International journal publications

- 1) Chong, J. R.; Le, D. L.; Sato, H.; Sou, K., Nanocapsule pH Regulator: Sustained Continuous Alkali Release from Thermosensitive Liposomes Reduces Acid Erosion. ACS Applied Materials & Interfaces 2020.

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