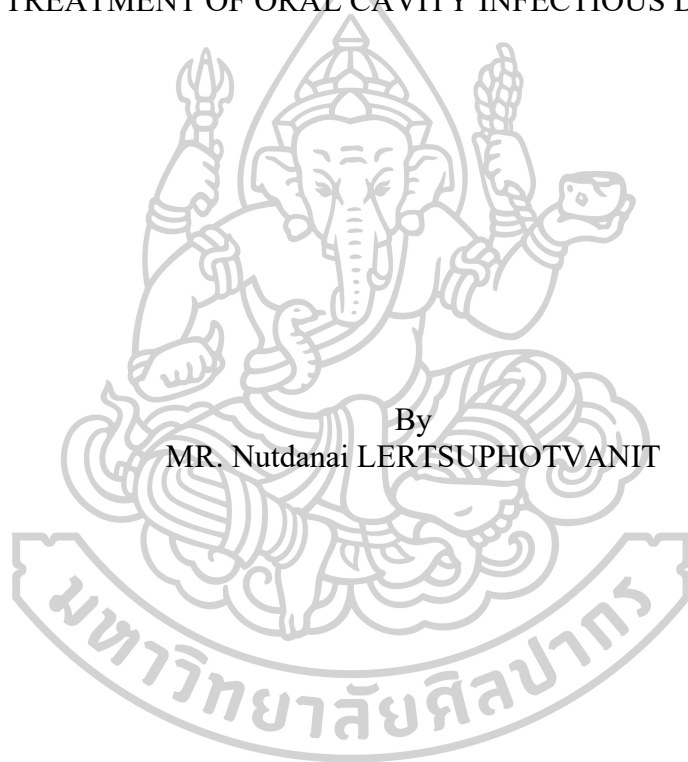




MECHANISTIC UNDERPINNING FORMATION OF NON-SOLVENT INDUCED
PHASE INVERSION-BASED BORNEOL *IN SITU* FORMING MATRIX FOR
TREATMENT OF ORAL CAVITY INFECTIOUS DISEASES



By
MR. Nutdanai LERTSUPHOTVANIT

A Thesis Submitted in Partial Fulfillment of the Requirements
for Doctor of Philosophy PHARMACEUTICAL TECHNOLOGY
(INTERNATIONAL PROGRAM)

Department of PHARMACEUTICAL TECHNOLOGY

Silpakorn University

Academic Year 2023

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Title	Mechanistic underpinning formation of non-solvent induced phase inversion-based borneol <i>in situ</i> forming matrix for treatment of oral cavity infectious diseases
By	MR. Nutdanai LERTSUPHOTVANIT
Field of Study	PHARMACEUTICAL TECHNOLOGY (INTERNATIONAL PROGRAM)
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Keyword : Borneol, *in situ* forming, periodontitis, oropharyngeal candidiasis, phase inversion, non-solvent induction

MR. Nutdanai LERTSUPHOTVANIT : Mechanistic underpinning formation of non-solvent induced phase inversion-based borneol *in situ* forming matrix for treatment of oral cavity infectious diseases Thesis advisor : Professor Thawatchai Phaechamud, Ph.D.

The mechanistic phase inversion, characterized by its ability to transform from a liquid to a solid-like matrix upon contact with an aqueous solution, has gained popularity as a simplified mechanism for *in situ* forming matrix (ISM). In this study, borneol was chosen as a matrix-forming agent for non-solvent induced phase inversion-based ISMs due to its safety and low aqueous solubility. The primary objective of this research is to conduct a comprehensive investigation into the mechanistic phase inversion and the development of antimicrobial-loaded borneol-based ISMs for the treatment of oral cavity diseases. The assessment included physicochemical testing, matrix morphology analysis, drug release, and antimicrobial activity. A 40% w/w borneol-based ISM was developed successfully for clotrimazole-loaded spray for oropharyngeal candidiasis treatment. Additionally, a vancomycin HCl-loaded 40% w/w borneol-based ISM (VDB) was formulated for periodontitis treatment, characterized by low viscosity (6.18 cP), favorable wettability, and solid-like borneol matrix formation. Moreover, VDB had achieved a 14-day release profile through Fickian diffusion, and dimethyl sulfoxide (DMSO) was confirmed as the suitable solvent without affecting the crystallization change of the borneol precipitate. Additionally, the 40%w/w borneol-based ISM with doxycycline hyclate (DH) and 5%w/w triacetin extended drug release *via* Fickian diffusion and effectively targeted periodontitis pathogens. In this research, the application of UV-visible imaging provided real-time insights into the mechanisms of phase inversion, DH release, and matrix formation. Borneol significantly retarded solvent diffusion and DH release. Initially, rate of solvent diffusion (0.030-0.048 molar/hour) and DH diffusion (0.22-0.27 mg/mL/hour) were high but gradually constant after 10 hours. Additionally, molecular dynamics (MD) modeling contributed valuable insights into the initial phase inversion processes that occurred on a millisecond scale upon contact between ISM and aqueous solutions. The drug diffusion constant of borneol-based ISM was significantly lower than that of borneol-free ISM (0.32 vs. 0.52 m²/s), underscoring the pivotal role of borneol in retarding DH release. Therefore, the mechanistic underpinnings of phase inversion bridged the gap between formulation development and analytical fields, offering direct visualization of dynamic processes previously challenging to monitor during the early stages of phase inversion. Understanding the mechanistic underpinning of phase inversion not only contributes to the ability to anticipate drug release patterns but also offers valuable insights for shaping the trajectory of future ISM formulations.

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Nutdanai LERTSUPHOTVANIT

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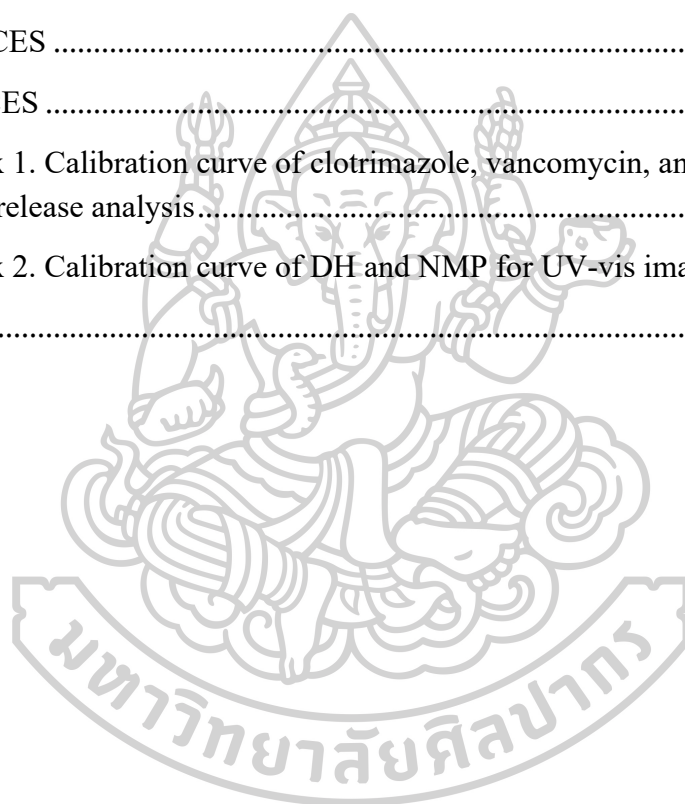
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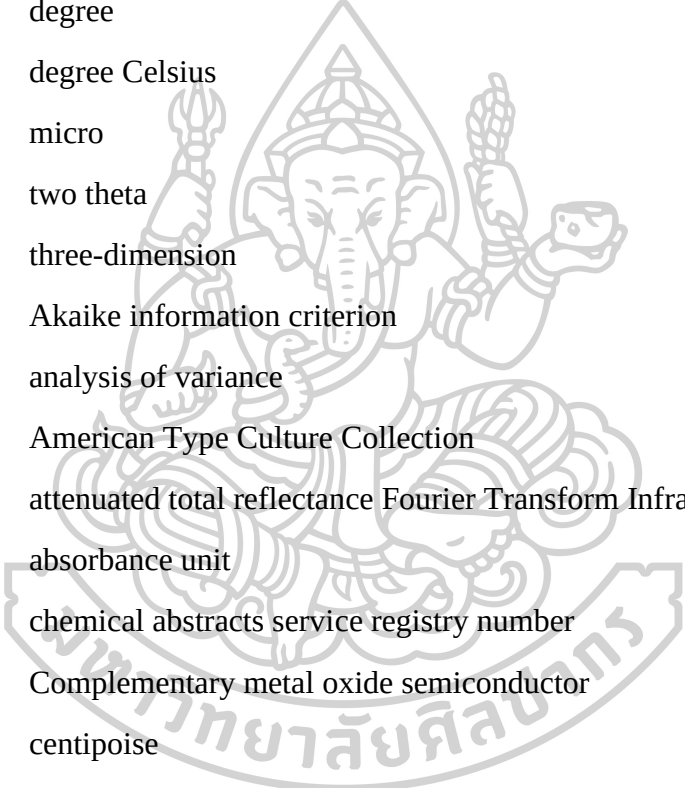
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LIST OF ABBREVIATIONS



%	percentage
%w/w	percent weight by weight
<	less than
>	more than
×	magnification
°	degree
°C	degree Celsius
μ	micro
2θ	two theta
3-D	three-dimension
AIC	Akaike information criterion
ANOVA	analysis of variance
ATCC	American Type Culture Collection
ATR-FTIR	attenuated total reflectance Fourier Transform Infrared Spectrometer
AU	absorbance unit
CAS No.	chemical abstracts service registry number
CMOS	Complementary metal oxide semiconductor
cP	centipoise
DH	Doxycycline hyclate
DMSO	Dimethyl sulfoxide
DMST	Department of medical sciences Thailand
DSC	differential scanning calorimetry
e.g. (Latin);	for example
Eq.	equation
et al.	and others
FDA	Food and Drug Administration
FTIR	Fourier Transform Infrared Spectrometer

g	gram
GRAS	generally regarded as safe
h	hour
H-bond	hydrogen bond
HCl	hydrochloride
HPLC	High performance liquid chromatography
HPMC	hydroxypropyl methylcellulose
ICH	International council on harmonization of technical requirements for registration of pharmaceuticals for human use
ISM	<i>in situ</i> forming matrix
k	release rate (related with each release equation)
k ₁	(for Peppas-Sahlin equation) kinetic constant for Fickian release,
k ₂	(for Peppas-Sahlin equation) kinetic constant for Case-II contribution
L	liter
LD ₅₀	median lethal dose
Log P	the logarithm of the octanol-water partition coefficient
M	molarity
m ² /s	square meter per second
MD	Molecular dynamics
MIC	minimum inhibition concentration
min	minute
mN	millinewton
msc	model selection criterion
Mw	molecular weight
n	(for Korsmeyer-Peppas equation) exponent of release value
n	(for viscosity testing) flow behavior index
N.D.	not determined
N/A	not applicable
N.C.	clear zone not found

nm	nanometer
NMP	<i>N</i> -methyl-2-pyrrolidone
No.	number
ns	nanosecond
p or p-value	probability value
PBS	phosphate buffer solution
pH	potentia hydrogenii (Latin); power of hydrogen
PLA	polylactide
PLGA	poly (lactic-co-glycolic acid)
PXRD	powder X-ray diffraction
PYR	2 -pyrrolidone
r^2	coefficient of determination
RMSD	root mean square deviation
rpm	revolutions per minute
S.D.	standard deviation
sec	second
SEM	scanning electron microscopy
SPSS	statistical package for social science
t	time
TGA	thermogravimetric analysis
TISTR	Thailand institute of scientific and technological research
UV	Ultraviolet
UV-vis	Ultraviolet-visible

CHAPTER 1:

INTRODUCTION

Rational and problem statement

Oral health refers to the health of the teeth, gums, and the entire oral-facial system that allows smile, speak, and chew [1]. It has a direct connection to overall health, and poor oral health can be detrimental and costly. Oral conditions are frequently considered and separated from other chronic conditions, but these are actually inter-related. Poor oral health is associated with other chronic diseases such as diabetes, heart and lung diseases, stroke, and preterm deliveries. Missing teeth, pain, and infection from oral diseases can limit food choices and worsen nutrition [2]. The 7 majority of oral health diseases reported by the World Health Organization (WHO) are dental caries (tooth decay), periodontal diseases, oral cancers, oral manifestations of HIV, oro-dental trauma, cleft lip and palate, and Noma (severe gangrenous disease starting in the mouth mostly affecting children). Most oral health conditions are largely preventable and can be treated in their early stages [3]. In this study, the focus will be on developing drug delivery systems for the treatment of periodontitis and oropharyngeal candidiasis diseases which are important diseases affecting oral health.

Periodontitis is mainly the result of infections and inflammation of the gums and bone that surround and support the teeth [1]. The disease is characterized by bleeding or swollen gums (gingivitis), pain and destruction of surrounding structures of teeth resulting in bone loss, gingival recession, increased tooth mobility and eventually leading to tooth loss [3, 4]. In its early stage, called gingivitis, the gums can become swollen and red with bleeding. In its more serious form, called periodontitis, the gum can come away from the tooth and supporting bone, causing teeth to become loose and sometimes fall out [1]. This is a major problem that affects a large number of patients in the society. Severe periodontal diseases are estimated to affect nearly 10% of the global population [3]. The microbial found in periodontal disease is complex and composed, the major bacteria that have been implicated in periodontitis such as *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Bacteroides forsythus*, *Prevotella intermedia*, *Campylobacter rectus*, *Eubacterium nodatum*, *Streptococcus intermedius* and *Treponema denticola* [5]. Thus, periodontal treatment is necessary to eliminate or inhibit the growth of bacteria on tooth surface and in periodontal pocket by mechanical scaling, root planning and medication [6, 7].

Antimicrobial agents are used in periodontal treatment for infection elimination [8]. Several antimicrobial agents have been evaluated for their clinical and microbiological efficacy in periodontal diseases such as chlorhexidine, tetracyclines (tetracycline, doxycycline and minocycline), metronidazole, ciprofloxacin, vancomycin and etc. [9-14]. The current microbiological treatment of periodontitis is through either the use of systemic antibiotics or the localized delivery system

incorporating an antibiotic. However, the large doses of systemic antibiotic must be taken in order to achieve sufficient concentrations in the gingival crevicular fluid of the periodontal pockets [15, 16], which may be associated with side effects of antibiotics and problems [17, 18] regarding antibiotic resistance [19, 20]. These disadvantages have evoked an interest in the development of local drug delivery systems for the treatment of periodontal diseases. Therefore, a more satisfactory approach to administering antimicrobial drugs directly into the pocket involves the use of a controlled release device. Local drug delivery limits the drug to its target site, with little or no systemic uptake; thus, a much smaller dose is required for effective therapy and harmful side effects can be reduced or eliminated [21]. In the present study, vancomycin HCl and doxycycline hyclate were used as the model drugs for local drug administration while the chosen reasons of these drugs will be discussed later.

Vancomycin is a potent glycopeptide antibiotic used primarily in the treatment of resistant gram-positive bacterial infections such as *Staphylococcus aureus* [22]. It currently represents the gold standard against methicillin-resistant *Staphylococcus aureus* (MRSA) infections. Furthermore, it could treat the periodontitis which caused by *P. gingivalis*, *A. actinomycetemcomitans*, *Bacteroides forsythus*, *Prevotella intermedia* and *Streptococcus intermedius* [23-26]. Thus, vancomycin HCl was selected as an antimicrobial model drug for periodontitis treatment in this study due to its broad spectrum and supported the drug resistance of other traditional antibiotics in the future. However, it is associated with low oral bioavailability and severe side effects upon systemic administration [27]. The administration into the body of this drug by injection is suitable for the application. Therefore, it is interesting to use 1%w/w vancomycin HCl [28] for periodontitis treatment.

Doxycycline hyclate (DH) belongs to the tetracycline group and serves as a broad-spectrum antibiotic extensively utilized in periodontal treatment. It demonstrates efficacy against both Gram-negative and Gram-positive bacteria, including strains producing beta-lactamase found in deep periodontal pockets [29]. Administering DH orally at a low dose has proven effective in substantially diminishing gum inflammation. However, this approach may lead to undesirable side effects such as nausea, and rash [30]. To address these drawbacks, a localized delivery method for DH has been implemented. Additionally, studies indicate that utilizing a DH-loaded delivery system for a period of 4 months in the treatment of periodontitis can significantly reduce the depth of periodontal pockets, presenting an efficacy comparable to scaling or root planning [31].

Nowadays, many commercial products in injectable local dosage form have been launched in the market such as Atridox[®], Dentomycin[®] and Periocline[®]. One of the popular products namely Atridox[®] containing DH as active drug dissolved in polymer solution is an injectable dosage form by gel forming to prolong a drug release [32]. It was claimed that this product can control the drug release for 7 days with efficient antimicrobial activities [33]. However, this commercial product is very expensive owing to using PLGA as polymer; thus, this research work aims to

investigate for employing borneol as matrix former in *in situ* forming matrix (ISM) because it is affordable, safe, biocompatible and biodegradable.

Oropharyngeal candidiasis represents a significant oral cavity affliction, characterized by opportunistic infection from the fungus *Candida albicans*, resulting in painful red or white lesions in the oropharynx that adversely impact taste, speech, and eating. The majority of infections are endogenously acquired [34]. This condition affects 15%–60% of individuals with hematological or oncological malignancies during periods of immunosuppression, and it is prevalent in 7%–48% of people with HIV infection, rising to over 90% in those with advanced disease [34, 35]. Various antifungal drugs, such as nystatin, amphotericin B, clotrimazole, ketoconazole, and fluconazole, are presently employed in oropharyngeal candidiasis treatment, available in both topical and systemic forms. Despite the array of options, polyenes and azoles, particularly clotrimazole, stand out as the most conventional and effective drug groups. The preference for topical dosage forms with local administration stems from their enhanced compliance and reduced side effects [36]. Consequently, clotrimazole emerges as a suitable antifungal model drug for this study.

Clotrimazole is an imidazole antimycotic drug for treating fungal infections of skin and mucus diseases. Its mechanism is disturbing amino acid transport into the fungus by acting on the cell membrane [37]. Nowadays, it is available topically in commercial product as troches, solutions, or cream forms. Systemic use is avoided because of modest systemic absorption, gastrointestinal and neurologic toxicities [37–39]. Oral clotrimazole is an alternative to nystatin, offering a more favorable taste and, more importantly, exhibiting anti-staphylococcal activity [37]. In this study, clotrimazole was expected to be formulated as an oral topical antifungal spray system for oral cavity.

The primary challenges inherent in the local drug delivery system within the oral cavity lie in formulating a product that possesses the capacity to adhere to the target organ. This is crucial for facilitating prolonged drug release while concurrently maintaining potent antimicrobial activities. The ISM system stands out as a satisfactory approach for controlled drug delivery. The liquid state, allowing for facilitated administration, can be transformed into the solid state, a phenomenon known as “phase inversion” [40–42]. This transformation allows for controlled drug release over prolonged periods of time and can be induced by various mechanistic phenomena, including solvent exchange, changes in pH, or temperature. Regarding solvent exchange, the typical process involves dissolving a water-insoluble matrix-forming agent in a biocompatible, water-miscible organic solvent such as *N*-methyl-2-pyrrolidone (NMP), 2-pyrrolidone (PYR), and dimethyl sulfoxide (DMSO). The drug is dissolved in this solution. Upon injection of this solution into an aqueous environment, like saliva, the organic solvent diffuses outward into the surrounding environment due to its miscibility with water, concurrently allowing water to diffuse into the system. Since the polymer is water insoluble, it separates *via* phase inversion and forms into the solid depot. The drug molecules or particles are trapped within this matrix depot and slowly released over time [41, 42].

The key substance of *in situ* forming system is matrix forming agent which should be a water insoluble matter with biocompatible, biodegradable, safe (non-toxic) characters and it should be miscible with solvent and drug. Borneol is chosen as a key matrix-forming agent in this study due to its noteworthy properties. This bicyclic monoterpene is naturally present in citrus peel oils and various spices, including nutmeg, ginger, and thyme. Widely utilized in food flavoring, air care products, cosmetics, and traditional Thai medicine, borneol has found application in the pharmaceutical field for its ability to alleviate local itching, bronchitis, minor muscle and joint pains. Additionally, it exhibits antibacterial and antinociceptive effects [43]. Notably, borneol can be incorporated into ganciclovir-loaded solid lipid nanoparticles (SLNs) using a modified microemulsion method. Borneol-modified SLNs represent an efficient drug delivery system for transporting drugs to the brain [44]. It's therefore believed to be an effective adjuvant that can improve drug delivery to the brain. [45]. According to the basic theories of traditional Chinese medicine, borneol can guide other components to the targeting tissues or organs in the upper part of the body, especially in the brain. It can enhance the blood-brain barrier permeability, improve the transportation of kaempferol to brain [46]. Besides, it can promote the drug absorption from the gastrointestinal tract and facilitate the distribution to the brain [47]. Borneol emerges as a safe and effective penetration enhancer for ocular drug administration, enhancing the transcorneal penetration of both hydrophilic and hydrophobic compounds without inducing toxic reactions [48]. Furthermore, it demonstrates anti-fibrotic activity, potentially inhibiting fibroblast mitosis, collagen production, and TIMP-1 production. This makes it a considered safe option for the treatment of oral submucous fibrosis [49]. Borneol was approved by the Food and Drug Administration as a flavor because of its non-toxicity. It's slightly denser than water, insoluble in water, but soluble in alcohol [43, 50, 51]. The low toxicity of borneol has been reported for skin irritation [50]. Due to its safety, apparent low aqueous solubility, and the absence of prior utilization in this context, borneol may serve as a suitable matrix-forming component for the ISMs in this research.

The aims of this thesis are to design and develop ISM formulation by using borneol as the matrix former of non-solvent induced phase inversion-based borneol ISM for the treatments of oropharyngeal candidiasis and periodontitis. To facilitate formulation development, it was imperative to comprehend the matrix-forming behavior of borneol. Consequently, the impact of varying concentrations of borneol was investigated and discussed regarding physicochemical properties, matrix-forming behavior, and the efficacy concerning each model drug. In addition, the role of some additive solvent such as triacetin for retardation of drug release was studied in this research. The physicochemical characters of prepared vancomycin HCl, DH and clotrimazole-incorporated borneol ISM formulations were investigated in terms of pH density, rheology behavior, viscosity, injectability, matrix formation behavior, morphology of matrix, interfacial phenomena, drug release and antimicrobial activities. The novel modified experiments conducted in this study aim to enhance our understanding and address the phase inversion phenomenon that has occurred. UV-

visible (UV-vis) imaging has been employed to track and visualize the complex processes of phase inversion, solvent diffusion, and drug diffusion within the prepared ISM. This technique is gaining popularity as a powerful tool for tracking and visualizing complex phenomena within pharmaceutical formulations. It offers non-invasive methods, allowing simultaneous observation of phase inversion, solvent diffusion, and drug diffusion in real-time—a feat traditional experiments cannot achieve. Additionally, computer dynamics simulation is applied to understand the mechanism of phase inversion in the early stages of solvent exchange and to support the development of this research. Furthermore, contact angle studies on the formulation's interaction with tissue surfaces simulate the matrix formation via a phase inversion process that mimics the oral cavity. This is expressed as wettability on the porcine mucosa in experimental conditions. A comprehensive understanding of the intricacies surrounding the phase inversion process and its associated mechanism can assist in predicting drug release patterns and provide essential insights for the future development of ISM formulations.

Objectives

1. To determine the mechanistic phase inversion of non-solvent induced phase inversion-based borneol ISM
2. To investigate the physicochemical properties and matrix forming behavior of non-solvent induced phase inversion-based borneol ISM formulation
3. To develop vancomycin HCl-loaded non-solvent induced phase inversion-based borneol ISM for crevicular pocket delivery
4. To develop clotrimazole-loaded non-solvent induced phase inversion-based borneol ISM spray for oropharyngeal candidiasis treatment
5. To study the role of triacetin on DH-loaded non-solvent induced phase inversion-based borneol ISM for periodontitis treatment

Hypothesis

1. The concentration of borneol influences the physicochemical properties and matrix forming behavior of non-solvent induced phase inversion-based borneol ISM.
2. The concentration of borneol influences the release pattern of vancomycin HCl, clotrimazole and DH from non-solvent induced phase inversion-based borneol ISM.
3. Triacetin influences the release pattern of DH-loaded non-solvent-induced phase inversion-based borneol ISM.
4. The borneol concentration, hydrophobicity of solvent and incorporated-drugs influences mechanistic phase inversion and the antimicrobial activities of non-solvent induced phase inversion-based borneol ISM.
5. UV-vis imaging can reveal phase inversion mechanistic of borneol-based ISM inversion phenomena and related phenomena in real time.
6. The dynamic computerized simulation could explain and support the mechanistic phase inversion of non-solvent induced phase inversion-based borneol ISM.

CHAPTER 2:

REVIEW LITERATURES

***In situ* forming matrix (ISM) systems**

Controlled drug delivery systems are capable for regulating rate of drug delivery, maintaining drug concentration within the therapeutic range with prolonged duration of drug release, and/or targeting drug to a specific tissue [52]. Of the many controlled drug release technologies, ISM systems have risen popularity over decades due to a range of advantages which include the site-specification, less invasive application, prolonged drug release, reduction in side effects associated with systemic delivery and also improvement of the patient compliance and comfort [53, 54]. Importantly, an administration by this method allows the injection of a relatively low viscosity material which then solidifies to a solid-like matrix form that controls the drug delivery to improve long-term therapeutic action [54]. The mechanism of *in-situ* forming systems can be classified into different types such as phase separation systems (*via* thermo-responsive, solvent exchange and pH change), crosslinked systems (*via* photo-initiated, chemical and physical) and solidifying organogels (*via* solubility change) [52]. The phase inversion-based or non-solvent induced ISM technology has attracted worldwide interest among pharmaceutical companies, which led to the development of commercial therapeutic products for a wide range of clinical applications [52]. This system consists of a water-insoluble polymer dissolved in an organic, water-miscible, biocompatible solvent, incorporating a drug through dissolution. Upon injection of this ISM solution into an aqueous environment, like saliva, the organic solvent diffuses outward into the surrounding environment due to its miscibility with water, while water permeates into the system. As the polymer is poor water soluble, it undergoes a gradual or rapid solidification process, transforming into a solid-like matrix [41, 42, 55]. The process where a single-phase homogenous solution is critically quenched, causing separation into a two phases can call “phase inversion” or “phase separation” [56]. The drug molecules or particles trapped within the matrix slowly release over time. This mechanism results in solvent exchange causing a phase inversion that leads to matrix formation, which in turn controls the rate of drug release [42]. For a clearer understanding, Figure 1 illustrates the schematic diagram of drug-loaded borneol ISM administered into the crevicular pocket. It highlights the transformation of the solution into a solid-like matrix through the phase inversion mechanism, effectively modulating drug release.

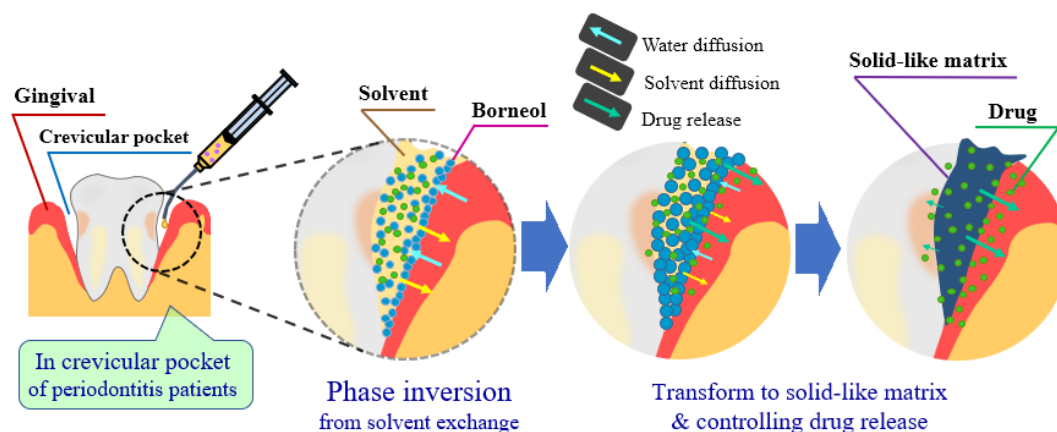


Figure 1. Schematic diagram of drug-loaded borneol ISM administered into the crevicular pocket and its phase transformation

Phase inversion is classified into two types based on the rate of phase inversion [52]. The first type, fast-forming phase inversion, undergoes phase inversion at a rapid rate (from seconds to minutes), resulting in the formation of a thin membrane with an inner porous matrix structure [55]. This change is a result of formulation using solvents that are strong and hydrophilic in nature (e.g. NMP, DMSO, benzyl alcohol) [57]. This system is of a lower viscosity and it requires lower force of injection [58]. In contrast, a slow forming phase inversion undergoes the phase inversion at a slow rate (from hours to days). Solvents used in this system is weaker and are hydrophobic in nature, therefore a matrix formation occurs at a slow rate [57]. The viscosities of this solutions system is usually of an order that makes an injection difficult unless the system is pre-emulsified or preheated to 37 °C [58]. Moreover, the matrix conformation can be described as being uniformly dense with a limited number of pores [54, 57] Limited pores result in slower drug release from these implants than that from fast forming phase inversion matrix but burst release is notably reduced. Thus, this system is not suitable to be used for sustainable dosage form due to the extended period of time for solidification of matrix formation [52].

Nowadays, ISM holds great promise in the field of drug delivery, with some commercial products recently approved by the FDA and others currently undergoing clinical trials. The most attractive feature of ISM systems is their applicability to a wide range of clinical indications. For instance, Atridox[®] contains DH for the treatment of chronic periodontal disease, Eligard[®] contains leuprolide acetate for advanced prostate cancer treatment, Sandostatin[®] contains octreotide for acromegaly treatment, and Relday[™] contains risperidone for schizophrenia and bipolar disorder treatments [52]. In the 1990s, the first ISM commercial product, namely Atridox[®], was released onto the market and was approved by the FDA in late 1998. It consists of DH as the active ingredient, using poly DL-lactide as the matrix former dissolved in NMP. This product is licensed for the treatment of chronic periodontal disease and utilizes the initially liquid state of the polymer solution to allow the injection of the antibiotic into the periodontal pocket. This system has been reported to allow the

release of DH over 21 days [59]. Atridox[®] allows for the local treatment of periodontal disease via the direct application of the antibiotic, and it does not require removal as it is a bioabsorbable system. Clinical research determined that the local delivery of DH resulted in a gain in clinical attachment and also a reduction in probing depths [31]. Atridox[®] is designed as the two-syringe system to ensure that the drug stability is maintained for the shelf life, that based on Atrigel[®] technology [60]. One syringe contains the dried drug powder, whilst the other contains the required amount of polymer solution. Before injection, couple the syringes and mix both components thoroughly by cycling between the two syringes approximately 100 times to form a homogeneous mixture [61]. Nevertheless, this method can be a lengthy process and is obviously not ideal. A two-component system could therefore be detrimental in terms of progress towards a patient self-administered formulation [52].

In most ISM formulations, the matrix-forming materials play a crucial role and are considered a key component of this system. These materials should be water-insoluble, biocompatible, biodegradable, safe (non-toxic), and miscible with both the solvent and the drug [52]. In recent times, aqueous insoluble polymers have found application as matrix-forming components in ISM. Examples of such polymers include PLGA, HPMC, Eudragit[®] L, zein, ethyl cellulose, and cholesterol [62-66].

Borneol

Borneol (C₁₀H₁₈O, molecular weight 154.25) is a highly lipid-soluble (Log K_{o/w}=2.85) bicyclic monoterpene alcohol with fragrant odor and bitter tastes. It occurs naturally in over 260 plants and found in citrus peel oils, spices such as nutmeg, ginger and thyme. It has melting point and boiling point at 202°C and 212°C, respectively [43, 50]. It's slightly denser than water, insoluble in water (1,186 mg/L), soluble in alcohol and ether and slightly soluble in propylene glycol [51, 67]. It can be analyzed by microwave-assisted extraction (MAE) followed by gas chromatography with flame ionization detector (GC-FID) or gas chromatography-mass spectrometry (GC-MS) method. Nowadays, it has been used as the food flavoring, air care products, decorative cosmetics and etc. [50]. Borneol has been approved by the Food and Drug Administration as a flavor because of its non-toxicity [50]. However, it can be metabolized to camphor; meanwhile, camphor could be absorbed rapidly and reported to cause the seizures, respiratory failure, myocarditis and hepatotoxicity; thus, borneol should not be applied in children and pregnant women without caution [68].

Borneol is classified as natural borneol and synthetic borneol. Traditional natural borneol originated from the resin of *Dipterocarpus aromatica* Gaertn.f. in the forest of Southeast Asia. Because of its rare resource and expensive price, the essential oil extracted from the fresh branches and leaves of *Cinnamomum camphora* (L.) J.Presl. is widely used as a substitute of natural borneol in recent years. In contrast, synthetic borneol is obtained by simple chemical transformation of camphor and turpentine oil [46].

The structure of borneol consists of one hydroxy group as showed in Figure 2. In addition, it can be verified by the Attenuated total reflectance Fourier transform infrared (ATR-FTIR) spectra in which sharp peaks are visible for pure crystalline borneol at 2950, 1454, and 1055 cm^{-1} (Figure 3). Moreover, the physical status of borneol is determined by differential scanning calorimetry (DSC) analyses and X-ray diffraction analyses (XRD) as showed in Figure 4. The DSC curve of pure borneol exhibits a single endothermic response corresponding to melting points of 198.2°C, whereas the presence of distinct peaks in the XRD patterns indicated that borneol is present as crystalline materials with characteristic diffraction peaks at 15.1° and 16.1° [69].

In the pharmaceutical field, borneol has been reported to treat cancer, ischemic stroke, heal of skin wound. Additionally, it could relieve the local itching, minor aches and pains of muscles and joints. Furthermore, it has antibacterial effect, antinociceptive effect and anti-inflammatory activity. In traditional Chinese medicine, it has been used for treatment of ocular disease, preventing and curing cardiovascular and cerebrovascular diseases [46]. Borneol has been claimed to co-administer with an active compound for enhancing their delivery manner to the brain; thus, it is a promising new agent that deserves further use as a blood brain barrier (BBB) permeation enhancer for drug delivery to central nervous system [44-47]. Borneol can enhance the BBB permeability and improve the transportation of kaempferol in rats by *in vivo* microdialysis sampling to brain which the brain bioavailability evidently increased with the co-administration with borneol of 15 and 30 mg/kg [46]. Borneol-modified solid lipid nanoparticles were effective for transporting drugs to the brain [44]. It was considered as a safe and effective penetration enhancer for ocular drug administration because of improving the transcorneal penetration of both hydrophilic and lipophilic compounds without causing toxic reactions [48]. It has also been reported to enhance an intestinal absorption of drug [70]. Electrospun borneol-polyvinylpyrrolidone (PVP) nanocomposites enhanced the borneol dissolution and increased its physical stability against sublimation for long-time storage by immobilization of borneol molecules with PVP through hydrogen bonding; however, borneol weakened the tensile properties of nanofibers [69]. Borneol significantly reduced the nociceptive behavior at the early and late phases of mice paw licking and reduced the writhing reflex in mice and borneol-treated mice reduced the carrageenan-induced leukocytes migration to the peritoneal cavity without impairing motor coordination [71]. High dose of borneol could cause a stomach irritation [72]; thus, the sustained-release solid dispersions (SRSDs) for co-loading gastrodin and borneol were prepared as a sustained release matrix [47]. Compared with the free mixture of gastrodin and borneol, brain targeting of SRSDs was slightly weaker, but stomach irritation caused by borneol was obviously minimized. Combined bismuth subgallate and borneol evenly applied to cheesecloth and placed over the lesion areas on the back of Sprague–Dawley rats had a synergistic effect on the skin wound restoration by decreasing lesion area, while increasing granulation tissue formation, re-epithelialization, eating behavior and reconstitution of skin appendages [73]. Borneol showed an anti-fibrosis activity which its mechanism of action might partly

be relevant to inhibiting effects on fibroblasts mitosis, collagen and TIMP-1 production; thus, It is recommended for safe use as a penetration enhancer for the treatment of oral submucous fibrosis [49]. Borneol has been used as sublimable agent for highly porous and low-density non-effervescent floating tablets [74]. Borneol at concentrations of 0.5 and 5 $\mu\text{g/mL}$ did not induce a significant toxicity on the immune system cells. A significant increase of cell viability was detected when the thymocytes were cultivated with borneol at concentrations of 0.5 and 5 $\mu\text{g/mL}$ [75]. Low toxicity of borneol has been reported for skin irritation on human volunteer and mice/mouse [50]. Given its safety profile and evident low aqueous solubility coupled with miscibility in organic solvents, borneol stands out as a potential matrix-forming component for ISMs, particularly in applications targeting the oral cavity and crevicular pocket.

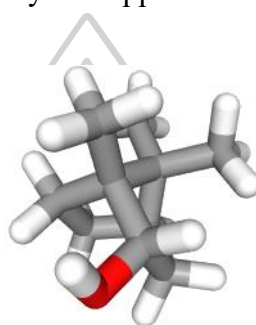


Figure 2. 3-D structure of borneol
(gray, white and red sticks represent C, H and O atom, respectively)

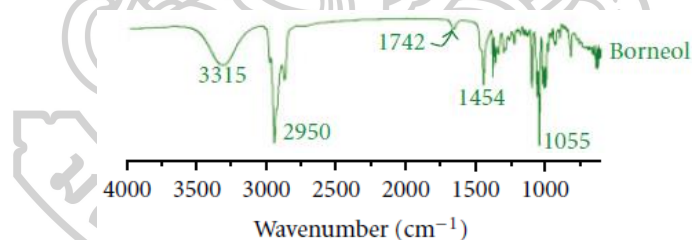


Figure 3. ATR-FTIR spectra of the borneol

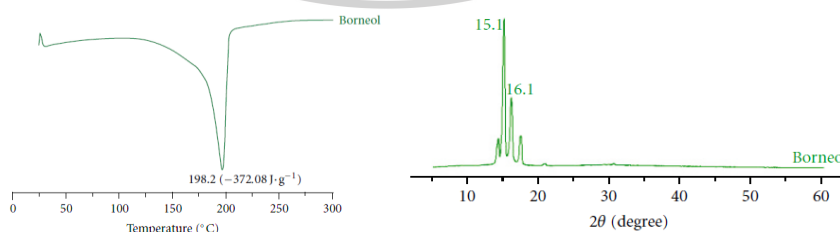


Figure 4. DSC thermograms (**left**) and X-ray diffraction patterns (**right**) of borneol

Solvent and additive in ISM

Practically, the solvents or vehicles of the ISM system are water miscible, biocompatible and organic in nature. Importantly, solvents should efficiently dissolve the matrix forming material and active compound, and be miscible with water and bodily fluids. Polarity of the solvent should be such that at least 10% should be

soluble in water [52, 76]. The viscosity of solvent should be within the range that is injectable. Solvent strength and its affinity for water directly influence the nature of phase inversion and matrix formation. For example, solvents having a high water affinity such as NMP and DMSO provoke a fast forming phase inversion [77]. Whereas, the hydrophobic solvents such as triacetin and ethyl benzoate promote a slow forming phase inversion [78]. Solvents that possess a water solubility of below 7% w/w have been shown to result in a slower drug release due to a reduction in water uptake [79].

ISM systems typically incorporate DMSO and NMP as solvents, and this choice is attributed to their established pharmaceutical precedence compared to other options [52]. NMP, with a molecular weight of 99.13, serves as a potent solubilizing agent with low viscosity. It is recognized as a solubilizing excipient employed in both parenteral and oral medications, with numerous commercial pharmaceutical products containing NMP already available in the market. The European Commission Scientific Committee on Consumer Safety has determined that NMP exhibits low acute toxicity through oral, dermal, and inhalation routes of administration [80-82]. DMSO, molecular weight of 78.13 is a non-aqueous dipolar aprotic solvent with colorless, oyster-like odor and relatively low toxicity (LD₅₀: 2.5 to 8.9 g/kg) [83, 84]. It is safe if used less than 100 mg/day [84]. It is miscible with most common solvents such as alcohol, ether, aromatics and water [85]. It readily penetrates into the cellular membranes; thus, it has been employed as a skin penetration enhancer [83, 86, 87]. It is approved by US-FDA to treat the interstitial cystitis [86]. In addition, it shows various pharmacological actions including analgesic, antioxidant and anti-inflammatory activities [87, 88]. In results of many studies found that DMSO is freely soluble in water which can breaks down the water structure and forms the strong hydrogen bond complexes with water molecules due to stronger interaction between DMSO-water [83, 86].

Normally, slow phase inversion systems may have a limited burst release, but they take a lengthier period of time to form a matrix, compared to that of fast inversion systems. As a way of compromising, the hydrophilicity of main solvents such as NMP and DMSO is reduced by the addition of more hydrophobic solvents such as triacetin, ethyl benzoate, and clove oil) to act as a co-solvent. As water miscibility decreases, drug release transitions from a high burst release profile to a zero-order release kinetics. This can be attributed to a change in the implant/depot morphology from a porous structure to a more uniformly formed dense structure [52, 57]. Similarly, the modification of the release rate is possible by selecting appropriate ratio of hydrophilic solvent and hydrophobic solvent [52].

Triacetin (C₉H₁₄O₆, molecular weight of 218.2) is one of additive solvents which has been used for retardation of a drug release [89, 90]. It has also known that triacetate, is a harmless edible fatty acid [38,39] and generally recognized as safe (GRAS) [91]. It is moderately soluble in water (7 g/100ml at 25 °C) but soluble in organic solvents[89]. It is relatively hydrophobic (log K_{o/w}=0.25) as it is a short chain triglyceride with a limited water miscibility (~7%) [78] and the structure of triacetin

as showed in Figure 5. The melting point and boiling point of triacetin are 3°C and 258-260°C, respectively [52].

The movement towards the use of solvent mixtures can be observed through literature in order to obtain the most suitable “solvent strength” to enable predictable and modifiable controlled release [52]. In the ISM system, the incorporation of triacetin serves to diminish the solvent quality and the affinity between water and polymer, leading to a decelerated phase inversion. This, in turn, results in a slower release rate, impacting not only the burst release but the overall release rate [55]. A recent research introduced a solvent combination comprising glycerol formal (hydrophilic) and triacetin (hydrophobic), demonstrating sustained blood levels of fipronil (flea adulticide) for 12 months following subcutaneous injection [92]. From the research of Tang and Singh, they found that initial burst release was increased to 65% from 36% by the addition of triacetin to a PLGA/NMP system. This was attributed to the limited solubility of hydrophilic aspirin in hydrophobic triacetin resulting in the aspirin only being in suspension that caused high burst release [90]. Therefore, triacetin is chosen as additive in borneol-based ISM formulation in this study due to the properties as mentioned above.

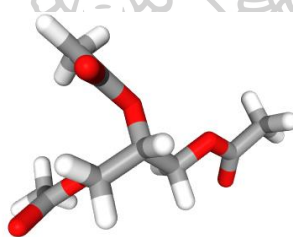


Figure 5. 3-D structure of triacetin
(gray, white and red sticks represent C, H and O atom, respectively)

Periodontitis

Nowadays, periodontal diseases affect about 20-50% of global population [93]. The 25.9% of an adult and 36.3% of an elder in Thailand have a health problem on the periodontitis with 4-5 mm depth of periodontal pocket. Over 19.8% and 12.2% of those adult and elder have the severe periodontitis (>6mm deep of periodontal pocket) which can cause more risk of infection, inflammatory, losing teeth and also affect the quality of life [94]. Moreover, it is a risk factor for cardiovascular disease such as coronary heart disease and stroke [95]. The main causes of periodontal disease are poor oral hygiene and tobacco use [3]. This disease is characterized by bleeding or swollen gums (gingivitis), pain, bad breath and destruction of surrounding structures of teeth resulting in alveolar bone loss, periodontal ligament destruction, attachment loss, gingival recession, bacteria accumulation, increased tooth mobility and eventually leading to tooth loss [3, 4]. In its early stage, called “gingivitis”, the gums can become swollen and red, and they may bleed. In its more serious form, called “periodontitis”, the gum can come away from the tooth and supporting bone, causing teeth to become loose and sometimes fall out [1]. Stages of gum disease from healthy

gums to advanced periodontitis are shown in Figure 6. Most of the pathogen of this disease are gram-negative bacteria and some of them are anaerobe bacteria, for example, *A. actinomycetemcomitans*, *P. gingivalis*, *Bacteroides forsythus*, *Prevotella intermedia*, *Campylobacter rectus*, *Eubacterium nodatum*, *Streptococcus intermedius* and *Treponema denticola* [5]. These bacterial infection can cause the periodontium (gingiva, periodontal ligament, dental cementum, and alveolar bone) damages by the bacterial by-product and immune activation [7]. Some of microbes is related to the inflammation and tissue loss including enterobacteria (such as *E. coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*), *C. albicans*, *Neisseria* spp., *Olsenella uli*, *Hafnia alvei*, *Serratia marcescens* and *Filifactor alocis* [96, 97]. Moreover, *S. aureus* could be found in periodontal pocket [98]. In healthy man, the gap between gingival and tooth is around 1 mm. However, the periodontal pocket of periodontitis patient is usually exceeding 5 mm [99]. Typically, the pH of the normal oral cavity in humans is kept close to neutrality (approximately 7.06) by saliva. In contrast, the average pH of the oral cavity in individuals with chronic periodontitis is 6.85, suggesting a slight acidemia [100].

The treatment techniques for these diseases are recommended as following: mechanical (scaling and root planning), systemic and local antibiotic treatment [101]. Several antimicrobial agents have been evaluated for their clinical and microbiological efficacy in periodontal diseases such as chlorhexidine, tetracyclines (tetracycline, doxycycline and minocycline), metronidazole, ciprofloxacin, vancomycin and etc. [9-14]. The large doses of systemic antibiotic must be taken in order to achieve sufficient concentrations in the gingival crevicular fluid of the periodontal pockets [15, 16], which may have side effects of antibiotics [17, 18] and antibiotic resistance [19, 20]. The local antibiotics delivery systems are interesting to keep a high and effective drug concentration at the target site with minimizing the side effect [102]. Nowadays, several dosage form such as films [103, 104], gel [105] including ISM systems [42, 63] have been reported for carrying antibiotics as the local drug delivery systems for periodontitis treatment. In the present study, vancomycin HCl and DH are chosen as the model drugs for local drug administration.

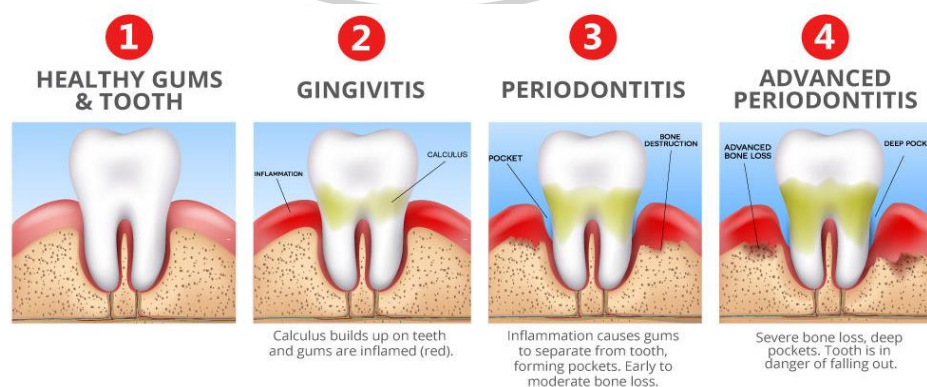


Figure 6. Stages of gum disease from healthy gums to advanced periodontitis

(Ref: <https://hydrofloss.com/blogs/hydro-floss-blog/h2-class-title-stages-of-gum-disease-h2>)

Oropharyngeal candidiasis

Oropharyngeal candidiasis is an opportunistic mucosal infection primarily instigated by the fungus *C. albicans*, although it can also be induced by other species such as *Candida glabrata*, *Candida tropicalis*, and *Candida krusei* in certain cases. [34]. The four main types of oropharyngeal candidiasis (Figure 7) are: (1) pseudomembranous (thrush), consisting of white, curd-like, discrete plaques on an erythematous background and found on the buccal mucosa, throat, tongue, or gingivae; (2) erythematous, consisting of smooth red patches on the hard or soft palate, dorsum of tongue, or buccal mucosa; (3) hyperplastic, consisting of white, firmly adherent patches or plaques that cannot be removed, usually bilaterally distributed on the buccal mucosa, tongue, or palate; and (4) denture-induced stomatitis, presenting as either a smooth or a granular erythema confined to the denture-bearing area of the hard palate, which occurs as red, fissured lesions in the corners of the mouth [106]. *Candida* species are commensals in the gastrointestinal tract. Most infections are endogenously acquired, although infections in neonates can be primary infections. Transmission can also occur directly from infected people or on fomites (objects that can harbor pathogenic organisms) [34]. *Candida* is found in the mouth of 18%–60% of healthy people in high- and middle-income countries [107]. Oropharyngeal candidiasis has a notable impact on individuals with hematological or oncological malignancies, affecting a range of 15% to 60% during periods of immunosuppression [34, 35]. Additionally, it manifests in 7% to 48% of individuals with HIV infection, and in over 90% of those with advanced disease. Notably, in severely immunosuppressed individuals, relapse rates are elevated, ranging from 30% to 50%, and relapses typically occur within 14 days of discontinuing treatment [34, 108]. Risk factors associated with symptomatic oropharyngeal candidiasis include: local or systemic immunosuppression; haematological disorders; broad-spectrum antibiotic use; inhaled or systemic corticosteroids; diabetes; wearing dentures, obturators, or orthodontic appliances; and smoking [34]. Oropharyngeal candidiasis can impair speech, nutritional intake and quality of life. It is the most common oral manifestation of HIV infection causing have overall lower levels of oral health [34, 106].

Conventionally, topical antifungals are the preferred method of treating oral candidiasis. Locally administered antifungals provide the benefit of minimized systemic exposure, thereby reducing the likelihood of adverse drug reactions or interactions. The British National Formulary (BNF) lists two options: nystatin and miconazole [109]. However, the four-times-per-day administration makes patient adherence for the requisite 7–14 days challenging. Reiterating the importance of this regular administration to patients for preventing infection can improve the patient compliance [110]. Furthermore, other antifungal drugs such as amphotericin B, clotrimazole, ketoconazole and fluconazole are currently used in oropharyngeal candidiasis in both of topical and systemic dosage form [36]. For mild oropharyngeal candidiasis, the clotrimazole troches 10 mg are recommend to be used in 5 times daily, or miconazole mucoadhesive buccal 50-mg tablet applied to the mucosal

surface over the canine fossa once daily for 7–14 days. While, the oral fluconazole 100–200 mg daily for 7–14 days is recommended for moderating severe patients. For HIV-infected patients, antiretroviral therapy is strongly recommended to reduce the incidence of recurrent infections. Moreover, disinfection of the denture is recommended in addition to antifungal therapy for denture-related candidiasis [111]. The most conventional and effective drugs for treating conditions like oral candidiasis belong to the polyenes and azoles groups, with clotrimazole being a notable example [38]. Therefore, due to its effectiveness and widespread use, clotrimazole is an intriguing and suitable choice as an antifungal model drug for this study.

In the treatment of oropharyngeal candidiasis, topical therapies with local administration are gaining popularity due to enhanced patient compliance and reduced side effects. Various topical dosage forms, including suspensions, mouth rinses, gels, and troches, are utilized for oral candidiasis. However, the rapid salivary clearance of the drug can lead to a swift decrease in drug levels in the affected area. An ideal dosage form for oral candidiasis treatment should ensure sustained drug release, providing an antifungal effect over an extended period [36]. Consequently, the application of ISM system in this study becomes compelling, as it aims to prolong drug release and address this challenge.



Figure 7. Four main types of oropharyngeal candidiasis: (A) curd-like white plaques in the oral cavity, (B) smooth red patches on the hard or soft palate, (C) firmly adherent white plaques on the tongue, and (D) granular erythema on the hard palate

(Ref: https://en.wikipedia.org/wiki/Oral_candidiasis, https://www.researchgate.net/figure/Chronic-hyperplastic-Candida-in-a-hospitalized-immunosuppressed-renal-transplant-patient_fig5_256608843)

Clotrimazole

Clotrimazole is a broad-spectrum triazole-based antifungal agent. Its mode of action involves inhibiting the enzyme cytochrome P450 14 α -demethylase, essential for fungal cell membrane synthesis. This inhibition leads to damage and alterations in the permeability of the fungal cell membrane, resulting in leakage and loss of crucial intracellular compounds and ultimately causing cell lysis [36, 112]. Clotrimazole exhibits antimycotic activity against various pathogens, including *Candida* spp., *Cryptococcus* spp., dermatophytes, and *Aspergillus* spp. [113]. Moreover, it demonstrates activity against certain Gram-positive bacteria [114, 115]. Commonly employed for skin, oral, and vaginal candida infections, clotrimazole is typically administered topically or as oral or vaginal troches, with limited systemic absorption.

[114]. When used intraorally as a troche, only small amounts are absorbed and then metabolized in the liver [116]. Nevertheless, it has been associated with transient and asymptomatic serum aminotransferase elevations during therapy, but it has not been linked to instances of clinically apparent acute liver injury [112]. Whilst, topical forms of clotrimazole, available as over-the-counter medications, are generally considered safe with minimal serious side effects. In cases where local irritation occurs, it is typically not severe enough to necessitate discontinuation of therapy. [114, 115] Therefore, the recommended mode of administration for this drug is local, typically through 10-mg lozenges (troches). These troches are designed to be dissolved slowly in the mouth, and the usual frequency is five times daily for a duration of 7 to 14 days. Notably, these troches are FDA approved for use in children aged 3 years and older [116]. Clotrimazole is characterized by its lipophilicity, with a log P value of 0.5. It exhibits free solubility in alcohol and is soluble in polyethylene glycol 400, acetone, and chloroform [112]. However, it demonstrates poor aqueous solubility (0.49 µg/ml) [117], a factor that may impact its antimycotic activity. The molecular structure of clotrimazole is depicted in Figure 8.

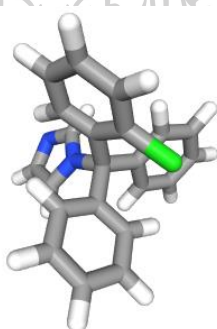


Figure 8. 3-D structure of clotrimazole

(gray, white, blue and green sticks represent C, H, N and Cl atom, respectively)

Vancomycin HCl

Vancomycin HCl is a hydrophilic molecule (log P= -3.1) with a molecular weight of approximately 1,450 Daltons. The structure of vancomycin HCl is showed in Figure 9. It appears as a white solid which is soluble in water (200 mg/ml); moderately soluble in methanol; insoluble in higher alcohols, acetone, [118, 119]. Vancomycin HCl diluted with water for injection (10 mg/mL) was stable for at least 84 days at 4°C and was stable at 25°C for 47 days [120]. Typically, UV-spectrophotometry and high-performance liquid chromatography (HPLC) with the UV wavelength of 280 nm are employed to identify the presence of vancomycin [121-123].

Vancomycin is a potent glycopeptide antibiotic used primarily in the treatment of gram-positive bacterial infections either susceptible or resistant Gram-positive bacteria especially, *S. aureus* (including MRSA), *Staphylococcus epidermidis*, *Streptococci* spp., viridans group *streptococci* and *Enterococcus* spp. The mechanism of antibacterial action of vancomycin is to inhibit bacterial cell wall synthesis, to

disturb the permeability of bacterial cell membrane and RNA synthesis. [22, 124-126]. It currently represents the gold standard against MRSA infections. At present, there is evidence indicating that vancomycin can inhibit periodontal microbes, including *A. actinomycetemcomitans*, *P. gingivalis*, *Bacteroides forsythus*, *Prevotella intermedia* and *S. intermedius* [23-26]. This drug has very low oral bioavailability (<10%) and its tissue distribution is 30% of serum [127]. Moreover, it can cause a severe side effects such as hematologic effects, cardiac arrest, shock, edema, phlebitis, nephrotoxic and red-man syndrome upon systemic administration [27]. Thus, the localized drug delivery system by injecting drug into the periodontal pocket seems more appropriate. In recent developments, ISM with vancomycin HCl-loaded utilizing lauric acid as the polymer have been reported for the treatment of periodontitis. These formulations demonstrated effective antimicrobial activity along with prolonged drug release lasting over 6 days, showcasing ease of use [128]. Additionally, fatty acid ISM with vancomycin HCl processed antimicrobial activities against various pathogens, including *E. coli*, *S. aureus*, including MRSA [129]. In addition, vancomycin HCl has been developed for knee joint infection treatment which sustainably released over 6 days and presented effective antimicrobial activities against *S. aureus* including MRSA [130]. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration of vancomycin for *S. aureus* is 12.5 and 25 $\mu\text{g/ml}$, respectively [131]. The MIC of vancomycin against MRSA is 2- 4 $\mu\text{g/mL}$ [126]. Furthermore, the susceptibility of *P. gingivalis* to Vancomycin HCl of 15.7 $\mu\text{g/ml}$ has been reported [132]. Therefore, vancomycin was chosen as the antimicrobial drug for the treatment of periodontitis owing to its broad-spectrum effectiveness and its potential to counteract drug resistance that may develop against other traditional antibiotics in the future.

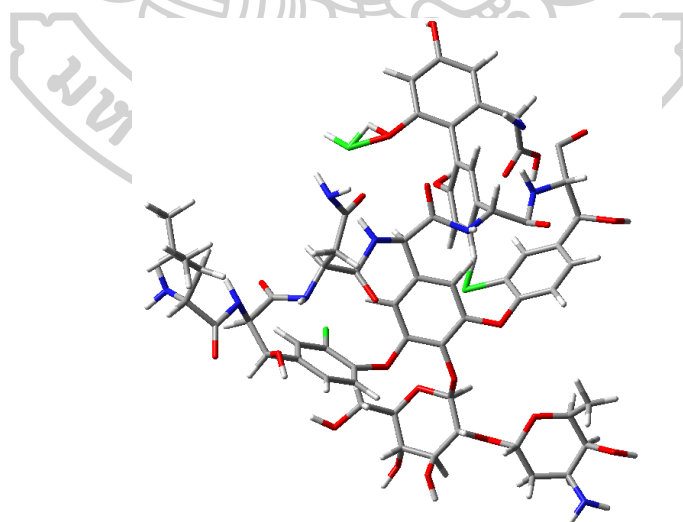


Figure 9. 3-D structure of vancomycin

(gray, white, blue, red and green sticks represent C, H, N, O and Cl atom, respectively)

Doxycycline hyclate

Doxycycline hyclate (DH) ($2C_{22}H_{24}N_2O_8 \cdot 2HCl \cdot H_2O \cdot C_2H_6O$; molecular weight = 545) is the hyclate salt form of doxycycline, broad-spectrum tetracycline antibiotic exhibiting antimicrobial activity. It is soluble >76.9 ug/mL in water and decomposed by photolysis, hydrolysis and thermal degradation. The structure of doxycycline is shown in Figure 10. DH is one of the most commonly used treatments for periodontitis. It is bacteriostatic drug which binds reversibly to the 30S ribosomal subunit, possibly to the 50S ribosomal subunit as well, thereby blocking the binding of aminoacyl-tRNA to the mRNA-ribosome complex. This leads to an inhibition of protein synthesis. In addition, this agent has exhibited inhibition of collagenase activity [133, 134]. It is given orally or parenterally and is absorbed by gastrointestinal tract and excreted by kidneys. DH can cause undesired side effects such as nausea, diarrhea, rash, and photosensitivity in both children and adult. It also causes the growth retardation and enamel hypoplasia. Furthermore, DH is contraindicated from second half of the pregnancy to seven years in children because it have harmful effects of the bone and tooth development [133].

Typically, the oral regimen for periodontitis is 250 mg of drug four time a day for up to 9 months whereas the dose of local treatment depends on the shape, size and number of pockets [135]. To reduce the frequency of drug administration, the sustainable drug delivery system has been developed. Atridox[®] is a one of the most popular commercial products which containing 5 or 10% of DH using poly (dl-lactide) as polymer dissolved in NMP allows for controlled release of DH for 7 days [8, 33]. The use of Atridox[®] could provide a high level of drug (250 mg/mL) in the gingival crevicular fluid for a period of 7 days and 10–20 mg/mL was still present for 3–5 days after the polymer had been removed [136]. Furthermore, Atrisorb-D[®] FreeFlowTM which containing 4% DH is licensed for local bacterial growth inhibition as healing takes place and can control release for a period of 7 days [52]. As previous studies, ISM formulation containing DH as model drug with various types of polymers such as Eudragit RS, ethyl cellulose and bleached shellac exhibited effective inhibition of *S. aureus*, *E. coli*, *S. mutans* and *P. gingivalis* [62, 63, 65, 66]. Furthermore, vancomycin release was achieved from various polymeric ISM, comprising 20% ethyl cellulose, 40% Eudragit RS, 30% bleached shellac, 10% cholesterol, along with 10% benzyl benzoate, over different durations—9 hours, 16 hours, 2 days, and 10 days, respectively [29, 63, 65, 66]. All these formulations were easy to inject into the target site because of their minimal force of injectability and could transform from solution into matrix-like structures for drug retention.

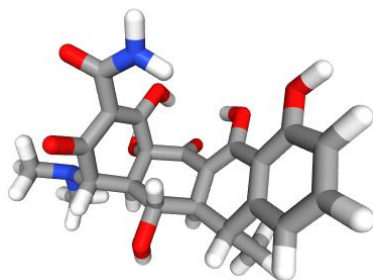


Figure 10. 3-D structure of doxycycline

(gray, white, blue and red sticks represent C, H, N and O atom, respectively)

Advanced techniques for exploring phase inversion mechanism

In the past, evaluations of ISM were limited to physicochemical tests, analyses of matrix formation morphology, and drug release assessments. Nowadays, several advanced imaging techniques have been developed to characterize the phase transformation mechanism in real-time such as including optical, spectroscopic, and medical imaging-based strategies. Imaging techniques that have found use in drug development include Raman spectroscopy and imaging, Magnetic Resonance Imaging (MRI), X-ray micro-Tomography (XmT), Fourier Transform Infrared (FTIR) imaging, Near Infra-red (NIR) imaging, fluorescence imaging, Terahertz (THz) imaging, Confocal Laser Scanning Microscopy (CLSM), and UV-vis imaging [137].

Diffusion weighted imaging (DWI), one type of MRI, is a method of signal contrast generation based on the differences in Brownian motion [138]. It is a functional imaging technique that displays information about the extent and direction of random water motion. Images can be sensitive to large or small displacements of water; therefore, macroscopic water flows and microscopic water displacements can be depicted [139]. Typically, this technique has been used to evaluate the molecular function and micro-architecture of the human body due to its noninvasive and safe technique [138]. It was developed to study the water direction in matrix diffusivity ISM *in situ* and provide novel functional information about matrix behavior through its ability to noninvasively characterize the transport properties within the matrix [140]. However, ISM in this study may not be investigated by this method since the solvent of formulation and the water in environment are both liquid and have a similar motion pattern. It may not be possible to differentiate the motions of their two solvents.

Additionally, CLSM has been utilized in a number of different pharmaceutical applications such as the visualization of the early gel layer formation in tablet matrices [141], visualization and identification of the percolation threshold [142], and the designing of matrices with improved resistance to dissolved and dietary sugars [143]. Fluorescence imaging has been applied to the swelling of polymers to investigate molecular diffusion and interactions between chromophores and solvent molecules [144]. Moreover, various methods can also be employed to uncover and comprehend phase inversion phenomena, including optical techniques, spectroscopy, and computer-based molecular dynamic (MD) simulation [138, 145]. Nevertheless,

some of these techniques are interesting for applying to characterize the mechanism of ISM. For this dissertation, the contact angle, surface tension, UV-vis imaging and MD simulation will be employed and conducted for mechanistic underpinning formation of non-solvent induced phase inversion-based borneol ISM.

Application of contact angle and surface tension measurement to simulate phase inversion mechanism.

Surface tension is a phenomenon wherein the surface of a liquid in contact with a gas behaves like a thin elastic sheet. This property is a potent parameter that offers insights into surfaces and intermolecular interactions. On the other hand, the contact angle is defined as the angle between the surface of the liquid and the contour of the contact surface. This angle serves as an indicator of the wetting and spreading ability of the liquid on the surface.

Nowadays, several researches mention about emerging role of surface tension and contact angle of the binary mixtures comprising DMSO/other solvents. For example, the surface tension decreased non-linearly when adding alcohol into DMSO mixtures. In addition, the intermolecular energy between alcohol and DMSO molecules decreased with longer alkyl chain of alcohol due to the lesser interaction between alcohol and DMSO molecules [85, 146]. The thermodynamic-based equations such as Li-Wang-Wang (LWW) model which proposed by C. Li, W. Wang and Z. Wang were selected to estimate the interaction energy between components [146, 147]. The dentist researcher mentioned the effect of DMSO on dentin collagen. DMSO-treated dentin collagen reduced the contact angle of water and promoted the wettability on dentin surface for infiltration. DMSO lowers water surface tension and cohesive forces by breaking down water self-associative tendencies and forming the strong hydrogen bond complexes with water molecules due to stronger interaction between DMSO-water [86].

These conceptual obtained data will be useful as a guide and inspire the idea of studying the effects of contact angle/surface tension of borneol-based formulations for periodontitis treatment. The understanding of these behaviors expects to provide novel information about molecular interaction between each component in formulation and wettability behavior of formulation on the different surfaces such as glass slide, acrylic, and plastic. The determination of contact angles was also performed on surfaces containing water, such as agarose gel, and on tissues including the tongue, gum, and buccal mucosa from porcine tissues, which mimic human oral cavity tissues due to their similarity to human tissue [148-151]. Determination of formulations wettability by using these tissues as the surface has not been reported previously.

UV-vis imaging

Traditional drug release testing relies on measuring the concentration of the active drug either in the overall solution or in periodic samples, often analyzed using UV spectrophotometry. These methods can disrupt the release process and do not

offer significant insights into how drug release occurs. Consequently, there has been a growing interest in real-time monitoring approaches to better understand drug release and phase transition processes. As a result, UV-vis spectrophotometry, in combination with advanced imaging methods, is now being used in drug release testing [137, 152]. UV-vis imaging, an emerging technology, enables the visualization of substance diffusion and related events at the interface between solid and liquid, while also providing concentration measurements. This technology yields valuable insights into drug diffusion behavior that are typically not attainable through offline measurements [137].

Many of the important applications of UV/Vis spectrophotometry in pharmaceutical contexts rely on light within the wavelength range of 190 nm to 800 nm, comprising UV light (190 to 400 nm) and visible light (400 to 800 nm) [153]. The fundamental principle of UV/Vis spectrophotometry and UV/vis imaging hinges on the ability of drug substances to absorb light. This absorption occurs when an electron is elevated to a higher energy state by the energy carried by an incident photon. The extent of light absorption within the UV/vis spectrum is determined by the chromophores within the molecule. The primary purpose of spectrophotometry is to quantify compounds within a solution. However, distinct molecular structures yield varying absorbance spectra, which can serve as a supplementary method for identifying compounds [153]. Quantification operates based on the absorption of light, following the Beer-Lambert law, which asserts that the amount of absorbed light is directly proportional to the concentration of absorbing species present in the medium. This law applies to monochromatic radiation. In practice, though, polychromatic radiation is utilized, which can result in deviations from the Beer-Lambert law [137, 153]. Other factors contributing to deviations from this law are linked to instrumentation, such as stray light, or characteristics of the analytes. Elevated analyte concentrations can induce changes in refractive index, chemical reactions, self-association, or interactions between molecules (e.g., drug-surfactant interactions) in the solution, leading to non-linearity. The Beer-Lambert law, unaffected by interactions affecting the absorbance spectra, is applicable to mixtures of molecules or analytes. This, along with the direct proportionality between absorbance and concentration, makes UV/vis spectrophotometry and imaging practical and relatively straightforward tools for quantifying drugs. Spatially and temporally resolved absorbance measurements are used in generating images through UV-vis imaging [137, 154].

The fundamental components of UV-vis imaging equipment typically consist of a light source and a complementary metal oxide semiconductor (CMOS) camera chip coated with a phosphorous layer. Depending on the instrument's design and the type of light source used (usually light-emitting diodes or LEDs), additional components may include bandpass filters for selecting specific wavelengths, fiber optic cables, collimating lenses to direct light to the measurement area, and a quartz cuvette [154]. Figure 11 illustrates the essential components of a UV imaging instrument. The phosphorous layer's role is to convert UV light into the visible

wavelength range, where the CMOS chip operates [155]. The Sirius SDI UV imaging system and its counterpart, the ActiPix D200, employ a pulsed LED lamp as the light source and a sensor chip measuring $25 \times 25 \text{ mm}^2$, CMOS chip with an effective pixel size of $13.75 \times 13.75 \mu\text{m}^2$. These instruments offer multiple bandpass filters for wavelength selection, which can be used simultaneously [152]. A noteworthy feature of these devices is their capability to capture images in the UV range. However, since the CMOS chip operates in the visible range, the system can function effectively at both UV and visible wavelengths, making the term Uv-vis imaging equally appropriate [152, 156].

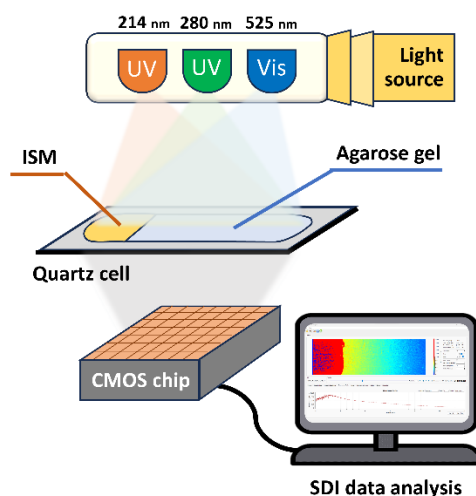


Figure 11. Schematic representation of the UV-vis imaging instrument components.

The UV-vis imaging technique has been applied to observe and analyze the intricate physicochemical events occurring within ISM formulations. It is a powerful tool for tracking and visualizing complex phenomena within pharmaceutical formulations [154]. Sun and colleagues [156] utilized UV-vis imaging along with light microscopy to simultaneously monitor drug release and the phase separation of ISM with PLGA implants from the same sample. This technique holds promise for studying phase inversion phenomena, including matrix formation and drug diffusion, within a reasonable timeframe. Moreover, this technique enables spatial mapping of substance distribution using unique spectral characteristics, leading to monitoring multiple phenomena like matrix formation, solvent diffusion, and drug release dynamics in real-time [154, 156]. Furthermore, the data gleaned from this technique enables an understanding of the fundamental mechanism that is crucial for tailoring ISM properties to achieve desired therapeutic outcomes.

Molecular dynamics simulation

Recently, Molecular Dynamics (MD) simulation has become an essential tool in studying biological and chemical systems, providing insights into the structure, dynamics, and thermodynamics of biomaterials [157]. MD is a computer simulation technique where atoms or molecules interact over a range of time scales, from

femtoseconds to milliseconds. Given the voluminous nature of molecular systems, analyzing the properties of complex systems using traditional methods becomes impractical. Hence, MD is applied to determine system properties [157-159]. This technique calculates the properties of the system by tracking the dynamics of atoms or molecules using the equations of motion and force fields, resulting in a substantial number of calculations. Various integrators, such as the Verlet method, Range-gatta method, and Gear predictor-corrector, can be employed for tracking dynamics [160].

In contemporary research, Molecular Dynamics (MD) has found extensive application in comprehending allostery, molecular docking, drug design, and refining structure predictions [161]. Understanding how a ligand binds to its molecular target is crucial for unraveling its function. In the pharmaceutical field, MD is practically applied in docking strategies, proving valuable during the drug design phase [162, 163]. MD has been instrumental in elucidating and exploring diverse phenomena, including the mechanism of hydrophobic gating in ion channels [159] and water permeation via aquaporin [164]. Moreover, MD has been employed to simulate the nucleation and crystallization of materials under various conditions. For instance, a couple of simulations were utilized to unveil the microscopic mechanism of crystal nucleation and the growth behavior of methane hydrate in porous media [165]. The arrangement of lauric acid at the water-hexane and water-carbon tetrachloride interfaces, influenced by interface-specific phenomena and the orientation and hydrogen bonding patterns of the interface/near-interface molecules, was also studied using MD simulation [166].

MD begins with obtaining initial atom coordinates, either from experimental structures or similar modeling data [167]. Subsequently, forces acting on each atom (force fields) are calculated as part of the initial model construction. These force fields account for various molecular features, such as springs for bond length and angles, periodic functions for bond rotations, and the Coulomb's law for van der Waals and electrostatic interactions [160]. Accelerations and velocities needed to rearrange atom positions are then calculated using the equations of motion (based on classical Newton's law of motion) through the force field. Several widely used simulation codes, including CHARMM [168], NAMD [169] and AMBER [170] have long been compatible with the messaging passing interface (MPI).

The term "Amber" encompasses a suite of programs designed to coordinate, execute, and analyze Molecular Dynamics (MD) simulations. The output of these programs consists of two text files: a coordinate (prmcord) file containing the Cartesian coordinates of all atoms, and a parameter-topology (prm-top) file that holds all other essential information necessary for computing energies and forces. This includes details such as atom names and masses, force field parameters, lists of bonds, angles, and dihedrals, along with additional bookkeeping information [171]. Despite MD simulations involving computations of properties for systems with very small numbers of atoms or molecules, recent studies have shown that the results obtained from MD simulations are not significantly different from macro-level experiments. [160].

Typically, it is difficult to understand the phase inversion mechanism of ISM and its phenomena through the traditional experimental process. While MD technique has developed to understand the movement of each component of formulation upon contact with water in molecular level including the structural changes and bonding of their component. MD simulation appears to be a more appealing approach for employing computer models to aid in comprehending phase inversion phenomena. MD simulations were successfully applied to investigate the diffusion of vancomycin through various fatty acid matrices in an ISM system [145]. Therefore, employing MD simulation techniques can provide support and establish connections with the macro-level properties of phase inversion process. This integration aids in fully understanding the phase inversion mechanism, elucidating the mechanistic underpinnings of the formation of non-solvent-induced phase inversion-based borneol ISM for the treatment of infectious diseases in the oral cavity.

Scope of research work

In this research, borneol was chosen as the matrix-forming agent for the ISM. Whereas, clotrimazole served as the model drug during the development of an oral ISM spray for oropharyngeal candidiasis treatment. A systematic evaluation was conducted to determine the optimal borneol concentration for the ISM spray. This evaluation encompassed a range of assessments, including physicochemical testing, analysis of matrix morphology, drug release studies, and antimicrobial activity testing. Additionally, a borneol-based ISM was developed for delivering vancomycin HCl to crevicular pockets for periodontitis treatment. The study explored various concentrations of the borneol to establish the most suitable concentration for this formulation, using conventional ISM testing methods. Furthermore, the investigation delved into the role of triacetin in doxycycline hyclate-loaded borneol ISM, aiming to extend drug release while preserving antimicrobial efficacy and matrix formation. In addition to formulation development, this research provided a thorough exploration of the mechanistic phase inversion and related phenomena associated with non-solvent induced phase inversion-based borneol ISM. To achieve this, advanced techniques such as UV-vis imaging and MD simulation were employed, offering a comprehensive understanding of the underlying mechanisms of this ISM formulation.

CHAPTER 3: MATERIALS AND METHODS

Equipment

1. Aluminum pans and covers (Perkin Elmer, Shelton, USA)
2. AMBER 20 software package (University of California, SF, USA)
3. Anaerobic incubator (Forma Anaerobic System, Thermo Scientific, Ohio, USA)
4. Analytical balance (CP224S, Sartorius, Germany)
5. Analytical balance (PA4102, Ohaus, USA)
6. Cylindrical cup (made from porcelain with diameter 1 cm and height 1.2 cm)
7. DD solver add-in program for Microsoft Excel (Redmond, WA, USA)
8. Demountable quartz cell (internal dimensions: $8.0 \times 40.0 \times 0.2 \text{ mm}^3$ (W $\times$ L $\times$ H)) (Starna Scientific Ltd., Hainault, Essex, UK)
9. Desiccators (Biologix Research Company, USA)
10. Differential scanning calorimeter (DSC) (DSC 8000, Perkin Elmer, USA)
11. Drop shape analyzer (FTA 1000, First Ten Angstroms, Newark, CA, USA)
12. Filter paper (Whatman no.1, United Kingdom)
13. Filter set (Nylon membrane, 47 mm, 0.45 μm , Lot 43920100, ANPEL Laboratory Technologies (Shanghai) Inc., China)
14. Fourier-transform infrared (FTIR) spectrophotometer (Nicolet 4700, Madison, USA)
15. Franz diffusion cell (DHC-6T, Logan instrument corp., Somerset NJ, USA)
16. Freeze dryer (Triad, Labconco, Missouri, USA)
17. Gauss View program (GaussView 26, Semichem Inc., Shawnee Mission KS, USA)
18. Glass slide
19. Heating incubator (Model NB-205, N-Biotek, Gyeonggi-do, Republic of Korea)
20. High-performance liquid chromatography (HPLC) (Agilent 1220 Infinity II, Agilent Tech, USA)
21. High-speed camera (Photron, FASTCAM-APX RS, Chiyodaku, Japan)
22. Incubator (BPH-9042, Shenzhen Kingdak, China)
23. Inverted stereomicroscope (Nikon Eclipse TE2000S, Nikon, Kawasaki, Japan)
24. LC Column (Eclipse XDB-C18, 4.6 x 150mm, 5 μm , Agilent Technologies, USA)
25. Magnetic stirrer (C-MAG-HS7, IKA, Germany)
26. Microbiological safety cabinet class II (safe 2010, Holten, EU)
27. Micropipette 2-20 μl , 20-200 μl , 100-1000 μl and micropipette tip
28. Needle (27 gauge)
29. Nylon membrane 0.45 μm . 47 mm. (WhatmanTM, M&P IMPEX Ltd., Bangkok, Thailand)
30. Petri dish glass (9 cm, 15cm) & petri dish plastic (15 cm)

31. pH meter (Seven compact, Mettler Toledo, Japan)
32. Plastic syringe (1 mL, 3 mL, 5 mL, 10 mL)
33. Powder X-ray diffractometer (PXRD) (Mini Flex II, Rigaku corp, Japan)
34. Pycnometer (Densito 30PX, Mettler Toledo Ltd., Vernon Hills, IL, USA)
35. Quartz cuvette (path length 3 mm) for UV-vis spectrophotometer
36. Rheometer (Brookfield DV-III Ultra, Brookfield Engineering Laboratories Inc., Middleboro, MA, USA)
37. Scanning electron microscope (SEM) (TESCAN MIRA3, Czech Republic)
38. SDI Data Analysis software (version 2.0, Paraytec Ltd, York, UK)
39. SG Ultra Clear 2002 water system (SG Water GmbH, Barsbüttel, Germany)
40. Sonicator (ELMA Transsonic Cleaning Baths 890/H, Germany)
41. Stainless cylinder cup (6 mm)
42. Stereomicroscope (SZX10, Olympus corp., Tokyo, Japan)
43. Surgical blade & surgical scissors
44. Syringe filter (Preclean™ 13 mm, Nylon membrane, 0.45 µm, Lot 41890480, ANPEL Laboratory Technologies (Shanghai) Inc., China)
45. Texture analyzer (TA.XT plus, Stable Micro Systems, UK)
46. Thermal gravimetric analysis (TGA) (Pyris TGA, Perkin Elmer, USA)
47. UCSF Chimera (University of California, SF, USA)
48. UV-vis imaging system (D200 ActiPix, Paraytec Ltd, York, UK)
49. UV-visible spectrophotometer (Cary 60 UV-vis, Model G6860A, Agilent, Selangor, Malaysia)
50. Visual Molecular Dynamics (VMD) software (University of Illinois Urbana-Champaign, IL, USA)
51. Vortex mixer (KMC-1300V, Vision scientific, Korea)

Materials

1. 2-pyrrolidone (PYR) (lot no. BCBP7338V, Fluka Analytical, Germany)
2. Acetonitrile, HPLC grade (lot no. K3H111, Fisher Chemical, Korea)
3. Agarose (lot no. C7031-17, Vivantis, Selangor Darul Ehsan, Malaysia)
4. *Aggregatibacter actinomycetemcomitans* ATCC 29522
5. Borneol (Chareorsuk-Osot, Nakhon Pathom, Thailand)
6. Brain heart infusion (BHI) (lot no. 0270845, Bacto, USA)
7. Brain heart infusion agar (BHA) (lot no. 0298038, Bacto, USA)
8. *Candida albicans* ATCC 17110, *C. krusei* TISTR 5259, *C. lusitaniae* TISTR 5156 and *C. tropicalis* TISTR 5306.
9. Chocolate agar (Department of medical science, Ministry of public health, Thailand)
10. Clotrimazole (lot no. 20001304, Lambrochem, Cologno Monzese, Italy)
11. Clove oil (lot no. 11910009, PC Drug Center, Thailand)
12. Dimethyl sulphoxide (DMSO) (lot no. 1862992, Fisher Chemical, UK)
13. Doxycycline hyclate (lot no. 20071121, Huashu Pharmaceutical Corporation, China)

14. *Escherichia coli* ATCC 8739
15. Ethanol, AR grade (lot no. 195125-1223, QReC, New Zealand)
16. Fully refined paraffin wax (lot no. 7119K18, Nippon Seiro, Japan)
17. Methanol, HPLC grade (lot no. 2040269, Fisher Chemical, UK)
18. Methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 43300,
19. *N*-methyl pyrrolidone (NMP) (lot no. 144560-118, QReC, New Zealand)
20. *Porphyromonas gingivalis* ATCC 33277
21. Potassium bromide, For IR spectroscopy (lot no. I297362002, Loba Chemie PVT, India)
22. Potassium dihydrogen orthophosphate (lot no. E23W60, Ajax Finechem, Australia)
23. Sabouraud dextrose agar (SDA) (lot no. 7312647, Difco, USA)
24. Sabouraud dextrose broth (SDB) (lot no. 6345690, Difco, USA)
25. Sheep blood agar (Department of medical science, Ministry of public health, Thailand)
26. Sodium hydroxide (lot no. AF310204, Ajax Finechem, Australia)
27. *Staphylococcus aureus* ATCC 25923, *S. aureus* ATCC 6923 and *S. aureus* ATCC 6538
28. The bulge of the porcine (general slaughterhouse, Nakhon Pathom, Thailand)
29. Triacetin (lot no. A0375797, Acros Organics, Germany)
30. Tryptic soy agar (TSA) (lot no. 7341698, Difco, USA),
31. Tryptic soy broth (TSB) (lot no. 8091999, Difco, USA),
32. Vancomycin HCl (lot no. WXBB5169V, Sigma-Aldrich Co., USA)

Experimental

1. Preparation of borneol-based ISMs

1.1. Clotrimazole-loaded borneol-based ISM as oral sprays for oropharyngeal candidiasis treatment

Various concentrations of borneol (B) solutions ranging from 20% to 50% w/w, mixed with 1% w/w clove oil (C) and 1% w/w clotrimazole (Z) dissolved in NMP (N) (abbreviated as 20BZCN-50BZCN), were prepared by mixing continuously for 3 hours with a magnetic stirrer until clear solutions were obtained. Moreover, CN (clove oil in NMP), ZN (clotrimazole in NMP), ZCN (clove oil and clotrimazole in NMP), and 40BN (40% w/w borneol in NMP) were prepared using the same method as the control group. The components of ISMs are shown in Table 1.

Table 1. Composition of clotrimazole-loaded ISM formulation

Formulation	Amount (%w/w)			
	Clotrimazole	Clove oil	Borneol	NMP
NMP	-	-	-	100
CN	-	1	-	99
ZN	1	-	-	99
ZCN	1	1	-	98
20BZCN	1	1	20	78
30BZCN	1	1	30	68
40BZCN	1	1	40	58
50BZCN	1	1	50	48
40BN	-	-	40	60

1.2. Borneol-based ISM in various solvents

40%w/w borneol was dissolved in different organic solvents including DMSO, NMP, PYR and ethanol, namely, DB, NB, PB and EB, respectively. These formulations were prepared by mixing continuously for 3 h with a magnetic stirrer until clear solutions were obtained.

1.3. Vancomycin HCl-loaded borneol-based ISM for periodontitis treatment

The different concentrations of borneol (ranging from 10% to 70% w/w) were dissolved in DMSO by continuous mixing for 3 hours with a magnetic stirrer until clear solutions were obtained. Additionally, 1% w/w vancomycin HCl (V) was introduced into the selected optimal formulation (40% borneol). The mixture was dissolved in a DMSO solution, abbreviated as VDB. As a control, a formulation consisting of 1% w/w V dissolved in DMSO (VD) was also prepared. The components of formulations are shown in Table 2.

Table 2. Composition of vancomycin HCl-loaded ISM formulation

Formulation	Amount (%w/w)		
	Vancomycin HCl	Borneol	DMSO
DB10	-	10	90
DB20	-	20	80
DB30	-	30	70
DB40	-	40	60
DB50	-	50	50
DB60	-	60	40
DB70	-	70	30
VDB	1	40	59
VD	1	-	99

1.4. DH-loaded borneol-based ISM using triacetin as additive solvent for periodontitis treatment

Various concentrations of triacetin between 2.5-25%w/w mixing with 40%w/w borneol and 5%w/w DH dissolved in NMP (N) and DMSO (D) (abbreviated as N3-N8 and D3-D8, respectively) were prepared by mixing continuously for 3 h with a magnetic stirrer until clear solutions were obtained. Moreover, 5% DH mixing with 40% borneol dissolved in NMP and DMSO (abbreviated as N2 and D2, respectively) and 5% DH dissolved in NMP and DMSO (abbreviated as N1 and D1, respectively) were prepared using the same method. The compositions of formulations are shown in Table 3.

Table 3. Composition of DH-loaded ISM formulation

Formulation*	Amount (%w/w)			
	Doxycycline hyclate	Borneol	Triacetin	Solvent (NMP/DMSO)
N1/D1	5	-	-	95
N2/D2	5	40	-	55
N3/D3	5	40	2.5	52.5
N4/D4	5	40	5	50
N5/D5	5	40	10	45
N6/D6	5	40	15	40
N7/D7	5	40	20	35
N8/D8	5	40	25	30

* The abbreviation N or D depends on the solvent (N = NMP and D = DMSO)

2. Physical properties evaluation of ISM

2.1. Physical appearance

The appearance of formulations such as clarity, homogeneity, and precipitation were observed by visual observation.

2.2. pH and density

The pH values of the formulations were determined using a pH meter (Seven compact, Mettler Toledo, Japan) with a sample size of $n = 3$. These values were then compared with those of the pure solvents (DMSO or NMP). Additionally, the densities of both the solvent and formulations were measured at room temperature using a pycnometer (Densito 30PX, Mettler Toledo, Japan) with a sample size of $n = 3$.

2.3. Viscosity and rheological behavior

The viscosities of all formulations were determined using a rheometer (Brookfield DV-III Ultra, Brookfield Engineering Laboratories Inc., Middleboro, MA, USA) with spindle number 40 at 150 rpm ($n = 3$). Additionally, the rheological

behavior of the formulations was assessed by examining the relationship between shear stress and shear rate ($n = 3$).

2.4. Surface tension, contact angle, and wettability

Surface tension was determined by observing the change in the shape of a pendant drop of the formulation suspended in air, utilizing a drop shape analyzer (FTA 1000, First Ten Angstroms, USA) with a sample size of $n = 3$. Additionally, the contact angle was measured five seconds after dropping formulation samples on various surfaces, including glass slides, paraffin, acrylic, low-density polyethylene (LDPE), agarose gel (0.6% agarose in PBS pH 6.8), and freshly porcine gum, tongue, and buccal mucosa. This was carried out using the same drop shape analyzer with a pump-out rate of $1.9 \mu\text{L/s}$ ($n = 3$). The contact angle degree was then calculated based on these images. The data collected from these experiments provided valuable insights into the wettability of ISM solutions on the different test surfaces.

The porcine cheek, gum, and tongue bulges were acquired from a local slaughterhouse (Nakhon Pathom, Thailand). The buccal mucosa was obtained by carefully removing the subcutaneous fatty layer and connective tissues from the porcine cheek bulge. The freshly prepared tissues underwent a wash with PBS (pH 6.8) and were wrapped in aluminum foil for subsequent use. Additionally, the freshly prepared porcine gum, tongue, and buccal mucosa were observed for their morphology using a stereomicroscope (Motic SMZ-171 Series). Following this, they were lyophilized in a freeze dryer (Triad, Labconco, Missouri, USA), coated with gold sputter, and examined under a scanning electron microscope (SEM) (TESCAN MIRA3, Czech Republic) at an accelerating voltage of 15 kV.

3. Evaluation of ISM Performance

3.1. Injectability and mechanical properties

The injectability test was measured to indicate the ease of injection. The compression mode of a texture analyzer (TA.XT plus, Stable Micro Systems, UK) was applied to determine an expelling force of formulation from a 1 mL plastic syringe coupled with a 27-gauge needle. The upper probe of this instrument pressed the syringe plunger at a constant force of 0.1 N and speed of 1.0 mm/s until the syringe barrel base was reached. The maximum expelling force, measured as the force required for injecting sample fluid from a syringe via the needle, was recorded. The energy required for expelling was determined from the force-displacement profiles.

The mechanical properties of the obtained borneol matrix after complete phase inversion were assessed using the same instrument. A 0.6% agarose solution, prepared by dissolving agarose in PBS (pH 6.8), was poured onto Petri dishes to create an agarose gel (height of gel = 1 cm). Once the gel set, a cylinder cup was used to cut a 300 μL hole (diameter of 7 mm) at the center of the Petri dish. The sample (150 μL) was dropped into this agarose hole and left for 3 days to ensure complete phase inversion and matrix formation. Using a texture analyzer, a spherical probe (diameter

= 5 mm) was slowly pressed (constant speed = 0.5 mm/s) into the matrix. Once the penetration depth reached 1.5 mm, the probe was held (hold time = 60 s) and then moved backward (speed = 10 mm/s). For analysis, the relationship between the applied force and the displacement of the probe was plotted over time. The maximum penetration force, remaining force and adhesion force were recorded. Furthermore, the mechanical property was calculated by a ratio of the remaining force to the maximum penetration force. All formulations were assessed in triplicates at room temperature.

3.2. Spray pattern

The test was exclusively applied to ISM spray formulations. It was not conducted for ISM formulations intended for delivery to the periodontal pocket. The high-speed camera (Photron, Fastcam-APX RS, Fujimi, Chiyodaku, Japan) was used to capture photos as the formulations were sprayed through the spray nozzle into the air. All formulations were tested for their spraying pattern with the high-speed camera and their spray angle was measured ($n = 3$) to indicate and compare the spray behavior. The speed for taking the photo was 1/3000 sec and the photo was selected at the order of 120 which the detection of spray angle was performed at the position close to the spray nozzle.

4. Characterization of matrix-forming behavior

4.1. Matrix formation behavior at the macroscopic level

In the case of using ISM in a crevicular pocket, a prepared ISM solution of 1 mL was injected through a 27-gauge stainless steel needle into a test tube containing 5 mL of PBS at pH 6.8. This procedure was performed to investigate the matrix formation behavior of borneol matrix at the macroscopic level following administration *via* a needle. The images were captured for 25 min after injection.

Additionally, the borneol matrix formation behavior in an artificial crevicular pocket was also observed at the macroscopic level. Agarose gel (0.6% w/v) was employed as a surrogate to simulate an artificial crevicular pocket in humans. It was prepared by dissolving 0.6 g of agarose powder in 100 mL of PBS (pH 6.8). Subsequently, the agarose mixture solution was heated on a hotplate at 120 °C for boiling until the agarose had fully dissolved. The resultant hot agarose solution was poured into the Petri dishes (agarose gel height of 1 cm in Petri dishes). After the gel set, a 300 μ L hole with a 7 mm diameter was created at the center of the petri dish using a cylinder cup. ISM formulation (150 μ L) was added into the cylindrical hole or well of the above agarose gel to compare the rate of matrix formation between formulations. Upon ISM contact with the aqueous phase (non-solvent), the formulation underwent phase inversion, transforming into an opaque solid-like matrix through a phase inversion mechanism. The matrix formation behavior was observed for 30 minutes using a stereo microscope (SZX10, Olympus Corp., Japan) with SZX10 Series software. The distance of matrix formation was measured in five zones, extending from the agarose rim to the inner area of the dish. The average rates

throughout the entire period from time 0 to 't' were used to calculate the rate of solvent diffusion into the agarose gel ($n = 3$) using Eq.1.

$$\text{Rate of matrix formation} = \frac{\text{Distance of opaque matrix (mm)}}{\text{Time (min)}} \quad [\text{Eq.1}]$$

4.2. Morphological change of matrix formation at the microscopic level

Microscopic changes in the matrix formation ability of the prepared ISMs were analyzed by using a drop shape analyzer (FTA 1000, First Ten Angstroms, USA). The ISM was pumped out at a rate of 5 $\mu\text{L/s}$ via a 18-gauge needle into PB (pH 6.8). The shape of matrix formation was observed for 90 sec.

4.3. Interfacial phenomena of matrix formation

The origin of matrix formation and its expansion at the interface between the formulation and agarose gel was observed using a stereo microscope (SZX10, Olympus Corp., Tokyo, Japan) to investigate the phase inversion phenomena at the initial phase of matrix formation. Initially, 20 μL of the prepared ISM solution was dropped onto a glass slide. Subsequently, 20 μL of PBS pH 6.8 was dispensed near the ISM solution, and the matrix formation at the interface was observed for 20 minutes following contact with PBS. Furthermore, the ISM was dropped close to the agarose gel on a glass slide to investigate the morphological aspects of the matrix at the microscopic level. This observation was conducted under an inverted stereomicroscope (Nikon Eclipse TE2000S, Nikon, Kawasaki, Japan) at various magnifications.

4.4. Water-induced matrix formation sensitivity

To determine the minimum amount of water required to induce the phase inversion of the ISM solution into a turbid dispersion, a 5 mL sample of the ISM solution was added to a 50 mL Erlenmeyer flask and titrated with distilled water from a burette. The sample was continuously swirled at 150 rpm while being gradually titrated at a constant rate with water (0.05 mL/s) until the clear solution changed into a milky white fluid. The amount of water titrated was recorded and calculated as a percentage of water capacity using the formula Eq. 2 ($n = 3$). A high value in this context reflects a high-water requirement to induce matrix formation, indicating that the ISM solution exhibits high-water resistance or low sensitivity to water-induced matrix formation.

$$\% \text{ water capacity} = \frac{\text{amount of water titrated (mL)}}{\text{amount of water titrated (mL)} + \text{amount of ISM solution (mL)}} \times 100 \quad [\text{Eq.2}]$$

5. Evaluation of *in vitro* drug release

5.1. Drug release and permeation of clotrimazole-loaded ISMs spray

The drug release of BZCN (selected from appropriate formulations ranging from 20BZCN to 50BZCN) was tested and compared with ZN. For this testing, a Franz-type diffusion cell (DHC-6T, Logan Instrument Corp., Somerset NJ, USA) was employed. The porcine cheek bulge obtained from a local slaughterhouse (Nakhon

Pathom, Thailand) was used as a membrane model to simulate the human cheek bulge. To obtain the buccal mucosa, the subcutaneous fatty layer and connective tissues were carefully removed from the porcine cheek bulge. The freshly prepared tissues underwent a wash with PBS (pH 6.8) and were wrapped in aluminum foil prior to use.

In the initial step, PBS was filled into the Franz receptor cells. The membranes were then mounted in the horizontal glass of Franz diffusion cells. Subsequently, 0.25 mL of the formulation was filled into the top section of the cell, above the membrane. The system was configured with controlled temperature (37 °C), water flowing through the cells, and constant speed stirring of the receptor solution to simulate the drug release behavior from the oral cavity. To monitor drug release, 1 mL aliquots of the solution from the receptor cells were sampled using a syringe, stored in vials, and replaced with 1 mL of fresh PBS in the receptor cells. The concentration of drug release was determined using high-performance liquid chromatography (HPLC) (Agilent 1220, Santa Clara, CA, USA). The instrument was coupled with a UV detector analyzed at 229 nm, and a C18 (150 × 4.6 mm) column was used. The mobile phase consisted of methanol: water (90:10), with a flow rate and injection volume of 1.0 mL/min and 20 µL, respectively. The cumulative mean drug release ± S.D. in percentage was then calculated with a sample size of $n = 3$.

Following the completion of the drug release experiment, the matrix formed on the tissue was collected and dissolved in ethanol for 1 hour. This solution was then analyzed using the HPLC method under the same conditions mentioned earlier to determine the remaining drug on the tissue. Additionally, the tissue was finely cut into pieces, sonicated, and soaked in ethanol for 1 hour. The resulting solution was also subjected to HPLC analysis under the same conditions to determine the remaining drug in the tissue. Ultimately, the drug content in three positions (in, on, and under the tissue) for both formulations was compared.

5.2. Drug release for vancomycin-loaded ISMs

The drug release of VDB was tested and compared with VD to evaluate the drug-entrapped properties of borneol. In this experiment, a cylindrical-shaped porcelain cup with a diameter of 1 cm and a height of 1.2 cm containing 0.4 g of the formulation was immersed in 50 mL of PBS (pH 6.8) at 37 °C. The rotation shaker was set at a speed of 50 rpm to simulate the drug release behavior from the crevicular pocket. The release medium was sampled (5 mL), and each sample was replaced with 5 mL of fresh PBS. The concentration of drug release was determined using a UV-Vis spectrophotometer (Cary 60 UV-Vis, Model G6860A, Agilent, Malaysia) at 280 nm. The cumulative mean drug release ± S.D. in percentage was calculated in triplicate. Additionally, the DD solver add-in program for Microsoft Excel (Redmond, WA, USA) was utilized to analyze the in vitro drug release data. Various mathematical release equations, including Krosmeier-Peppas, zero-order, first-order, and Higuchi's equations, were employed to fit the cumulative percentage of drug release profiles in the range of 10%-80%. The coefficient of determination or r^2 was measured to assess the fitting of the data.

5.3. Drug release for DH-loaded ISMs

This test was evaluated using the same method as mentioned above for measuring the DH-release by using the cup method. Additionally, the matrix in the cup, which occurred after drug release testing, was collected, and kept in a desiccator overnight. The morphology of the matrix was observed under a stereomicroscope (SZX10, Olympus Corp., Japan) at magnifications of 2.6x and 13x.

Furthermore, the DD solver add-in program for Microsoft Excel (Redmond, WA, USA) was utilized for curve fitting to scrutinize the drug release kinetics from the obtained drug release profiles. Various mathematical equations, including zero-order, first-order, Higuchi's, Korsmeyer-Peppas's, Hixon's, Hofenberg's, and Peppas-Sahlin's equations, were used to fit the release profiles. The estimated release parameters such as the coefficient of determination (r^2), Akaike information criterion (AIC), and model selection criterion (MSC) were recorded. Furthermore, kinetic parameters were reported such as k_{KP} (the constant of incorporation of structural modifications and geometrical characteristics of the system) and n (exponent of release) from the Korsmeyer-Peppas model. In this model, $n = 0.5$ indicates that drug release was governed by diffusion or the Fickian model (Case I), whereas $n = 1.0$ corresponds to zero-order release kinetics of the drug release rate, and the mechanism relates to the swelling or relaxation of the gel (Case II non-Fickian relaxational model). A value of n between 0.5 and 1.0 was applied to the non-Fickian model, and the parameter from the Peppas-Sahlin's model typically provides more detail regarding the drug release mechanism. The parameters k_1 (constant of Fickian diffusional contribution), k_2 (constant of Case II), and m (the purely Fickian diffusion exponent) from Peppas-Sahlin's model were practically employed to describe the drug release kinetics. A higher value for k_1 or k_2 indicates the mean of the dominant release mechanism of that formulation.

6. Evaluation of antimicrobial activity

The standard strains used for clotrimazole-loaded ISMs were *S. aureus* ATCC 6538, *E. coli* ATCC 25922, *C. albicans* ATCC 17110, *C. krusei* TISTR 5259, *C. lusitaniae* TISTR 5156, and *C. tropicalis* TISTR 5306. Whereas, the standard strains used for vancomycin-loaded ISMs were *S. aureus* ATCC 25923, *S. aureus* ATCC 6923, MRSA ATCC 43300, *E. coli* ATCC 8739, *C. albicans* ATCC 17110, and *P. gingivalis* ATCC 33277. Moreover, the standard strains used for DH-loaded ISMs were *S. aureus* ATCC 25923, MRSA ATCC 43300, *E. coli* ATCC 8739, *C. albicans* ATCC 17110, *C. krusei* TISTR 5259, *C. lusitaniae* TISTR 5156, *C. tropicalis* TISTR 5306, *A. actinomycetemcomitans* ATCC 29522, and *P. gingivalis* ATCC 33277.

The antimicrobial activities of the ISM formulation were tested using the cylinder plate method. This technique is based on the diffusion of the formulation from a stainless-steel cylinder cup with an inside diameter of 6 mm and a height of 10 mm through agar gel inoculated with the test microorganism. Bacterial and fungal strains were inoculated onto TSA and SDA agar plates, respectively, with an

approximate spread corresponding to a 0.5 McFarland turbidity standard. For *A. actinomycetemcomitans* and *P. gingivalis*, the inoculation was done on chocolate agar and sheep blood agar, respectively. A 100 μ L aliquot of each formulation was dropped into the cylinder cap, which was placed on the surface of an inoculated medium plate. Normal saline solution was used as a negative control. For *P. gingivalis*, the test was performed in an anaerobic incubator at 37 °C for 18 h. After incubation for 2 days, the diameter of the inhibition zone against the tested organism was measured (n = 3).

7. Evaluation of borneol precipitates

Borneol-based ISMs in various solvents including DB, NB, PB, and EB were selected to evaluate borneol precipitation. The excess water was added into each formulation for phase inversion-induced and causing opaque solid-like precipitates. These precipitates were filtered *via* paper filtration and kept in a desiccator overnight to ensure that free of water in precipitates.

7.1. Determination of precipitate morphology

The precipitated samples were soaked with glycerin and photographed with an inverted stereomicroscope (TE2000S, Nikon Instruments Inc., New York, USA) under various magnification.

7.2. Identification of precipitated borneol

The analysis of borneol precipitated from a variety of solvents was conducted with the objective of identifying significant functional groups using Fourier-transform infrared (FT-IR) spectroscopy. The precipitated samples were blended with potassium bromide (AR grade for IR) and plunged with a plunger and die to make a pellet using a hydraulic press at compression force of 4 tons. The % FT-IR transmittance of the sample was recorded using FT-IR (Nicolet 4700, Madison, USA). Moreover, the powder X-ray diffractometer (PXRD) (Mini Flex II, Rigaku corp, Japan) was used to investigate the crystalline properties of the intact borneol and the ISM precipitates. The test samples were ground into fine particles by mortar and pestle and loaded into the aluminum holder. All samples were scanned in the degree of 2θ in the range of 5 to 30 $^{\circ}2\theta$

7.3. Thermal analysis of precipitated borneol

Differential scanning calorimetry (DSC) was undertaken to determine the melting point of intact borneol and borneol precipitates. All precipitated samples were weighed at 2.5-4 mg and then placed in a non-hermitically aluminum pan. The pan was sealed then the thermal property was determined by DSC (DSC 8000, Perkin Elmer, USA). The samples were investigated under 10 $^{\circ}$ C/min of heating rate and the heating range was 30-100 $^{\circ}$ C under 40 mL/min of nitrogen gas flow. Additionally, thermogravimetric analysis (TGA) was conducted to ascertain the highest temperature at which borneol degradation occurred, for both intact borneol and precipitated borneol samples. Five milligrams (5 mg) of each compound were accurately weighed

and placed into non-hermetically sealed aluminum pans. TGA (Pyris TGA, Perkin Elmer, USA) was performed. The samples were subjected to controlled heating, and weight loss measurements were recorded. The heating rate was 10°C/min with a heating range was 30-300°C under 200 mL/min of nitrogen gas flow. While DTGA was calculated by the rate of %weight change at each time.

8. Unraveling mechanistic phase inversion of borneol-based ISM

8.1. Utilizing UV-vis imaging to investigate the phase inversion mechanism of ISM

8.1.1. Preparation of ISM and agarose gel

ISM solutions were prepared, containing 0.5%w/w DH as a model drug and 40% w/w borneol as a matrix-forming agent, by dissolving them in NMP as primary solvent. To explore the impact of varying cosolvent content within the ISM, two distinct triacetin concentrations—5%w/w and 25%w/w—were added as F3 and F4, respectively. Additionally, F2 represented a DH-loaded borneol-based ISM without triacetin, dissolved in NMP. The control group, F1, consisted of DH loaded in NMP. The DH content was reduced within the formulations from 5% w/w to 0.5% w/w. This adjustment was necessitated due to the inability to measure absorbance at high DH concentrations. In the case of the 5%w/w DH-loaded ISM, the high DH concentration was predominantly present in the agarose gel component, rendering its absorbance undetectable by the CMOS as it surpassed the analytical concentration range. Given these constraints, lowering the DH concentration emerged as the optimal resolution. Nevertheless, the comparative assessment of diffusion among the various ISMs, a critical aspect of this study, remained feasible. This was because the drug concentrations were still sufficient for diffusion into the agarose gel, and the concentration of DH was uniformly reduced in all formulations. To simulate the gingival tissue environment, a 0.6% w/v agarose gel was prepared by dissolving 0.6 g of agarose powder in 100 mL of PBS (pH 6.8). Subsequently, the agarose mixture solution was heated in a microwave at 900 watts for 7 seconds until the agarose had fully dissolved. The resultant hot agarose solution was poured into a demountable quartz cell (internal dimensions: 8.0 × 40.0 × 0.2 mm³ (W × L × H)) sourced from Starna Scientific Ltd., Hainault, Essex, UK. Following this, the quartz cell was sealed and left to undergo gelation for a duration of 2 hours at room temperature.

8.1.2. UV-vis imaging

Schematic of the experiment pertaining to this subject matter is provided in Figure 12. Initially, a portion of the prepared gel within the demountable quartz cell (situated 12.5 mm from the left-edge of the internal cell) was excised to create a straight interface. Subsequently, PBS (pH 6.8) was introduced into the void resulting from the gel removal, and the cell was then sealed. This quartz cell,

now containing PBS as the reference solution, was placed within the UV-vis imaging system (D200 ActiPix, Paraytec Ltd, York, UK) for calibration. After capturing reference images with PBS, the prepared ISM sample ($\sim 18.5 \mu\text{L}$) was positioned within the void that was previously occupied by PBS, initiating the imaging process for drug release. The D200 imaging system featured a $25 \times 25 \text{ mm}^2$ (width $\times$ length) CMOS chip with an effective pixel size of $13.75 \times 13.75 \mu\text{m}^2$. UV and Vis imaging measurements were carried out at wavelengths of 214 nm, 280 nm, and 525 nm to monitor solvent diffusion, DH diffusion, and matrix formation, respectively. Furthermore, a solution of DH in NMP (F1) was performed for comparison with the other formulation of ISM.

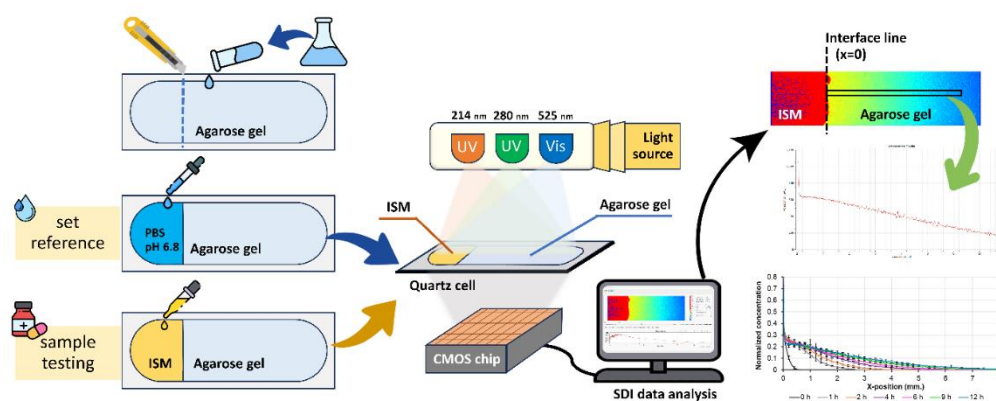


Figure 12. Schematic representation of the UV-vis imaging setup for monitoring drug release, solvent diffusion, and matrix formation of ISM

8.1.3. UV-vis imaging data analysis

The images were captured at a rate of 3 frames per minute with a file subsample of 50, continuously for 12 hours, in a room maintained at temperatures of 20-23°C. The cabinet covered the D200 ActiPix and ensured protection from external light. The SDI Data Analysis software (version 2.0, Paraytec Ltd, York, UK) was employed to analyze and interpret the acquired images. By utilizing UV-vis imaging, the absorbance areas within the agarose gel segment were measured at wavelengths of 214 nm, 280 nm, and 525 nm, and subsequently presented as absorbance maps. From the processed image data using the SDI Data Analysis software, a black horizontal bar ($0.3 \times 8.0 \text{ mm}$, $W \times L$, for solvent and DH analysis and $0.3 \times 6.0 \text{ mm}$, $W \times L$, for matrix analysis) was placed in the center of the cell and positioned adjacent to the interface line between the ISM and agarose (except the case of matrix analysis, horizontal bars were placed between the interface line). The absorbance measured from this bar was calculated and illustrated as concentration-distance (x-position) curves using a calibration curve. The $x = 0$ position was set at the interface line. Additionally, the diffusivities of DH and the solvent were determined by tracking changes in concentration within the agarose gel over time. These concentrations, measured in the agarose section, were plotted against elapsed time, generating concentration-time profiles for both DH and the solvent. Furthermore, the

cumulative amount of DH and solvent was plotted over varying time periods, illustrating the percentage of DH release and solvent diffusion. Moreover, the interplay between phase inversion and drug release was explored by correlating solvent diffusion with drug diffusion, revealing the relationship between these phenomena.

8.1.4. Calibration curves for UV-vis imaging analysis

A DH calibration curve ranging from 0.18 to 3.33 mg/mL was established in a demountable quartz cell (0.2 mm light path) with 0.6% w/v agarose gel. Similarly, NMP calibration curves spanned 0.01 to 5.20 M, employing the same cell and gel. The D200 imaging system was utilized for imaging at 280 nm and 214 nm for DH and NMP, respectively. These calibration curves were subsequently employed to calculate the concentrations of DH and NMP at different positions and time points.

8.2. Computer dynamics simulation for understanding mechanistic phase inversion

8.2.1. MD simulation of ISM formulation

To comprehend and explain the phenomena of phase inversion and molecular movement on a molecular scale upon contact with aqueous fluid, computer dynamics modeling was undertaken. Initially, the molecular coordinates of DH, borneol, triacetin, and NMP were retrieved from the Cambridge Crystallographic Data Centre (CCDC) and the PubChem database. The AMBER program, version 20, was employed to prepare the topology file and the parameter modification file (frcmod) for all molecules. Models F1-F4 were designed (calculated based on the molar ratio) and formulated as three-dimensional square box models. The molar ratio of each molecule and the size of each box are detailed in Table 4. Subsequently, GaussView06 and UCSF Chimera were utilized to rearrange the molecules in each system, and the models were created using the LEAP module in Amber Tools. The energy of the system was then minimized and heated to 310 K using the Sander module under constant pressure for 200 ns.

8.2.2. MD simulation of interfacial behavior of ISM with water

A preliminary study on water sensitivity for matrix formation indicated that the minimum water content required to induce ISM solution and transform it into the matrix was 20% w/w of the total substance. Therefore, the ratio of formulation to water was 4:1, calculated based on weight. This ratio was employed to design and arrange molecules in a 3D box model for each formulation with water to observe the phase inversion phenomena at the interface when the formulation came into contact with water. The amount of each substance and their ratio were calculated and are presented in Table 5. The geometry of the last snapshot of each simulated drug–borneol–NMP/triacetin system was collected and placed next to the box of TIP3P water. Additionally,

the models were built using the LEAP module in Amber Tools. Subsequently, energy minimization was conducted, followed by heating up to 310 K using the Sander module, and MD simulation at 310 K with constant pressure for 200 ns using Amber20. Moreover, Visual Molecular Dynamics (VMD) software was utilized to analyze and visualize all simulations.

Table 4. Box design details of all models used in the simulation processes before contact with water box

Details	F1	F2	F3	F4
Number of Dox molecules	80	80	80	80
Number of Bor molecules	0	1840	1840	1840
Number of Tri molecules	0	0	160	800
Number of NMP molecules	6800	4000	3600	2160
Mole ratio of Dox:Bor:Tri:NMP	1:0:0:85	1:23:0:50	1:23:2:45	1:23:10:27
Total number of molecules in system	6,880	5,920	5,680	4,880
Total number of atom in system	113,280	121,840	120,080	115,600
Size of box (x, y, z)	112, 104, 120	140, 116, 132	140, 132, 140	140, 116, 124

Remark: Dox=doxycycline, Bor=borneol and Tri=triacetin

Table 5. Box design details of all models used in the simulation processes after contact with water box

Details	F1	F2	F3	F4
Number of Dox molecules	80	80	80	80
Number of Bor molecules	0	1840	1840	1840
Number of Tri molecules	0	0	160	800
Number of NMP molecules	6800	4000	3600	2160
Number of Wat molecules	7920	7920	7920	7920
Mole ratio of Dox:Bor:Tri:NMP:Wat	1:0:0:85:99	1:23:0:50:99	1:23:2:45:99	1:23:10:27:99
Total number of molecule in system	14,800	13,840	13,600	12,800
Total number of molecule in system	137,040	145,600	143,840	139,360
Size of box (x, y, z)	112, 104, 147	140, 116, 159	140, 132, 167	140, 116, 151

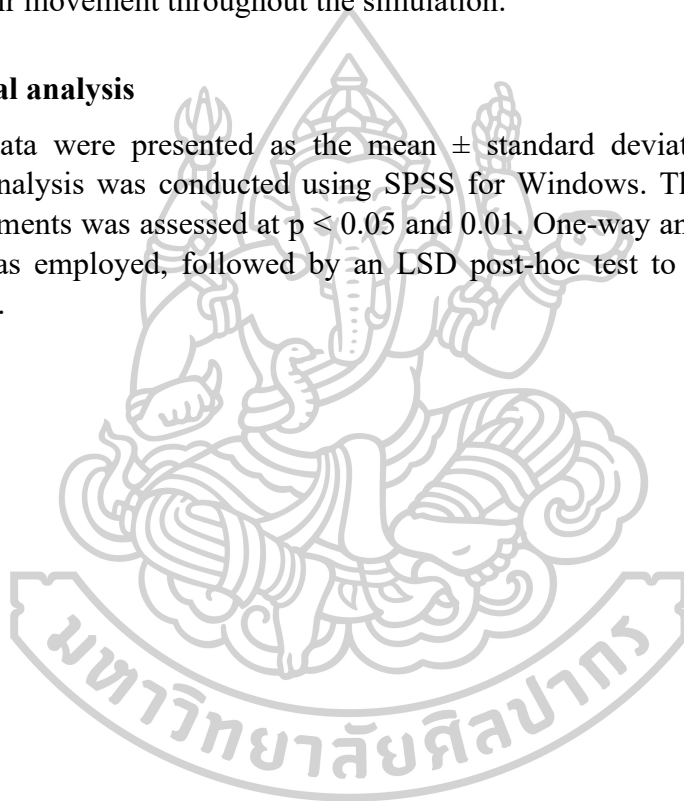
Remark: Dox=doxycycline, Bor=borneol, Tri=triacetin and Wat=water

8.2.3. Simulation analysis

The hydrogen bond (H-bond) occupancy was determined using VMD software, employing criteria that include a distance less than 3.5 Å between the acceptor and donor atoms and an angle less than 60° formed by the acceptor, donor, and hydrogen atoms. The root-mean-squared deviation (RMSD) of matrix, borneol, NMP, and doxycycline was calculated using the “*cpptraj*” module. The “*ptraj*” module with the simulation time-evolved trajectory was utilized to compute the diffusion constants, representing the distance migrated from the initial position. The diffusion constants highlight the ability of doxycycline, borneol, NMP, triacetin, and water to migrate outflux/influx, providing insight into their movement throughout the simulation.

9. Statistical analysis

The data were presented as the mean $\pm$ standard deviation (mean $\pm$ S.D.). Statistical analysis was conducted using SPSS for Windows. The significance level for measurements was assessed at $p < 0.05$ and 0.01. One-way analysis of variance or ANOVA was employed, followed by an LSD post-hoc test to determine statistical significance.



CHAPTER 4:

RESULTS AND DISCUSSIONS

1. Clotrimazole-loaded borneol-based ISM as oral sprays for oropharyngeal candidiasis treatment

1.1. Physical appearance

The successful preparation of ISM solutions was achieved with varying concentrations of borneol (20-50% w/w) combined with 1% w/w clove oil and 1% w/w clotrimazole dissolved in NMP. Homogeneous and clear solutions of CN (clove oil in NMP), ZN (clotrimazole in NMP), ZCN (clotrimazole and clove oil in NMP), and 40BN (40% w/w borneol in NMP) were also obtained for comparative purposes. Clotrimazole and clove oil readily dissolved in NMP during preparation through simple mixing. Borneol concentrations exceeding 50% w/w faced difficulty in dissolution, leading to the appearance of precipitates of excess borneol powders. The successful formulation of clotrimazole-loaded borneol-based ISMs was confirmed by transparent, colorless, and low-viscosity solutions. The visual appearance did not significantly change with the incorporation of clotrimazole or increasing borneol content in ISM solutions. The addition of clove oil to borneol-based ISMs only imparted a clear yellowish color to the otherwise colorless solution, as clove oil itself has a yellowish hue.

1.2. pH and density

The pH values of the ISMs were examined to assess their potential for irritating contact tissue. The pH values for the solvent, drug solutions, and ISMs are provided in Table 6. Notably, NMP and CN exhibited significantly higher pH values compared to the other formulations ($p < 0.05$). The pH decreased after adding clotrimazole or clove oil and further decreased gradually with an increase in borneol content, attributed to the reduced amount of NMP in the formulation [172]. The pH values of drug-loaded borneol-based ISMs fell within the range of 5.59–6.61, which is deemed acceptable as these values are close to the pH of saliva in the oral cavity (pH 6.8). Additionally, the typical spray dosage form of around 0.5 mL applied to the oral cavity is expected to be diluted by the relatively high flow rate of saliva, helping maintain the local environmental pH at 6.8 [173].

The density value of NMP was $1.03 \pm 0.01 \text{ g/cm}^3$ and remained nearly constant after adding clove oil and clotrimazole (Table 6). Comparing the density values of NMP and the other formulations, no significant difference was observed. A slight decrease in density was noted with the addition of more borneol to the formulation, likely due to the density of borneol being 1.01 g/cm^3 [174]. These formulations were slightly heavier than water; hence, they should not float after being sprayed into an aqueous medium and should submerge while matrix formation occurs at the targeting site. Additionally, these density values were further utilized as a necessary parameter for determining surface tension.

Table 6. Physical properties of borneol ISM with clotrimazole-loaded and related formulations. Results are presented as mean $\pm$ SD, with $n = 3$.

Formulation	pH	Density (g/cm ³)	Surface tension (mN/m)	Viscosity (cP)
NMP	11.07 $\pm$ 0.07 ^a	1.03 $\pm$ 0.01	37.36 $\pm$ 0.74 ^c	2.40 $\pm$ 0.06 ^d
CN	10.68 $\pm$ 0.04 ^b	1.03 $\pm$ 0.01	37.47 $\pm$ 0.79 ^c	2.54 $\pm$ 0.07 ^d
ZN	7.59 $\pm$ 0.01 ^{a,b}	1.03 $\pm$ 0.00	38.34 $\pm$ 0.95 ^c	2.62 $\pm$ 0.10 ^d
ZCN	7.43 $\pm$ 0.02 ^{a,b}	1.03 $\pm$ 0.00	37.77 $\pm$ 0.86 ^c	2.44 $\pm$ 0.03 ^d
20BZCN	6.61 $\pm$ 0.01 ^{a,b}	1.02 $\pm$ 0.01	33.32 $\pm$ 0.93	3.83 $\pm$ 0.02
30BZCN	6.22 $\pm$ 0.01 ^{a,b}	1.01 $\pm$ 0.01	32.39 $\pm$ 1.10	4.31 $\pm$ 0.01
40BZCN	5.89 $\pm$ 0.02 ^{a,b}	1.01 $\pm$ 0.00	31.42 $\pm$ 0.92	5.35 $\pm$ 0.03
50BZCN	5.59 $\pm$ 0.01 ^{a,b}	1.00 $\pm$ 0.01	30.19 $\pm$ 0.61	7.91 $\pm$ 0.11 ^c
40BN	5.91 $\pm$ 0.03 ^{a,b}	1.01 $\pm$ 0.01	32.19 $\pm$ 0.70	5.02 $\pm$ 0.05

The superscripts a, b, c, and d represent $p < 0.05$ within the tested formulations.

1.3. Surface tension

Surface tension analysis reveals that a fluid suspended in air contracts to the smallest possible surface area of a droplet due to cohesive forces among liquid molecules [175]. The presence of additional excipients with cohesive or adhesive properties can influence the surface tension of the fluid [176, 177]. A lower surface tension promotes the spreadability of formulations on tissue surfaces as it reduces the cohesive forces of the fluid droplet. The surface tension values of the formulations are presented in Table 6. The addition of clotrimazole increased the surface tension more than clove oil, while a gradual decrease in this value was observed with the addition of borneol. The surface tension of borneol-based ISM solutions was significantly ($p < 0.05$) higher than that of non-loaded borneol formulations. This suggests that clove oil and clotrimazole may interact with NMP molecules and submerge in the bulk fluid phase rather than interacting on the droplet surface. The reduction in surface tension with increased borneol addition indicates that adhesion forces between NMP-borneol molecules disturb the cohesive forces among NMP molecules around the surface region. Polar aprotic solvents like NMP exhibit stronger hydrogen bonding interactions between molecules compared to borneol-borneol or borneol-NMP interactions [176, 177]. Therefore, the tendency of decreased surface tension with borneol addition could facilitate the ease of spraying and enhance the spreadability of the formulation for delivery to the target site, such as the oral cavity membrane for treatment.

1.4. Contact angle and wettability

The usual method for measuring contact angle involves using a drop shape analyzer on a standard glass slide. However, this study extended the assessment of contact angle to ISMs on agarose gel and porcine buccal membrane, aiming to evaluate the wettability or spreadability of the ISM on the target surface. On the glass

surface, borneol-free solutions exhibited similar relatively high contact angle values in the range of 33-38° (Figure 13). The inclusion of borneol decreased this value on the glass surface, indicating increased hydrophobicity of the formula and improved adhesive properties. However, the gradual increase in viscosity led to an enhanced contact angle due to the increased difficulty in fluid spreadability [178, 179]. Moreover, the higher viscosity of 40BZCN resulted in a greater contact angle value than 40BN (Figure 13). The relatively high contact angle values of 30% and 40% IBU-based ISMs, attributed to their high viscosities impeding formulation spreadability on the glass surface, have been reported previously [178]. A similar observation of higher contact angle values for more concentrated rosin-based ISMs in DMSO has also been reported [179].

To simulate the environment of buccal mucosa covered with saliva, this study also utilized the surface of an agarose gel containing phosphate ion species and water as another target surface. The smooth planar appearance of the upper part of the agarose gel made it a suitable surface for assessing the spreadability of ISMs [178]. Remarkably, the contact angle of borneol ISMs was significantly ($p < 0.05$) higher than that of all borneol-free solutions. The phase inversion upon contact with water from agarose gel changed the solubilized borneol phase to a matrix-like structure, reducing their spreadability. The higher the amount of borneol incorporated, the greater the contact angle on the surface of agarose gel. Meanwhile, the good miscibility of NMP and water led to an apparent lowering of the contact angle for borneol-free formulations (Figure 13). Similar results were observed on the surface of porcine buccal mucosa, even though significantly higher contact angle values ($p < 0.05$) were obtained when compared to the surfaces of agarose gel and borneol-free formulations. The direct contact of borneol-loaded ISMs on buccal mucosa soaked with PBS could suddenly provoke more rapid phase inversion from formulation than inducing with water from viscous/dense mass of agarose gel. The higher viscous fluid of 40BZCN could retard a phase inversion and showed slightly less value of contact angle than that of 40BN [180]. This consequence could be also attributed to the fact that the incorporated other solutes could minimize the cohesive force between NMP molecules as previously presented in surface tension; thereafter, the extent of spreading increased, whereas the contact angle decreased, indicating the difficulty in matrix formation. This evidence was corresponded with the ibuprofen-based ISMs in DMSO and NMP that increased ibuprofen loading decreased the contact angle on agarose gel [178]. Even though there were the rather high values of contact angle of developed ISMs, they were apparently less than 60°, manifesting their good wettability on these test surfaces [178, 181]. Typically, the wettability of ISMs is required for their adhesive quality onto buccal mucosa to prevent the undesirable dissipation of the transformed clotrimazole-loaded borneol matrix [182].

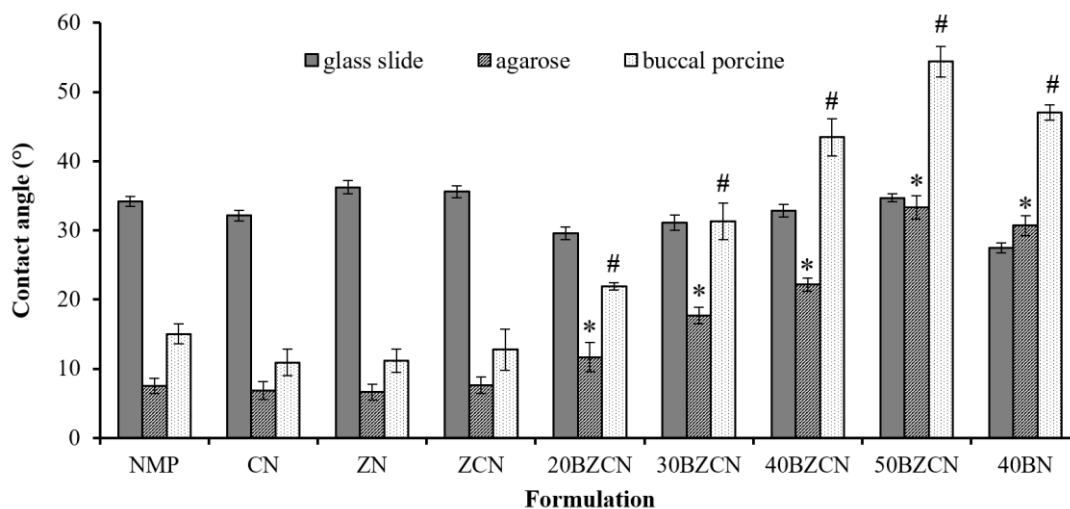


Figure 13. contact angle of borneol ISM with clotrimazole-loaded and related formulations on the surface of the glass slide, agarose gel, and buccal porcine at room temperature. Results are presented as mean $\pm$ SD, with $n = 3$. *, # represent $p < 0.05$.

1.5. Viscosity and rheological behavior

The viscosity of borneol-based ISMs was significantly higher than that of non-loading borneol formulations, as presented in Table 6 and Figure 14(A). The addition of clove oil, clotrimazole, and increasing borneol content increased the viscosity of the systems due to the decrease in solvent content resulting from the incorporation of these compounds, leading to increased solute concentration and environmental viscosity. Nonetheless, all of them exhibited prominently low viscosity (< 8 cP) compared to typical polymer-based ISMs because of the low density, small size, and unsophisticated structure of loaded borneol in NMP [29, 62, 63, 65]. This obtained viscosity data also corresponds with other ISMs reported; for example, the viscosity of vancomycin HCl-loaded borneol-based ISM using DMSO as a solvent for periodontal pocket delivery was notably lower than 20 cP [183]. NMP is claimed to be an important vehicle in pharmaceuticals, used for drug extraction and crystallization, and mentioned to enhance the solubility and permeability of drugs by simultaneously acting as a co-solvent and a complexing agent. The strong hydrophilic interaction between the hydroxyl group of borneol and the keto group of NMP decreased NMP molecular movement and increased viscosity [184]. Significantly, the viscosities of clotrimazole-loaded borneol-based ISMs were remarkably lower than those reported for polymer-based ISMs [62, 63, 65]. In addition, these developed ISMs also showed lower viscosities than natural resin-based ISMs [181, 185]. These natural resins including rosin, benzoin, propolis and aloe dissolved in DMSO and NMP and employed as the gelling agents of ISMs in which the viscosities of benzoin in NMP and DMSO were 6.61-171.46 and 19.77-137.03 cP, respectively, compared with propolis (i.e., 119-155 and 80.96-577.90 cP, respectively). The rather low viscosities (< 20 cP) of ibuprofen-based ISMs has recently reported using NMP and DMSO as the solvents [178]. The advantage of the remarkably low viscosity of these clotrimazole-loaded borneol-based ISMs includes the simplicity of mixing for

fabrication and ease of use through spraying. Similar favorable characteristics were also reported for ISMs fabricated using lauric acid as a matrix-forming agent [129, 130]. Moreover, they exhibited Newtonian flow, as shown in Figure 14(B), which is considered one of the appropriate flow behaviors for injection dosage forms. This behavior is observed when a compression force is applied to a syringe plunger to discharge the formulation through the spray nozzle into the oral cavity [186].

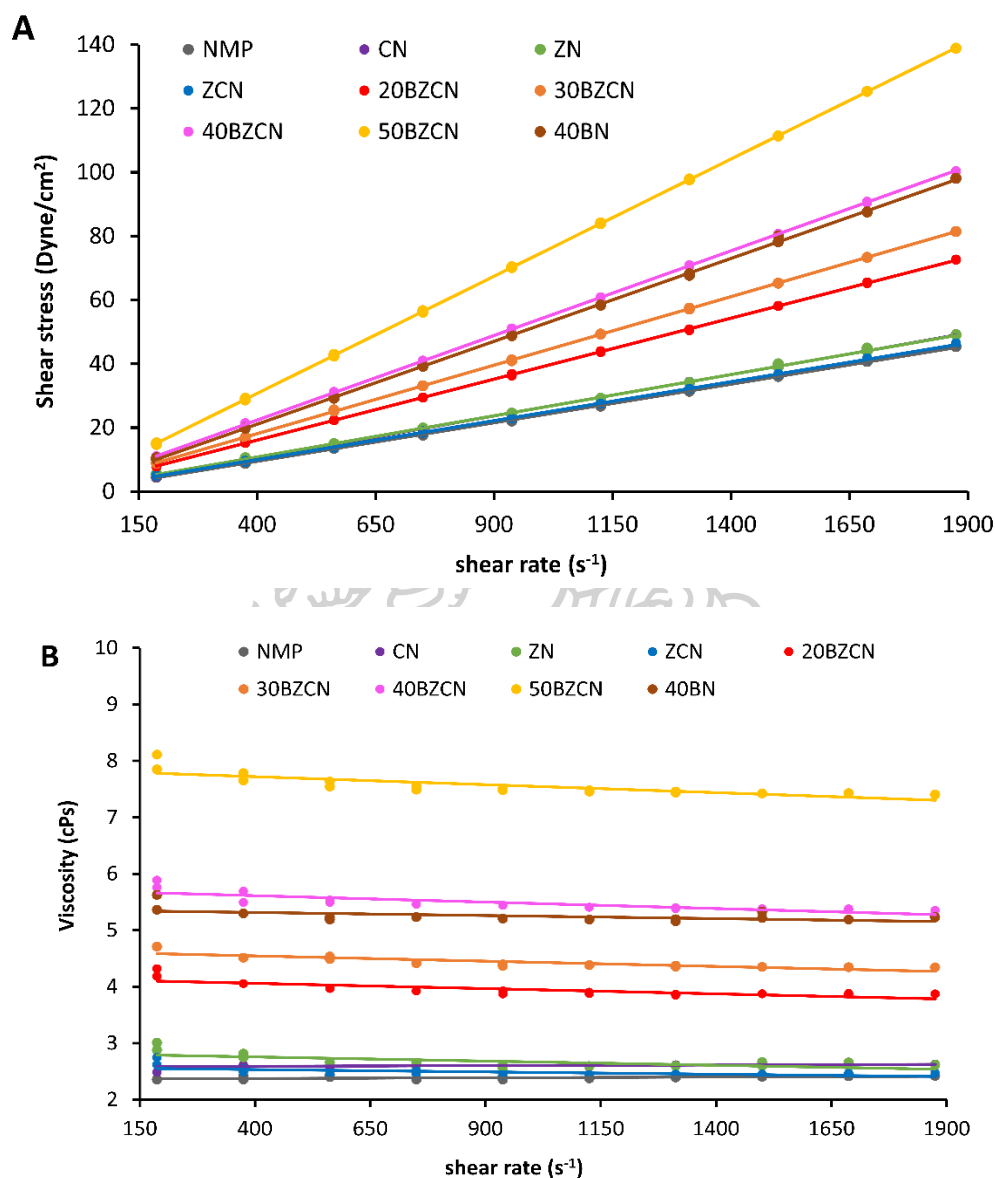


Figure 14. Rheological behavior of borneol ISM with clotrimazole-loaded and related formulations plotting between (A) shear stress against shear rate and (B) viscosity against shear rate at room temperature.

1.6. Matrix formation behavior at the macroscopic level

The snapshots of borneol matrix after formulation contact with PBS pH 6.8 are shown in Figure 15(A). NMP and ZN sank to the bottom of the test tube due to their higher densities, while CN and ZCN exhibited a colloid-like appearance in PBS because clove oil dispersed in the aqueous medium. The more insoluble compounds of ZCN, including clotrimazole and clove oil, promoted a cloudier liquid. The cloudy matrix gradually occurred over time for 40BN. The drug-loaded ISMs comprising borneol less than 40% w/w showed a loose, cloudy mass that expanded upward, while the lower part remained liquid. This result indicates that low-concentration borneol-based ISM may not form into an interconnected dense matrix properly. The 50BZCN presented a more rapid phase transformation to a dense, opaque matrix than 40BZCN. The abrupt matrix formation of 50BZCN was evident, whereas the step-by-step change from a solution to a matrix state and cloudier matrix was observed for 40BZCN. This occurrence was also noted in other ISMs comprising different concentrations of polymers [62, 63, 65], natural resins [181, 185] and fatty acids [129, 130, 145]. In addition, the appropriate concentration of matrix forming agents from these previously reports were nearly to 40% w/w of them because of their fast dense matrix formation but not too rapid before formulation spreading. The sudden change from solution to matrix of 50BZCN might not spread sufficiently on buccal mucosa. By comparison, 40BZCN had the greater mass of obtained matrix than 40BN because the drug-loaded formulation composed of more aqueous insoluble ingredients.

The cross-sectional view of the phase transformation of drug-loaded borneol-based ISMs in the agarose well is depicted in Figure 15(B). The 20BZCN showed a rather transparent matrix occurring around the agarose rim and gradually changed to be cloudier over time, signifying its relatively low dense matrix formation due to the lower amount of borneol. A thicker matrix formation was evident for 30BZCN than the others, as the higher concentration of borneol created a denser matrix as a barrier for phase inversion and slowed further phase transformation. This characteristic result has been previously mentioned in the case of fatty-based ISM [129, 130, 145]. Theoretically, various factors, such as the presence of new interfacial energy from the initial crystal, aqueous surface, and bubble surface, can interrupt thermodynamic stability. This is coupled with supersaturation resulting from continuous phase inversion, such as NMP leakage. Subsequently, the ISM stabilizes itself, leading to further matrix formation [187]. Larger cloudy mass in liquid phase appeared inner part of 50BZCN formulation was seen indicated its ability to later formed into matrix. The trend of diminishing transformation rate by time of 40BZCN and 50BZCN resulted from the restriction of solvent transfusion through interfacial network of initiate borneol matrix, which became denser with higher tortuosity and lower porosity by time. Similarly, the dense gelation network also lowered the water diffusion coefficient [188]. This results from macroscopic matrix formations were beneficial to demonstrate the performance of 40BZCN to gradually phase transformation to matrix with sufficient amount of matrix mass for spreading on target area.

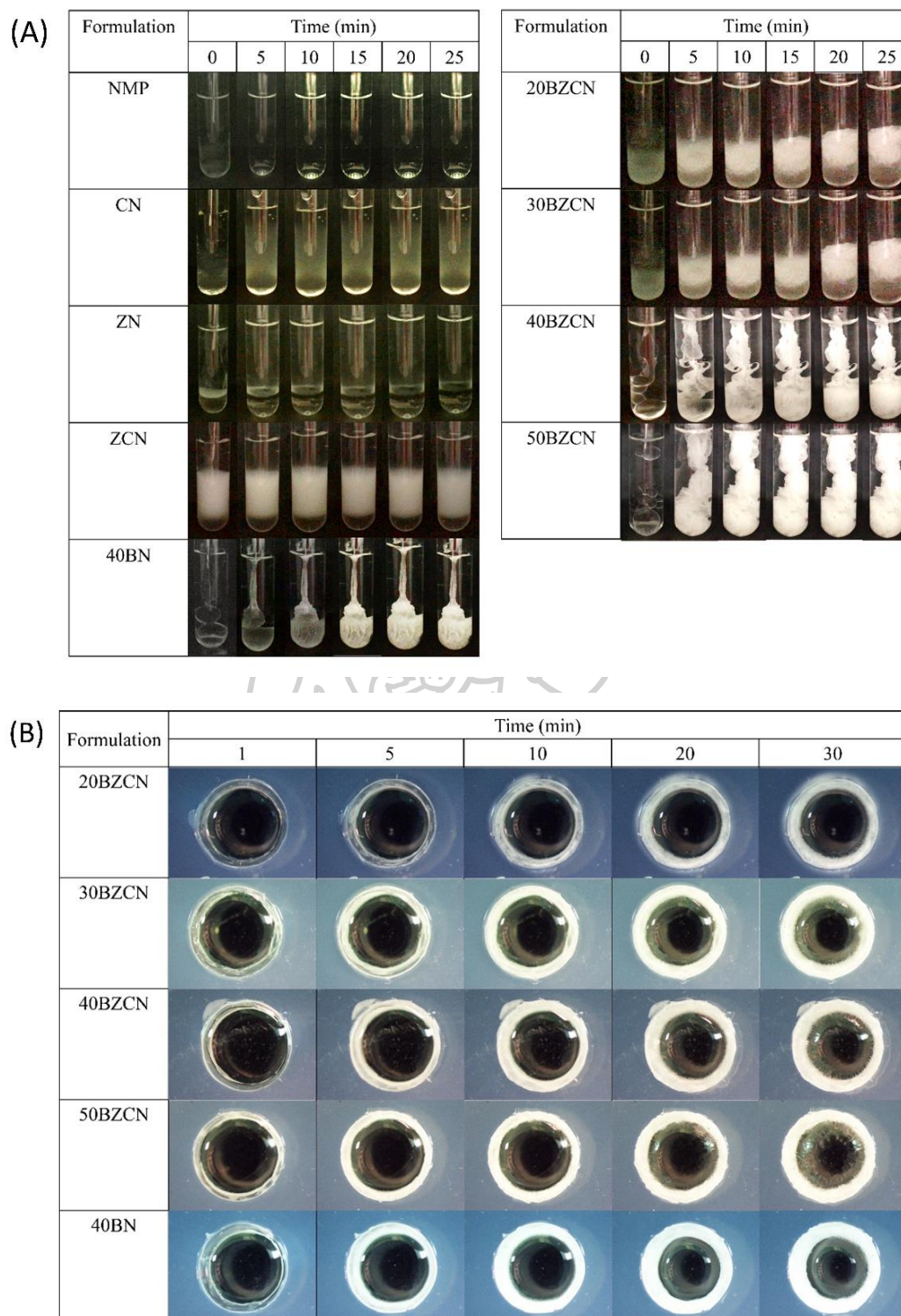


Figure 15 (A) *In vitro* matrix formation behavior of ISMs through a 27-gauge needle into PBS pH 6.8. (B) Matrix formation behavior of ISMs after contact with agarose gel containing PBS pH 6.8 in an artificial crevicular pocket under a stereo microscope at (magnification of 4×).

1.7. Morphological change of matrix formation at the microscopic level

The morphological change of borneol matrix from drug-loaded ISMs after pulling out and contact PBS surrounding was shown in Figure 16. The 20BZCN flowed to the bottom of the medium due to gravity because it did not transform sufficiently into matrix droplet. This low concentrations of borneol in the ISM led to relatively slow phase transformation because of the low affinity of its formulation to environmental water and the small amount of matrix forming agent available [129]. Initially, 30BZCN formed irregularly shaped droplets, and a significant amount of its liquid formulation flowed downward initially and then decreased over time. Furthermore, the shrinkage of the matrix droplet was evident, and it remained suspended at the needle tip. This behavior of matrix formation was observed for 40BZCN with less leakage of liquid from the initial droplet. Additionally, a spherical droplet was generated and remained stable for the initial 20 seconds, then shrank into an obovate matrix with a rough surface. A similar transformation platform occurred for 50BZCN; however, a smaller spherical droplet with slight liquid leakage was obtained. Therefore, increasing the amount of borneol in the ISM hastened the transformation into a hard matrix with smaller and more spherical-shaped droplets before subsequent shrinkage into smaller, obovate, and rough matrices. The greater mass of the borneol matrix could retain the inner liquid and prevent it from leaking out of the droplet. The leakage of liquid from the 40BN droplet was like that of 40BZCN; nevertheless, the size of the obtained droplet of 40BN was apparently smaller. This droplet shrinkage also indicated the phase inversion from ISM during its phase transformation. Therefore, the initially spherical-shaped droplet could not retain its shape after more NMP diffused outward into PBS due to the high affinity and good miscibility with the water phase of this solvent. Unlike lauric acid-based ISM, there were no floating droplets due to the higher density of borneol-based ISM [129]. This study not only offers a novel experimental approach for tracking ISM systems but also provides a valuable assessment tool for evaluating the self-forming behavior of ISM dosage forms at the microscopic scale.

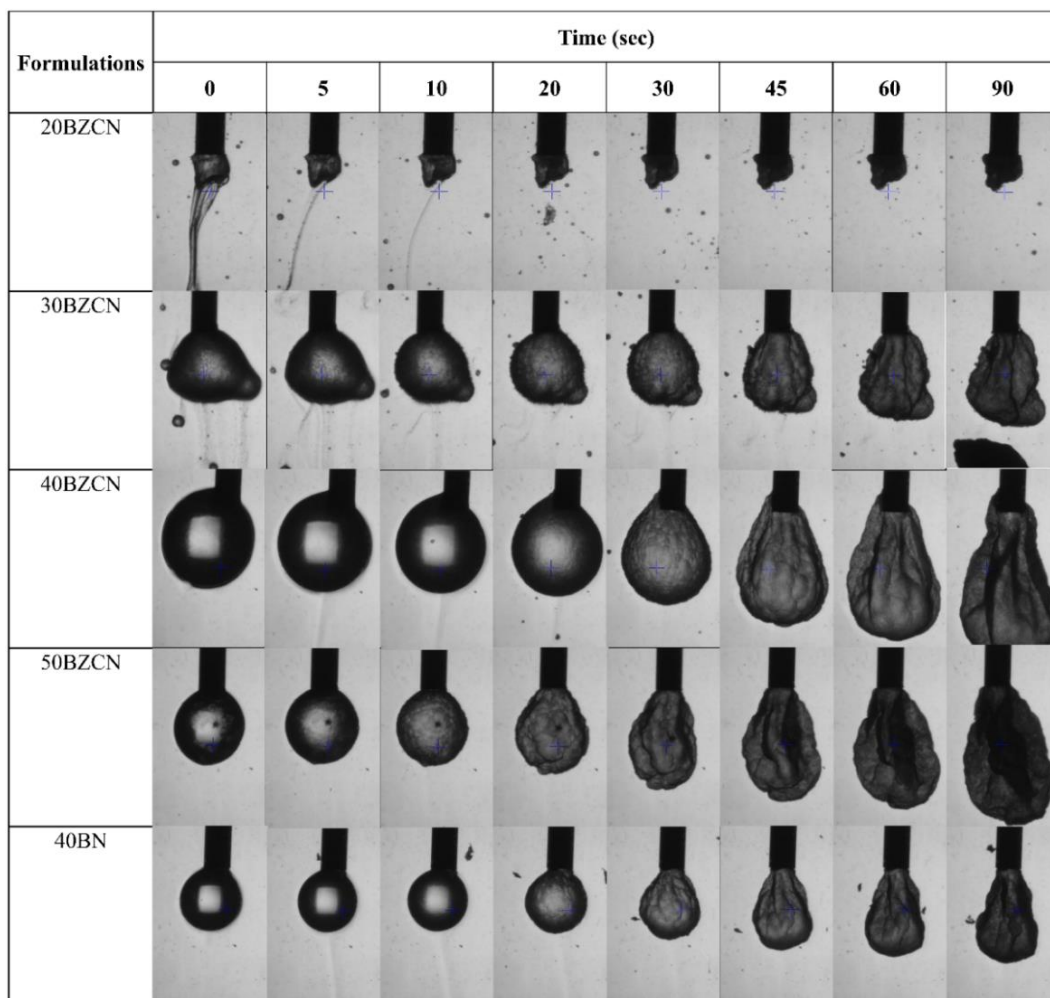


Figure 16. Shape of matrix formation with time from clotrimazole-loaded borneol-based ISMs and borneol solution in PBS pH 6.8.

1.8. Spray pattern

The determination of the spray angle serves as a crucial method for evaluating the efficacy of ISM formulations. This measurement provides insights into the formulation's capacity to distribute and cover the affected surface effectively. While specific criteria for establishing an optimal value might not be defined, it is generally preferred to have a distribution value that is suitable and not excessively low. This ensures that the formulation can adequately cover the targeted area, promoting its therapeutic effectiveness. NMP exhibited the widest spray angle ($61.70 \pm 0.94^\circ$) and scatter among all solutions. Whilst, the ISM containing both clotrimazole and clove oil (ZCN) showed a narrower spray angle ($48.60 \pm 1.36^\circ$) compared to ISM containing only clove oil (CN) ($52.83 \pm 0.81^\circ$) and clotrimazole (ZN) ($56.20 \pm 2.44^\circ$). The addition of borneol, such as in 20BZCN, resulted in a rather wider spray angle ($51.94 \pm 1.19^\circ$) than ZCN, possibly due to the lubricating effect of borneol. However, the higher concentration of borneol increased viscosity, minimizing the spray angle and droplet scatter. There was a gradual decrease in spray angle with increasing borneol concentration. The spray angles for 30BZCN, 40BZCN, and

50BZCN were $48.96 \pm 0.69^\circ$, $46.72 \pm 1.05^\circ$, and $40.46 \pm 0.72^\circ$, respectively. Whereas, borneol ISM with clove oil and clotrimazole-free (40BN) displayed a spray angle of $41.81 \pm 1.43^\circ$. Notwithstanding, the spray angle of 40BZCN ($46.72 \pm 1.05^\circ$) could be acceptable for application in the oral cavity.

1.9. Water-induced matrix formation sensitivity

The transition of the clear solution of clotrimazole-loaded borneol-based ISM solutions to a turbid fluid due to phase separation induced by the addition of water is depicted in Figure 17 through the variation in water capacity percentage. The data collected provides insights into the formulations' susceptibility to water and their tendency to undergo phase inversion when exposed to a more polar dispersed medium. Consequently, a lower percentage of water capacity indicates the formulation's sensitivity to water [129]. The observed reduction in % water capacity, leading to potential phase separation, was notably prominent with an increase in borneol concentration, as depicted in Figure 17. Significantly ($p < 0.05$), the % water capacity for 50BZCB was lower compared to the other formulations. The heightened presence of the hydrophobic compound, borneol, renders the formulation more susceptible to phase separation in the aqueous environment. Surprisingly, 40BZCN and 40BN exhibited a similar % water capacity, suggesting comparable sensitivities to phase separation, despite the introduction of clotrimazole, clove oil, and borneol in 40BZCN, which reduced the solvent content. Furthermore, the lipophilicity of the system declined with increased water content, given the higher dielectric constant of water (78.54) compared to NMP (32) [188, 189]. The strong affinity and miscibility between NMP and water can be attributed to hydrogen bonding and dipole-dipole interactions. Iamir et al. reported that this bond is stronger than that between water molecules and is influenced by a complex with a molal fraction ratio of 2:1 in the water-NMP solution; however, an increase in temperature may intensify this interaction [190]. The advantageous miscibility between water and NMP facilitated significant phase inversion potential, promoting the outward diffusion of NMP from ISM into the aqueous phase. This solvent exchange within the ISM system facilitated phase inversion and matrix formation upon application to the target area, such as the buccal mucosa. A comparable outcome was reported in antibiotic drug-loaded ISMs utilizing ibuprofen and lauric acid as matrix-forming agents, where a reduction in water capacity was observed with an increase in the concentration of the matrix-forming material [178]. Nevertheless, this technique for measurement of water capacity was difficult to apply to polymeric or natural resin-based ISMs because the added water did not diffuse homogeneously in these rather viscous formulations. Consequently, only localized phase separation would happen at the upper portion of these formulations, making it difficult to accurately determine the % water capacity value. In contrast, less viscous ISMs utilizing a non-polymeric or non-resin matrix-forming agent experienced uniform phase separation due to moderate mixing, allowing for a more reliable determination of their percentage of water capacity.

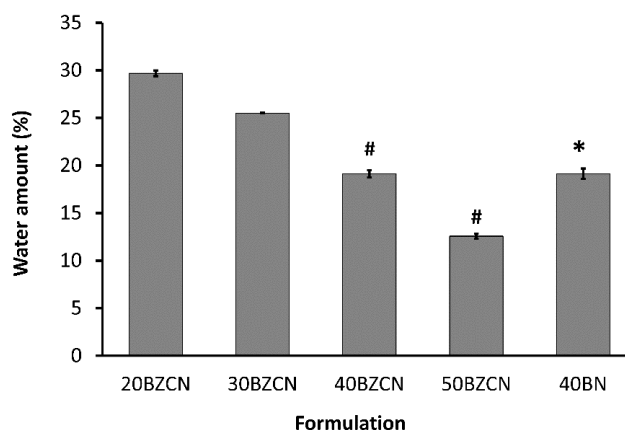


Figure 17. Percentage of water inducing phase separation of borneol ISMs with clotrimazole-loaded and related formulation. Results are presented as mean $\pm$ SD, with $n = 3$. *,# represent $p < 0.05$.

1.10. Drug release and permeation

C. albicans, a commonly occurring commensal microorganism, usually establishes itself on the oral mucosa. The various morphological states of *C. albicans* play a significant role in the stages of colonization, growth, and spreading. The yeast form is linked with both the initial attachment and dissemination, while the hyphal form facilitates *C. albicans*' ability to invade host tissues. [190, 191]. In addition to active penetration, *C. albicans* utilizes endocytosis as another complementary mechanism for host cell invasion. This passive process, driven by the host cell but induced by the fungus, involves lytic enzymes and invasions expressed on hyphae binding to and degrading inter-epithelial cell junctional proteins, such as E-cadherin. This enables the fungus to penetrate between epithelial cells [192]. Consequently, it is crucial to investigate the diffusion ability of antifungal drugs from a formulation through the oral cavity mucosa, such as the buccal membrane. The release behaviors of clotrimazole from ZN and 40BZCN through a nylon membrane using the Franz diffusion apparatus are depicted in Figure 18(A). Clotrimazole exhibited rapid diffusion from ZN through the nylon membrane, reaching a plateau state at 2 hours, notably faster than 40BZCN. The maximum flux value of 40BZCN was $370 \mu\text{g}/\text{cm}^2$ at 2 days. The formation of the borneol matrix, initiated upon contact with the soaked nylon membrane, slowed down the drug diffusion from 40BZCN. The gradual phase inversion process and greater tortuosity of the borneol matrix contributed to the retardation of clotrimazole diffusion into the receiving medium in the receptor. Notably, 40BZCN effectively prolonged the release of clotrimazole. The observed decrease in the release rate of propranolol HCl in the presence of halloysite nanotubes in a polyvinyl alcohol transdermal gel was attributed to drug loading into the material lumen or strong ion interaction between the drug and halloysite nanotubes. Similarly, the higher viscosity of topically used clotrimazole-loaded microemulsion played a role in slowing down drug release [193]. This result confirmed the capability of the developed phase inversion-based ISM, such as 40BZCN, to sustain the release of clotrimazole.

The permeation behaviors of ZN and 40BZCN through the porcine buccal membrane are illustrated in Figure 18(B). Significantly more clotrimazole was released and penetrated from ZN through the porcine buccal membrane into the receiving medium in the receptor compared to the performance of 40BZCN. Additionally, the lag time of drug release for ZN was shorter than that for 40BZCN, which was 7 h and 26 h, respectively. Therefore, the buccal membrane acted as a barrier for drug penetration, and the borneol matrix generated from 40BZCN ostensibly controlled the drug penetration. This drug's permeation through the porcine buccal membrane was conducted in parallel with drug release through the nylon membrane for 48 h. The clotrimazole's release and permeation behaviors could be compared between selected test formulations over the duration of these studies. Saliva flow and meal intake could limit its adhesion to the oral mucosa; nevertheless, the prolonged ability for drug release and permeation of the developed ISM should be useful for efficient treatment of oropharyngeal candidiasis. Additionally, the ability of drug permeation could enhance the antimicrobial activities against invading microbes in the mucosa tissue. A biocompatible and biodegradable spray is the most appropriate dosage form for buccal application due to its versatility, adaptability, physical flexibility, comfort, lightness, acceptability, and adjustable size; therefore, it enhances patient compliance compared to bio-adhesive tablets [194].

The accumulations of clotrimazole in the donor buccal membrane and the receiving medium are shown in Table 7. There was no residue remaining for analyzing the drug content in a donor or in the formulation of ZN, indicating this solution's notable ability for drug penetration. NMP has been claimed as a skin penetration enhancer due to its ability to increase lipid fluidity and has shown a substantial increase in the penetration of various drugs [195]. It could support the penetration of clotrimazole; therefore, a significantly higher amount ($p < 0.01$) of drug retention was found in both the buccal mucosa and ISM compared to the receiving medium. However, this hydrophobic drug accumulated significantly more in tissue than in the aqueous medium ($p < 0.01$). For 40BZCN, the drug amount remained significantly higher in the borneol matrix at the donor part than in the buccal membrane and medium part as shown in Table 7. This finding aligns with the earlier observation in drug release, illustrating the borneol matrix's capability to impede the diffusion and penetration of the drug through the buccal membrane. However, the presence of accumulated clotrimazole in the buccal mucosa indicates its potential antifungal activity against invading microbes in the host tissue, particularly in the buccal area. Oral candidiasis typically involves fungal infections affecting the tongue and other oral mucosal sites, characterized by fungal overgrowth and invasion of superficial oral tissues [34, 38]. Hence, the penetration of clotrimazole serves as a beneficial factor for antifungal activity against invading microbes. The minimal presence of the drug in the receiving medium indicates a limited penetration of clotrimazole into the bloodstream, which aligns with the intended purpose of the developed ISM. Specifically designed as a localized drug delivery system for treating oropharyngeal candidiasis in the oral cavity, the clotrimazole-loaded borneol-ISM aims for drug accumulation in porcine

buccal tissue and the borneol matrix, demonstrating the effectiveness of the 40BZCN formulation against oropharyngeal candidiasis pathogens.

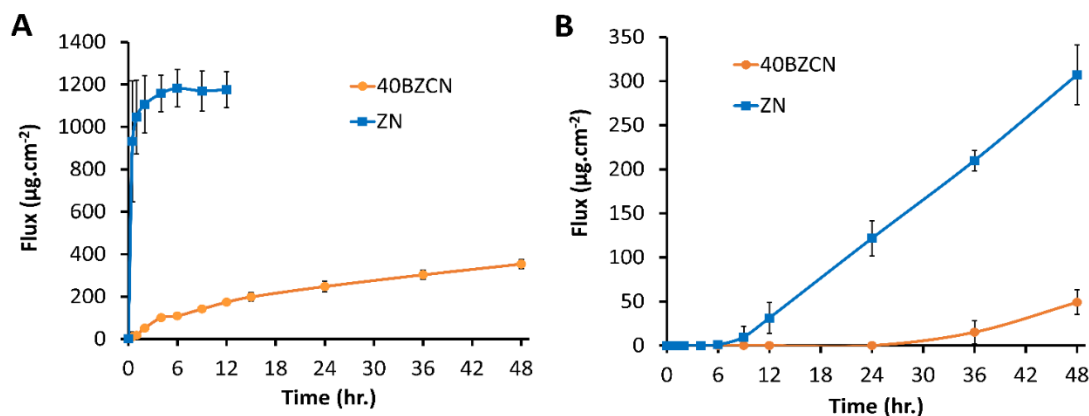


Figure 18. Clotrimazole permeability from 40BZCN compared with ZN through (A) nylon membrane and (B) porcine buccal membrane, with $n = 6$.

Table 7. Clotrimazole remains of BZCN compared with ZN in different parts of Franz cell equipment. Results are presented as mean $\pm$ SD, with $n = 6$.

Place of drug for analyzing	Clotrimazole remains (%)	
	BZCN	ZN
Borneol matrix (upper buccal porcine)	77.10 ± 5.72^a	N/A
Buccal porcine (membrane part)	$16.39 \pm 5.65^{a,b}$	75.51 ± 9.81^b
Received medium (lower buccal porcine)	$3.44 \pm 1.56^{a,b}$	21.56 ± 4.73^b

N/A represents not applicable. The superscript of a and b represent $p < 0.01$.

1.11. Antimicrobial activities

The measurements of the inhibition zone sizes for the developed formula's antimicrobial activity against *S. aureus*, *E. coli*, *C. albicans*, *C. krusei*, *C. lusitaniae*, and *C. tropicalis* as determined through the cup agar diffusion method, can be found in Table 8(A) and Table 8(B). The photographs of inhibition zone of the developed formula against *S. aureus*, *E. Coli* and *C. albicans* are presented in Figure 19. *S. aureus* and *E. coli* were employed as standard Gram-positive and Gram-negative bacteria, respectively. All the tested *candida* species were included in the study because they are all associated with oropharyngeal candidiasis. NMP, a colorless dipolar aprotic solvent, exhibits compatibility with both water and the most frequently employed organic solvents. This is because of its nonvolatility and very good performance for dissolving diverse materials; therefore, it is used as a component in some pharmaceutical dosage forms because of its safety with low toxicity (LD_{50} of 4.15 g/kg) [42, 190, 196]. Additionally, NMP has been employed as a co-solvent to

enhance the saturation solubility of various pharmaceuticals and drug-related substances [196]. Commercial products for periodontitis treatment such as Atridox[®] are composed of 33.3% w/w NMP to dissolve the polymer and DH [197]. Therefore, NMP was employed as a solvent for these developed ISM formulations. As shown in Table 8(B), NMP exhibited notable antimicrobial activities against all test microbes, especially *C. albicans*, *C. lusitaniae*, and *C. tropicalis*. The effective suppression of *C. albicans* and bacterial growth by NMP has been previously documented [198]; thus, it might promote the antimicrobial activities of developed ISMs. The addition of clove oil increased antimicrobial activities against all test microbes except *C. krusei*. Clove oil has been noted for its strong antibacterial properties, capacity to expedite wound healing, and capability to reduce the microbial presence in wounds. [199]. It causes a considerable reduction in the quantity of ergosterol, a specific fungal cell membrane component [200]. Furthermore, clove oil almost completely inhibited the germ tube formation by *C. albicans*. Thus, clove oil demonstrates significant antifungal activity against clinically relevant fungi, including strains resistant to fluconazole. Lauric acid ISMs, which contain a higher concentration of clove oil, show a larger inhibition zone, particularly against *C. albicans*, as reported previously [201].

The notable increase in the clear zone against *S. aureus* was observed because clotrimazole exhibits activity against specific Gram-positive bacteria. ZN significantly inhibited all tested *Candida* species, resulting in clear zones larger than 35 mm. The introduction of clove oil and clotrimazole in NMP (ZCN) caused a slight alteration in the inhibition clear zone compared to the ZN formulation. Specifically, 20BZCN expanded the clear zone, particularly against *Candida* species but not against *E. coli*. Previous studies have reported the antifungal activity of borneol against planktonic *C. albicans* cells and its inhibitory effect on preformed biofilms [202]. Borneol ISMs with vancomycin HCl-loaded have been reported for their efficient inhibition of various microbes, designed for the treatment of periodontitis [183]. However, the inclusion of borneol at concentrations higher than 20% w/w gradually decreased the inhibition zone against all tested microbes. The inhibition clear diameter of 20BZCN against *C. albicans* was significantly ($p < 0.05$) larger than that of 30BZCN, 40BZCN, and 50BZCN, respectively. Moreover, their values showed significant differences among each other, as indicated in Table 8(A). Meanwhile, the inhibition clear diameter of 20BZCN against *C. krusei* was significantly ($p < 0.05$) larger than those of 30BZCN and ZN. Similarly, the inhibition clear diameter of 20BZCN against *C. lusitaniae* was significantly ($p < 0.05$) larger than those of 30BZCN, ZN, and ZCN. Additionally, the inhibition clear diameter of 20BZCN against *C. tropicalis* was significantly ($p < 0.05$) larger than that of 30BZCN. Therefore, the borneol matrix formed after phase transformation *via* phase inversion, and it impeded the diffusion of antimicrobial agents and NMP from borneol ISMs. These results resembled other ISMs fabricated using rosin [185], ethyl cellulose [172], and some fatty acids [129, 130, 145] as matrix-forming materials. An increased concentration of these matrix-forming materials diminished the inhibition zone against pathogens of periodontitis due to their ability to retard drug diffusion

into inoculated agar media. Elevating the concentration of borneol facilitates the creation of a denser matrix, serving as a barrier to decelerate the diffusion of dissolved compounds and solvents, consequently leading to a diminished inhibition zone. Nevertheless, despite this effect, effective inhibition against *Candida* species was attained, characterized by a wide-diameter clear zone. Consequently, the designed localized clotrimazole-loaded borneol-based ISM should demonstrate proficient suppression of the pathogens and associated microbes causing oropharyngeal candidiasis, as intended. The minimum inhibitory concentration (MIC) values for clotrimazole against *S. aureus*, *E. coli*, *C. albicans*, *C. krusei*, *C. lusitaniae*, and *C. tropicalis* are 31.25, >32.00, 0.008, 0.125, 0.008, and 0.008 $\mu\text{g/mL}$, respectively [203-205]. Clotrimazole primarily acts as an antifungal agent with limited antibacterial effects, resulting in higher MIC values against *S. aureus* and *E. coli* compared to *Candida* spp. Typically, one puff of an oral spray releases approximately less than 200 μg . Assuming 100 μg per puff and two consecutive puffs per dose, an ISM (200 μg) is sprayed into the oral cavity, equivalent to 1.542 μg of clotrimazole in the formulation (1% w/w). This calculation is based on the clotrimazole remaining on the porcine buccal mucosa tissue (77.10%), as detailed in Table 8(A) and Table 8(B). The average volume of saliva in adults is 1.07 mL [206]. Consequently, the concentration of clotrimazole from ISM sprayed into the mouth is 1.44 $\mu\text{g/mL}$. These concentrations surpass the MIC values against all *Candida* spp., underscoring the antifungal effectiveness. Furthermore, the antibacterial properties of NMP and borneol within the formulations, as previously reported [199], contribute to a robust inhibitory effect against both bacteria and fungi.



Table 8. Inhibition zone diameter of borneol ISM (A) and related formulations (B) against *S. aureus*, *E. coli*, *C. albicans*, *C. krusei*, *C. lusitaniae* and *C. tropicalis*, with n = 3.

(A)

Microbes	Inhibition zone diameter (mean $\pm$ SD.) (mm.)			
	20BZCN	30BZCN	40BZCN	50BZCN
<i>S. aureus</i> ATCC 6538	20.33 $\pm$ 0.58	17.00 $\pm$ 1.00	16.00 $\pm$ 1.00	13.83 $\pm$ 1.26
<i>E. coli</i> ATCC 25922	14.00 $\pm$ 1.00	12.33 $\pm$ 1.53	11.00 $\pm$ 1.00	8.17 $\pm$ 0.29
<i>C. albicans</i> ATCC 17110	43.67 $\pm$ 1.15 ^a	42.67 $\pm$ 0.58 ^a	42.33 $\pm$ 0.58 ^a	42.67 $\pm$ 0.58 ^a
<i>C. krusei</i> TISTR 5259	38.67 $\pm$ 0.58 ^b	36.33 $\pm$ 1.53 ^b	34.67 $\pm$ 1.15	31.00 $\pm$ 1.73
<i>C. lusitaniae</i> TISTR 5156	39.33 $\pm$ 1.53 ^c	37.00 $\pm$ 1.00 ^c	35.33 $\pm$ 0.58	33.33 $\pm$ 0.58
<i>C. tropicalis</i> TISTR 5306	40.67 $\pm$ 1.15 ^d	39.33 $\pm$ 0.58 ^d	36.33 $\pm$ 0.58	35.00 $\pm$ 1.00

(B)

Microbes	Inhibition zone diameter (mean $\pm$ SD.) (mm.)			
	NMP	CN	ZN	ZCN
<i>S. aureus</i> ATCC 6538	10.67 $\pm$ 1.53	14.17 $\pm$ 1.04	21.33 $\pm$ 1.53	20.00 $\pm$ 1.00
<i>E. coli</i> ATCC 25922	17.67 $\pm$ 1.53	19.00 $\pm$ 1.00	18.00 $\pm$ 1.00	17.00 $\pm$ 1.00
<i>C. albicans</i> ATCC 17110	28.33 $\pm$ 1.53	30.33 $\pm$ 0.58	39.33 $\pm$ 0.58	39.00 $\pm$ 1.00
<i>C. krusei</i> TISTR 5259	14.33 $\pm$ 1.53	14.00 $\pm$ 2.00	37.00 $\pm$ 2.00 ^b	32.00 $\pm$ 1.00
<i>C. lusitaniae</i> TISTR 5156	30.00 $\pm$ 1.00	30.67 $\pm$ 0.58	38.33 $\pm$ 1.15 ^c	38.33 $\pm$ 0.58 ^c
<i>C. tropicalis</i> TISTR 5306	27.67 $\pm$ 0.58	29.33 $\pm$ 0.58	36.33 $\pm$ 0.58	37.00 $\pm$ 1.00

The superscript of a, b, c, and d represent p < 0.05.

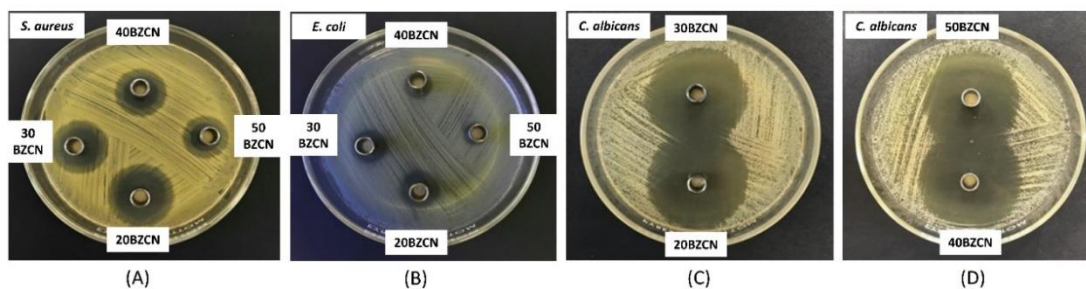


Figure 19. Photographs of agar plate after complete antimicrobial activities testing depicting the inhibition zones of 20BZCN, 30BZCN, 40BZCN, and 50BZCN against *S. aureus* (A), *E. coli* (B), and *C. albicans* (C,D), with $n = 3$.

Based on the results of all experiments in this section regarding clotrimazole-loaded borneol-based ISMs with the addition of clove oil, using NMP as the solvent for oral cavity application, it was found that all prepared formulations yielded clear, less viscous solutions with acceptable pH values. More adding borneol content slightly decreased the density, surface tension, water capacity and spray angle but increased the viscosity and matrix formation. Significantly higher contact angle on agarose gel and porcine buccal mucosa was promoted from NMP removal from these borneol-based ISMs. The clotrimazole-loaded ISM, containing 40% borneol, rapidly transforms into a matrix, making it suitable for spraying. The borneol matrix formed by ISM facilitated enhanced drug penetration *via* synthetic and porcine buccal membranes, signifying its potential antifungal activity against invading fungi. Borneol ISM with clotrimazole showed efficacious inhibition growth against *S. aureus*, *E. coli*, *C. albicans*, *C. krusei*, *C. lusitaniae* and *C. tropicalis* for oropharyngeal candidiasis treatment. Therefore, this ISM developed containing 40% w/w borneol with phase inversion process exhibited great potential as a promising clotrimazole-loaded spray for oropharyngeal candidiasis treatment.

2. Vancomycin HCl-loaded borneol-based ISM for periodontitis treatment

Previously, borneol has been successfully employed as a matrix forming agent in oral ISM sprays for oropharyngeal candidiasis treatment, with a concentration of 40% proving most suitable for matrix formation while effectively controlling drug release and inhibiting essential pathogens in oral cavity. However, extending the application of borneol to other types of formulations remains a challenge. In this section, the utilization of borneol as a matrix agent is interesting to develop ISM formulation for periodontitis treatment. Periodontal disease is a prevalent condition in the realm of ISM development for drug delivery, exemplified by well-established commercial products such as Atridox[®], which has been available for an extended period. This study starts by exploring the physical and physicochemical characteristics of borneol precipitates using various solvents to investigate their impact on borneol crystal formation and to determine the optimal solvent for ISM development. After obtaining the suitable solvent, a study is conducted to establish

the appropriate concentration of borneol for ISM development. Subsequently, vancomycin HCl is incorporated into the optimal ISM concentration, and further efficiency evaluation is performed.

2.1. Morphological and physicochemical behaviors of borneol precipitates

Since, borneol is a water-insoluble bicyclic monoterpene alcohol which could be soluble in some organic solvents, it is possible for using as matrix forming agent of non-solvent induced phase inversion-based ISM. In this study, borneol was dissolved in organic solvents of ISM including NMP, DMSO, PYR, and using ethanol as a controlled solvent. However, understanding the physicochemical properties of borneol after its transformation into a solid-like matrix through precipitation is essential. Therefore, the objective of this study is to characterize borneol precipitates obtained from biocompatible organic solvents, including NMP, DMSO, PYR, and ethanol, to identify the most suitable solvent for borneol-based ISM development.

2.1.1. Characterization of matrix formation

All prepared borneol ISMs were clear and homogeneous. The morphology of the borneol matrix from ISMs in different solvents after being introduced into PBS (pH 6.8) is shown in Figure 20. In the case of borneol dissolved in ethanol (EB), it was unable to undergo a transformation into an opaque matrix, unlike the other formulations. The log P values of ethanol, NMP, DMSO, and PYR are -0.31, -0.46, -1.35, and -0.85, respectively [207-209]. Borneol, a bicyclic monoterpene alcohol with a log P value of 2.85, is characterized as a non-polar substance [50]. It exhibits behavior more like ethanol in terms of hydrophobicity than other solvents due to their comparable polarities; therefore, borneol might dissolve in ethanol more easily than in other solvents. Additionally, the interaction force between borneol and ethanol molecules, characterized by strong H-bonds, could be achieved through the bonding between the -OH group on their molecules. In contrast, the interaction force between borneol and other solvents was a dipole-dipole force, which is weaker than the borneol-ethanol molecular interaction. It is possible that EB was induced by PBS more slowly than other ISM. Consequently, EB was unable to undergo a transformation into matrix within 25 minutes after contact with aqueous solution. On the other hand, borneol dissolved in PYR (PB) tended to form a gel-like matrix within 10 minutes. However, a strong opaque solid-like matrix could not be achieved within 25 minutes. The retardation of phase inversion of PB was due to the low solubility of borneol in PYR and the highest viscosity (14.03 cP) compared with other solvents, as mentioned previously [210]. In contrast, the solubilities of borneol in NMP and DMSO were lower than that in ethanol but higher than that in PYR. Consequently, borneol dissolved in NMP and DMSO (NB and DB) could undergo phase inversion, transforming from a homogeneous solution into an opaque solid-like matrix within 10 minutes, and gradually forming into a more turbid matrix over time.

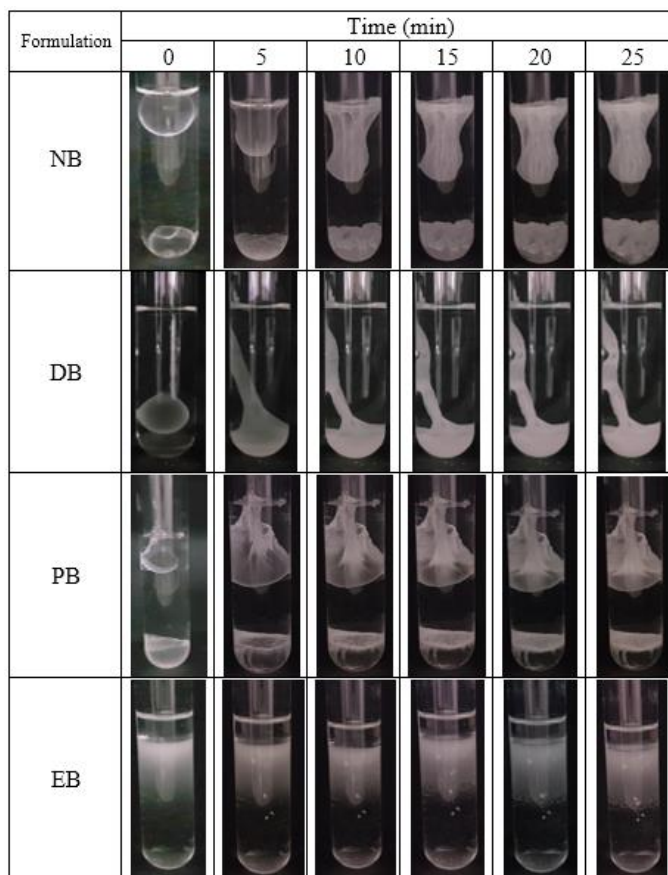


Figure 20. Change of matrix formation over time of different ISM

2.1.2. Determination of precipitate morphology

The morphology of borneol precipitates collected from different organic solvents after complete phase inversion is depicted in Figure 21. Crystals from all formulations exhibited irregular, translucent, and rough surfaces. The crystal size of NB, DB, and PB was 17.57 ± 3.89 , 25.34 ± 3.77 , and 18.21 ± 4.67 μm , respectively. These sizes were smaller than intact borneol (49.57 ± 8.04 μm). Whilst, the size of EB (41.04 ± 4.90 μm) was somewhat smaller than intact borneol but larger than the other borneol precipitates (NB, DB, and PB). The smaller precipitates were more prone to coagulate and form a stronger matrix, making NB, DB, and PB more amenable to transformation into a matrix than EB when the formulation encountered an aqueous environment. However, the scanning electron microscope (SEM) technique was not utilized to examine their topography in this research due to borneol sublimation during operation under vacuum settings with high voltage.

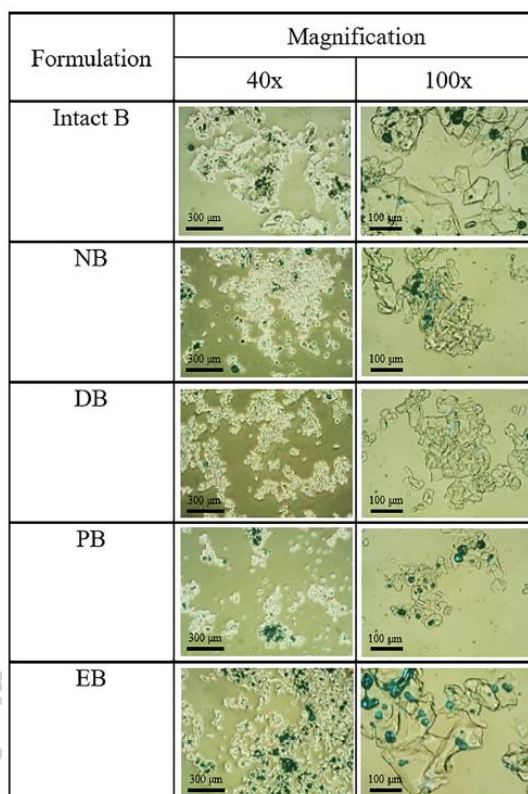


Figure 21. Morphology of different precipitates under an inverted microscope (magnification 40x and 100x).

2.1.3. FT-IR

The FT-IR spectrum of intact borneol revealed main characteristic bands at wavenumbers of 3455, 2955, 1458, and 1060 cm^{-1} . The hydroxy group of the borneol molecule exhibited a broad band at 3650-3200 cm^{-1} , corresponding to -OH stretching vibrations. Another band in the spectral region of 3050-2850 cm^{-1} corresponded to C-H stretching in $\text{CH}_2\text{-CH}_3$, which is connected to primary or secondary carbon. The band observed at 1458 cm^{-1} was related to CH_2 deformation, while the band at 1060 cm^{-1} was attributed to C-O of alcohols [69, 211]. In comparison, both intact borneol and all borneol precipitates exhibited similar dominant bands in the IR spectrum. This suggests that the solvents used for borneol precipitation during phase inversion did not alter the main functional groups of borneol.

2.1.4. DSC and TGA

DSC was conducted to determine the melting points of borneol in the form of intact borneol and borneol precipitates. The melting points of the precipitates (NB, DB, PB, and EB) exhibited a wider range and were somewhat higher than that of intact borneol (193°C), likely due to the interference of some remaining solvent. However, their polymorphism properties remained unchanged. Additionally, the degradation of borneol precipitates occurred at a lower temperature than intact borneol, as observed in TGA thermograms (Figure 22).

Both intact borneol and borneol precipitates showed degradation (more than 10%) when heated to 100 °C and completely degraded up to approximately 170 °C. The rate of degradation, calculated from TGA thermograms, was plotted as a function of temperature in the DTGA thermograms (Figure 22), and the maximum degradation temperature was reported in Table 9. It indicated that the maximum degradation temperature of intact borneol (170 °C) was higher than that of borneol precipitates, which appeared in a range of 150-159 °C. Considering the characterization of precipitate morphology described above, the crystal size of borneol precipitates was generally smaller than intact borneol. This could potentially contribute to a higher degradation rate due to the small crystals having a larger surface area. Moreover, the precipitation of borneol with phase inversion might interfere with the crystallization of borneol, decreasing the thermal stability of borneol [212]. The thermal stability of borneol precipitates was slightly less than that of intact borneol. However, they showed a minimal mass loss (less than 5%) in the temperature range from 30 °C to 100 °C. Therefore, borneol precipitates from organic solvents (except for EB) exhibited a relatively stable behavior up to 100 °C.

Table 9. Thermal properties of intact borneol and borneol precipitates result from DSC and TGA analysis.

Formulation	Melting point (°C)	Maximum degradation temperature (°C)
Intact B	192.69	170.47
NB	198.55	150.54
DB	197.42	156.77
PB	195.78	152.21
EB	197.85	159.14

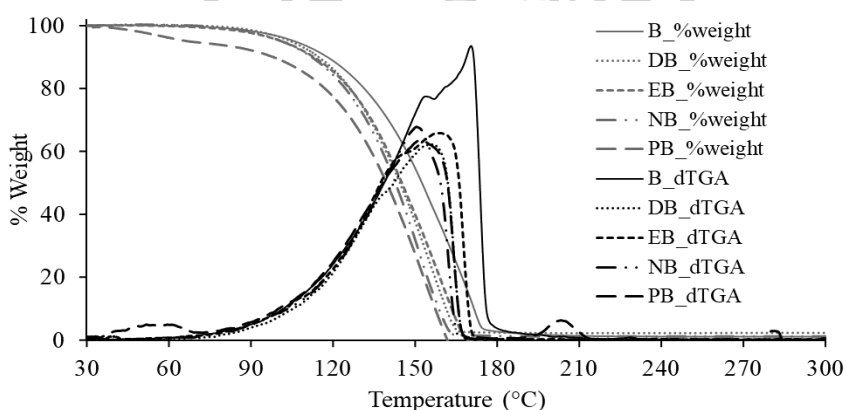


Figure 22. TGA and DTGA thermograms of intact borneol and borneol precipitates.

2.1.5. PXRD

As depicted in Figure 23, the distinct peaks in the PXRD patterns indicate that borneol was present as crystalline materials with characteristic diffraction peaks at 14.4 , 15.2 , and $16.3^\circ 2\theta$. Table 10 presents the intensity and difference in peak intensity values of three dominant peaks of borneol precipitates and those of intact borneol. The peak intensity at $15.2^\circ 2\theta$ of NB, PB, and EB was decreased. Additionally, the peak intensity at $16.3^\circ 2\theta$ of EB was very high. This suggests that the borneol crystal might be interfered with during phase inversion, especially with PYR and ethanol [69, 212]. However, it does not indicate a conversion of crystal form into an amorphous form, as the halo pattern is not found in the PXRD pattern. On the other hand, the PXRD pattern of DB shows little change compared with intact borneol. As a result, DMSO was chosen as the ISM solvent because it had the least influence on the crystallinity alteration of borneol compared to other solvents. The orderliness and strength of the crystals, indicative of a crystalline shape, contribute to the formation of a dense matrix. When a drug-loaded ISM was applied, the drug becomes entrapped within the dense matrix structure and was released gradually over time. Conversely, the absence of the crystal state may impeded the creation of dense and robust matrices.

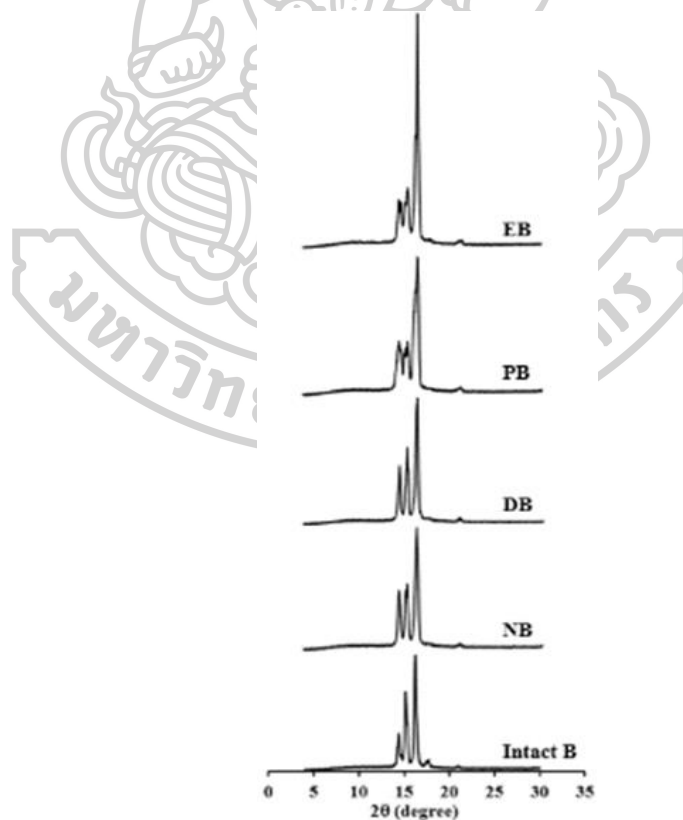


Figure 23. X-ray diffraction pattern of intact borneol and borneol precipitates.

Table 10. Intensity and difference of intensity of dominant peaks of intact borneol and borneol precipitates.

2θ (°)	Intensity (count/sec)					Difference of peak intensity			
	Intact borneol	NB	DB	PB	EB	NB	DB	PB	EB
14.4	6,513	8,567	8,793	7,493	7,173	2,054	2,280	980	660
15.2	14,840	9,680	11,543	7,846	8,510	- 5,160	- 3,297	- 6,994	- 6,330
16.3	21,680	18,150	18,880	19,733	36,180	- 3,530	- 2,800	- 1,947	14,500

The Borneol ISM prepared using ethanol as a solvent failed to transform into a solid-like matrix, while PB took a considerable amount of time and did not undergo complete transformation. The transformation of borneol ISM solution in NMP and DMSO to a solid-like matrix occurred within 10 minutes, and their borneol precipitates exhibited a smaller size than intact borneol. Borneol precipitates showed lower degradation temperatures than intact borneol, while their melting temperatures were rather higher than intact borneol, with a wider range. Nonetheless, borneol precipitates remained stable up to a temperature of 100°C, and their main functional groups were similar to those of intact borneol. The crystallinity of borneol precipitates decreased compared to intact borneol due to interference from some remaining solvent. Particularly, the borneol precipitate from DMSO exhibited the least crystallization change. Therefore, DMSO was considered the proper solvent for borneol ISM. For ISM development, solvent type is a crucial factor that can influence the physicochemical properties of borneol precipitate. Understanding borneol precipitates by physicochemical characterization is important for designing and developing borneol ISM drug delivery formulations.

2.2. Development of borneol-based non-solvent induced phase inversion-based ISM for periodontitis treatment of vancomycin HCl

Based on the experimental data from the previous section, it was determined that DMSO was the most suitable solvent for ISM formulation development, although NMP was not less efficient than DMSO. However, in the preliminary test, borneol as matrix forming agent and vancomycin HCl as model drug were dissolved in both DMSO and NMP. The prepared ISM using NMP as the solvent found the white-solid precipitated after 30 minutes of mixing, while ISM using DMSO resulted in a clear solution with no precipitates. Therefore, DMSO is the appropriate choice as the solvent for the borneol ISM using vancomycin HCl as model drug in this study. The aim of this study was prepared and evaluated borneol-based ISM for periodontitis treatment and find out the proper concentration of borneol for using in this ISM.

2.2.1. Appearance, pH, and density

All of prepared ISMs appeared as clear and homogeneous solutions. With increasing borneol content, the pH considerably lowers ($p < 0.01$) as shown in (Figure 24(A)) due to the reduction of DMSO solvent (pH of DMSO = 9.68 ± 0.23). Whilst, The pH of VDB (7.90 ± 0.06), was lower than that of DMSO

because of the acidic hydrochloride in the salt form of vancomycin HCl, although only 1%w/w vancomycin HCl was incorporated. Normally, the pH of oral cavity is maintained near neutrality, around 7.06, like the pH of saliva. In case of patient with chronic periodontitis, the average pH in the oral cavity is 6.85 (slightly acidemia) [100]. Additionally, the pathogens associated with periodontitis typically thrive in a mildly acidic pH environment. *P. gingivalis*, a principal pathogen of periodontitis, tends to grow within the pH range of 6.5-7.0 [213, 214]. On the contrary, excessive alkalinity can stimulate the formation of dental plaque crystals, contributing to periodontal disease [100]. Therefore, a slightly mild-alkaline of VDB was deemed suitable for application in the crevicular pocket as it can mitigate pathogenic stimulation. In practical terms, the resting fluid volume in the crevicular pocket of a patient with periodontitis is approximately 1.0 μL , with a crevicular clearance rate of 40 times/h, resulting in a fluid volume clearance rate of 20-40 $\mu\text{L/h}$ [215]. When small amounts of the formulation were injected into this pocket, it exhibited a close-to-physiological pH (around 7.90). Consequently, the likelihood of gingival tissue irritation is minimal due to the rapid transformation of the preparations into a solid mass and the application of a very small injection volume. This approach not only reduces the pathogenic stimulating factor but also avoids initiating gingival tissue irritation [100].

The density of borneol solutions gradually decreased ($p < 0.01$) in a narrow range of 1.00-1.10 g/cm^3 with borneol concentration dependence (Figure 24(B)), whereas the densities of DMSO and borneol were 1.09 and 1.01 g/cm^3 , respectively [174, 216]. Normally, the density is related and used to estimate viscosity and the nucleation rate of soft compound [217]. The higher quantities of lower density borneol in the formulations resulted in lower overall formulation density. In practical terms, the density of a binary mixture at a given temperature is the sum of the density of each pure compound under the respective mole fraction ratio [218]. Therefore, all ISM showed a tendency to settle in the crevicular pocket because their solution density values were higher than that of the saliva fluid (aqueous fluid).

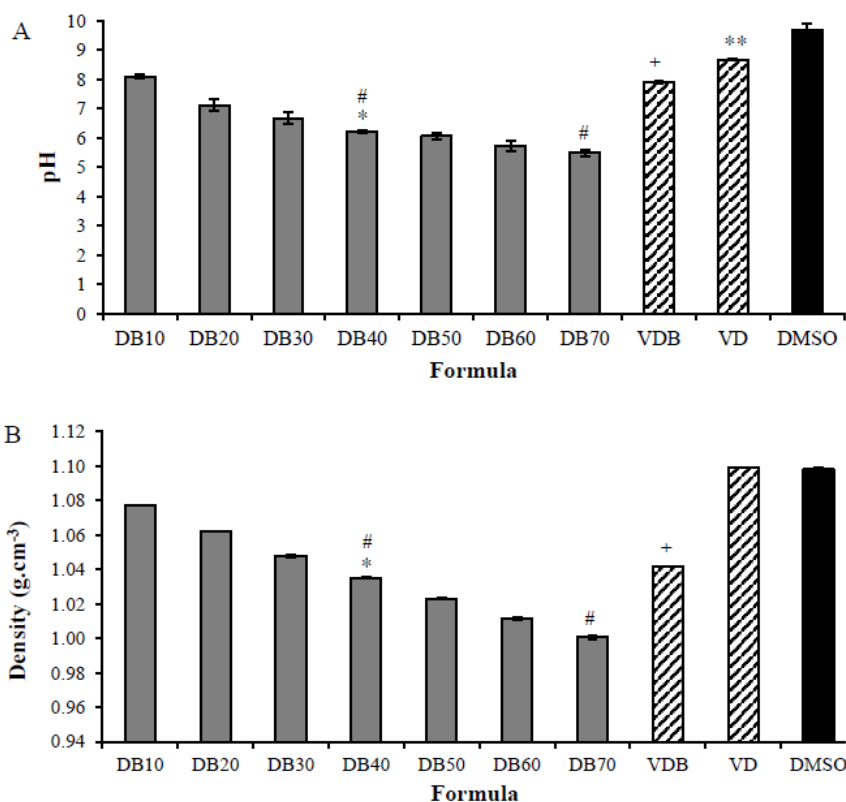


Figure 24. (A) pH and (B) density of different formulations. Results are presented as mean $\pm$ SD, with $n = 3$. *,** represent $p < 0.01$ and $p < 0.05$ compared with DMSO. #,## represent $p < 0.01$ compared with DB10 and $p < 0.01$ compared with DB40.

2.2.2. Viscosity and rheological behavior

The viscosity significantly increased ($p < 0.01$) with increasing borneol concentration (Figure 25(A)) because of the strong hydrophilic interaction between the hydroxyl group of borneol and the sulfoxide group of DMSO with diminishing DMSO molecular movement and decreasing solvent amount [217, 218]. However, the viscosity of each formulation was notably lower than 20 cP since the low density, small size, and unsophisticated structure of the borneol molecule minimized the molecular interactions indicating the slightly viscous manner with ease of injectability [86, 128]. Rheological behavior is commonly assessed through shear rate and stress parameters. Viscosity (η) or the exponential constant is a key parameter that describes flow behavior. The relationship between shear stress and shear rate profiles for the formulations is depicted in Figure 25(B). The exponential constant for each formulation fell within the range of 0.92-1.01, nearly reaching 1.00. The linearity trends of all formulations shifted towards higher shear stress values as the shear rate increased, indicating Newtonian flow behavior [219, 220]. The flow behavior of injectable ISM should be a Newtonian or pseudoplastic flow for easier injectability after pressing the syringe plunger [221]. In comparison, the viscosities of the borneol ISMs were noticeably lower than those of polymeric

ISMs. For instance, the viscosities of polymer ISMs consisting of 20% w/w ethyl cellulose, 30% w/w bleach shellac, and 35% w/w Eudragit RS were 2200, 120, and 300 cP, respectively. In contrast, formulations containing ethyl cellulose at 25% w/w, bleach shellac at 35% w/w, and Eudragit RS at 40% w/w were unable to flow [172, 219]. From this experiment, it was observed that the low viscosity characteristic of borneol ISMs could facilitate their ease of injectability, presenting a significant advantage over polymer ISMs. After injection, ISM is exposed to the aqueous environment in the periodontal pocket, where DMSO diffuses out and exchanges with the aqueous fluid, inducing the phase separation of the dissolved matrix-forming agent into a solid matrix. Therefore, there may be a period for diffusion in and out of the aqueous phase and solvent, resulting in a lag phase for the phase separation of the matrix-forming agent [172]. During this time, the ISM may remain in a solution state. The rapid solvent exchange and phase separation should be observed when the viscosity of the system in the periodontal pocket is low. Additionally, viscosity should be considered because it influences other parameters, such as contact angle, surface tension, the rate of matrix formation, and the force for expelling the formulation via a syringe.

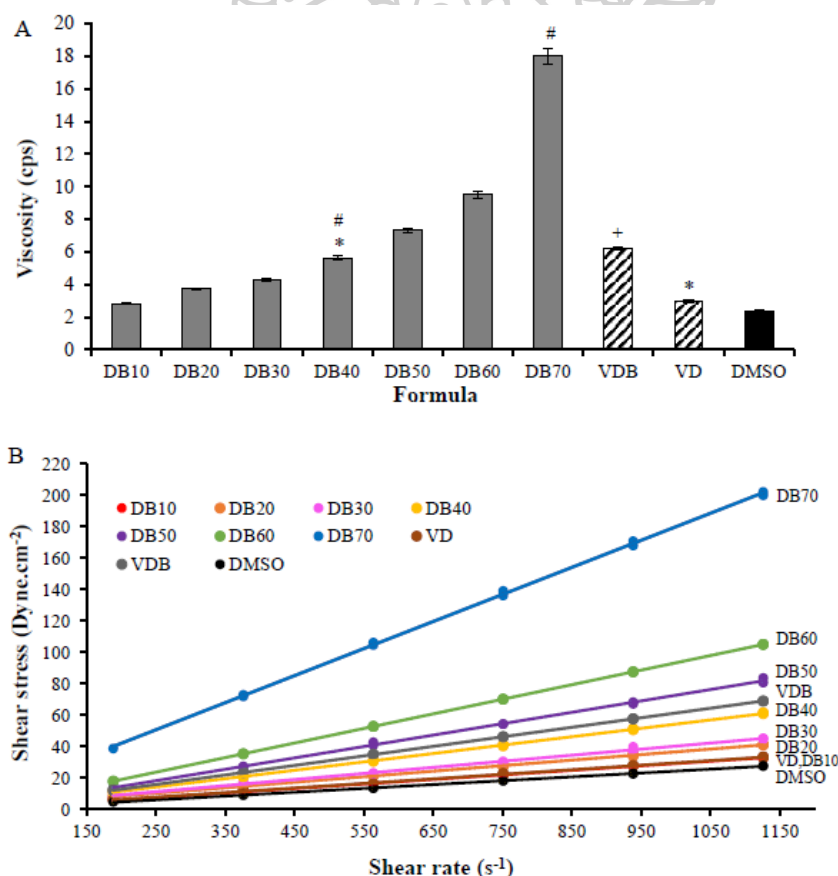


Figure 25. (A) Viscosity and (B) rheological behavior showing in shear stress and shear rate profile of different formulations at room temperature. Results are presented as mean $\pm$ SD, with $n = 3$. * $p < 0.01$ compared with DMSO, # $p < 0.01$ compared with DB10 and + $p < 0.01$ compared with DB40.

2.2.3. Surface tension

The wettability property typically corresponds to the well-fitted performance of dosage forms with any target sites, and some parameters such as surface tension and contact angle could signify these characteristics properly. Surface tension at liquid-air interfaces results from the greater attraction of liquid molecules to each other due to cohesion force, making the tendency for minimum liquid surfaces. The report on surface tension and contact angle of the borneol ISM has not been mentioned previously. The surface tension of the non-solvent induced borneol-based ISM formulations containing various borneol concentrations appeared in a narrow range of 33.09-37.99 mN/m, which was lower than that of DMSO (43.71 mN/m) as shown in Figure 26(A). The surface tensions of the solutions comprising low borneol concentrations (DB10 and DB20) significantly decreased ($p < 0.01$) in a borneol concentration-dependent manner, but they were only slightly altered in the solutions with a high borneol concentration. Typically, DMSO is a dipolar aprotic solvent with strong dipole force interactions between their molecules, whereas borneol is a bicyclic monoterpene alcohol whose structure could provide a steric hindrance effect [4, 86]. Hence, DMSO has a higher bond strength between molecules than borneol. In the binary mixture, the component with the strongest molecular interaction settles down in the bulk liquid phase instead of interacting on the surface of the droplet mass. Meanwhile, the compound with the lowest molecular interaction is expelled from the bulk to the surface of the droplet mass due to the attractive forces between solvent molecules [177, 222]. When a small amount of borneol was added to formulations (DB10 and DB20), the dissolved borneol molecules moved to the surface. Consequently, the surface tension of that binary system was reduced, primarily influenced by the deposition of borneol molecules at the droplet surface. The surface layer became saturated with borneol molecules as the borneol concentration increased to 30%. As a result, the surface tensions of the solutions with higher borneol concentrations did not change significantly. The schematic diagram illustrating this transition in surface tension behavior of the binary mixture due to different borneol concentration depositions is presented in Figure 26(B). This discovery provides new insights and potential applications, laying the groundwork for further experimental exploration of interactions between substances in the future. Additionally, the surfactant effect from incorporating borneol into the solution at a low concentration might decrease surface tension by minimizing the attractive forces within the solution, leading to an increase in free volume space [223].

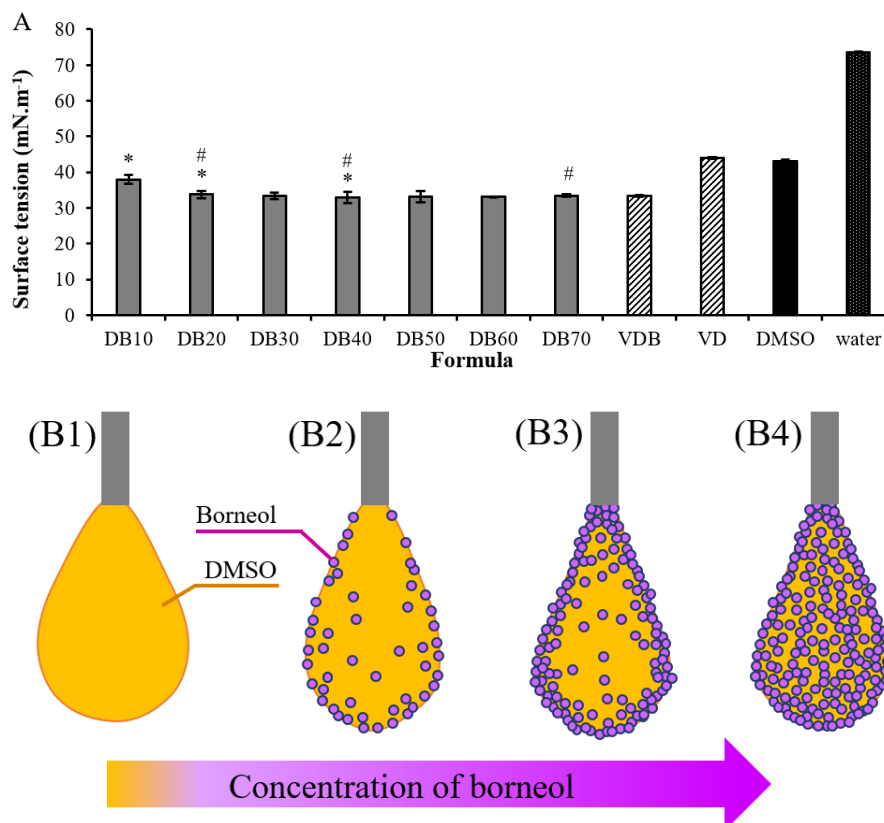


Figure 26. (A) Surface tension of borneol ISM. Results are presented as mean $\pm$ SD, with $n = 3$. * $p < 0.01$ compared with DMSO, # $p < 0.01$ compared with DB10 and **(B)** Schematic diagram exhibiting borneol molecules distribution in pendant drop in air of ISM containing: **(B1)** 0% borneol; **(B2)** 10%-20% borneol; **(B3)** 30% borneol (saturated concentration) and **(B4)** more than 30% borneol (excess concentration).

2.2.4. Contact angle and wettability

The contact angle, measured as the angle between the surface of the fluid and the outline of the contact surface base, was found to be minimal in the range of 16.00° to 28.68° for droplets of borneol solutions on a glass slide. This angle gradually decreased with borneol concentration dependence, as illustrated in Figure 27. Similar behavior was observed for the contact angle on surfaces of acrylic and LDPE. The dipole-dipole molecular force between DMSO molecules and the molecular interaction between these surfaces and the solvent were disrupted by the hydrophobic nature of borneol, especially with increasing borneol concentration. Consequently, the contact angle was markedly smaller for higher concentrated borneol solutions. However, a slightly smaller or stable contact angle was evident in the case of 70% borneol solution on the surfaces of glass slide, acrylic, and LDPE. This characteristic might be attributed to the high viscosity of the borneol solution, which impeded its spreadability. The contact angle of DB10 on glass slide, paraffin, acrylic, and LDPE was significantly higher than that of DB40 and DB70. There was a decrease in contact angles on

paraffin as borneol concentration increased, indicating some hydrophobic interaction between the hydrophobic paraffin surface and borneol molecules (Log P = 2.85) [224].

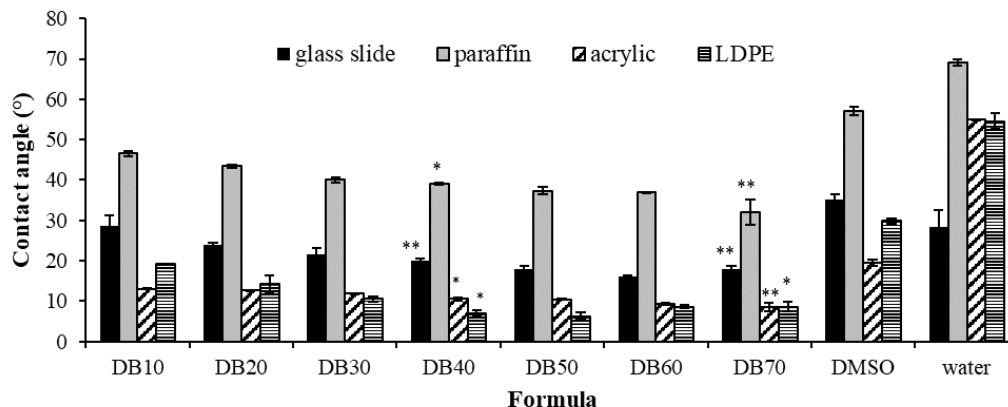


Figure 27. Contact angle of borneol ISM on glass slide, paraffin, acrylic, and plastic. Results are presented as mean $\pm$ SD, with $n = 3$. * $p < 0.01$ and ** $p < 0.05$ compared with DB10.

Moreover, the contact angle was determined on agarose gel and porcine oral cavity tissues, as presented in Figure 28. There was a trend for the enhancement of the contact angle on agarose gel and the gum/buccal mucosa of porcine for higher concentrated solutions. This increment behavior was provoked by the transformation with phase inversion of the borneol solution into a semi-solid state and thereafter into a matrix-like state induced by phase inversion. When borneol solutions were exposed to water from agarose gel or gum/buccal mucosa of porcine, borneol initiated phase inversion due to its water-insoluble characteristic, leading to diminished spreading ability on the tested material surfaces [225] as shown in the schematic diagram (Figure 29). The solutions comprising 60% and 70% borneol were not tested for their contact angle on porcine gum in this study. High-concentrated borneol-loaded solutions in DMSO could promote more rapid phase transformation from an aprotic solution into a solid-like matrix via phase inversion on the surfaces of both agarose and gum/buccal mucosa of porcine. However, this value of the binary mixture containing 40% - 70% borneol on buccal mucosa was rather stable. The values of the contact angle were rather higher with borneol concentration dependence; nevertheless, they were smaller than 90° , exhibiting their good wettability. Typically, the wetting ability of dosage forms is essential for the adhesiveness manner between the dosage form and the surface of the target site for improvement of contact time [226]. The contact angle of DB10 on agarose gel and these porcine tissues was significantly lower than that of DB40 and DB70. However, DMSO, water, DB10, and DB20 did not exhibit a contact angle on the tongue surface because they are absorbed into this tissue. Typically, DMSO readily penetrates the skin after cutaneous application [227]. In addition, an increase in surface roughness has been widely suggested to decrease contact angles because the liquid is likely to penetrate into the roughness grooves [228].

Figure 30(A) and Figure 31(A) depict the markedly rough morphology of the porcine tongue, while Figures 30(B) and 31(B) reveal a relatively smooth topography on the porcine gum. Additionally, Figures 30(C) and 31(C) exhibit a smooth surface on the buccal mucosa. Understanding the surface tension and contact angle behaviors of the borneol-DMSO binary mixture is valuable for its potential development into an ISM for drug delivery within the oral cavity.

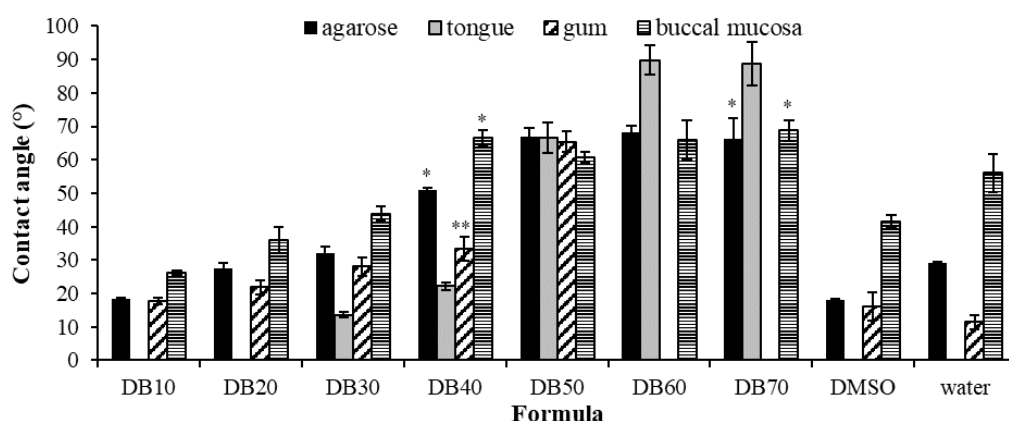


Figure 28. Contact angle of borneol ISM on agarose gel and difference type of porcine tissues. Results are presented as mean $\pm$ SD, with $n = 3$. * $p < 0.01$ and ** $p < 0.05$ compared with DB10.

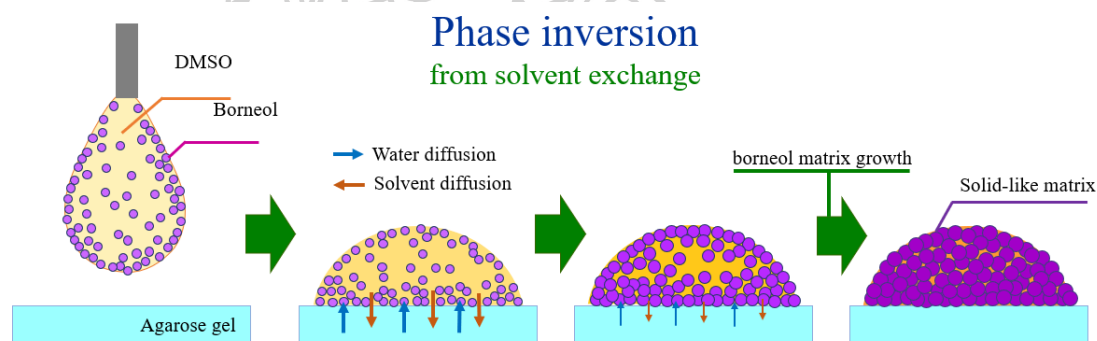


Figure 29. Schematic diagram of the phase inversion phenomena of borneol ISM, transitioning from a solution to a solid matrix upon contact with agarose gel.

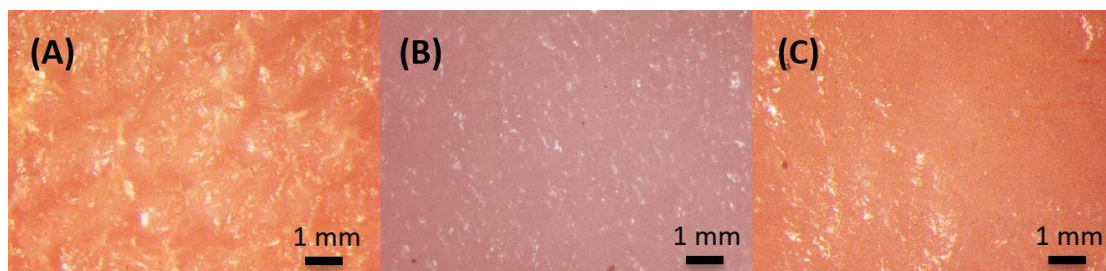


Figure 30. Surface morphology of tongue (A), gum (B), and buccal mucosa (C) under inverted microscope (magnification of $5\times$).

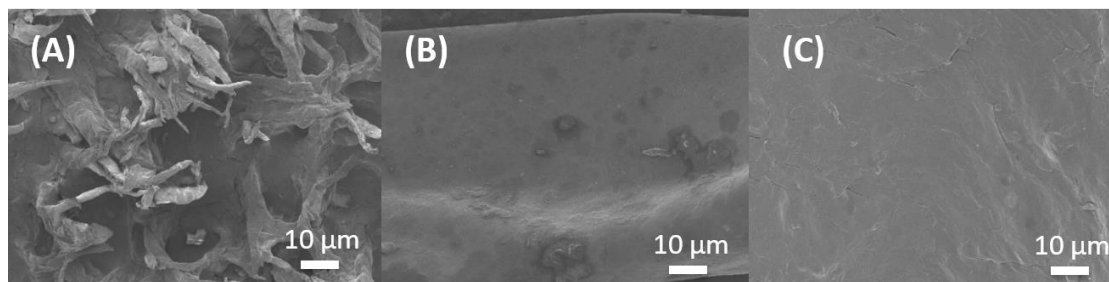


Figure 31. SEM images of tongue (A), gum (B), and buccal mucosa (C) (magnification 500×).

2.2.5. Matrix formation behavior at the macroscopic level

The PBS was used to monitor the matrix development of borneol ISMs. The prepared ISM was transformed into a solid-like matrix with an opaque mass over time, as shown in Figure 32. The ISM was injected into a test tube containing PBS at pH 6.8 via a needle, and subsequently, they were drawn from the bottom to the surface of the PBS to showcase the discernible changes in the matrix following contact with PBS. Interestingly, within 25 minutes, DB10 and DB20 did not manifest an evident solid-like matrix, attributed to an insufficient concentration of borneol for the formation of a dense matrix. For DB10 and DB20, a turbid colloid dispersion in the PBS was observed, characterized by loosely packed solid particles at the bottom of the test tube. The density of matrix formation increased with higher borneol concentrations. Notably, DB60 and DB70 encountered challenges during needle injection, sticking more readily in needles due to their heightened viscosity. Consequently, some sections of the matrices of these formulations were observed to float to the surface, influenced by the needle tip drag. Additionally, the lower density of these formulations contributed to the floating of certain matrix parts. In contrast, DB30 to DB50 tended to form solid-like matrices without floating and remained submerged over time, exhibiting characteristics suitable for application as an ISM for periodontitis injection. These observations suggest that patients can expect a comfortable administration process due to the formulations' rapid transformation. Importantly, ensuring patient compliance remains a crucial factor in the overall treatment process [40, 229]. The matrix formation behavior of lauric acid ISM occurred in PBS from the solution state to a gel-like matrix and then to a solid-like matrix over time [129]. Remarkably, an accelerated transformation was observed with an increasing amount of lauric acid. In contrast, ISM formulations containing less than 10%, 20%, and 30% w/w of ethyl cellulose, bleach shellac, and Eudragit RS, respectively, exhibited no discernible matrix formation. The matrix formed by the ethyl cellulose ISM was notably thick, while the one prepared with bleach shellac displayed elasticity. Conversely, the matrix of Eudragit RS was found to be fragile [219]. Hence, the matrix formation of borneol-ISM demonstrated similarities to that of ethyl cellulose, bleach shellac, and Eudragit RS ISMs. The rate of matrix formation for VDB mirrored that of DB40, indicating that vancomycin HCl does not impact the rate of matrix formation. This experiment

affirms the ease with which borneol-ISM can be injected into the crevicular pocket, undergoing an instantaneous transformation into a matrix. The transformation process of borneol ISM commenced upon contact with the PBS. Subsequently, DMSO from the formulation diffused concurrently with water, leading to the development of a solid-like borneol matrix characterized by hardening, white, opaque solid. This process followed a non-solvent-induced mechanism with initiated phase inversion [220]. In summary, the matrix with a higher borneol concentration exhibited greater density than the matrix with a lower borneol concentration (Figure 32). The mechanism underlying matrix formation can be elucidated through the phase inversion behaviour, wherein dissolved borneol undergoes a phase inversion, forming a solid matrix while its solvent exits the system [42].

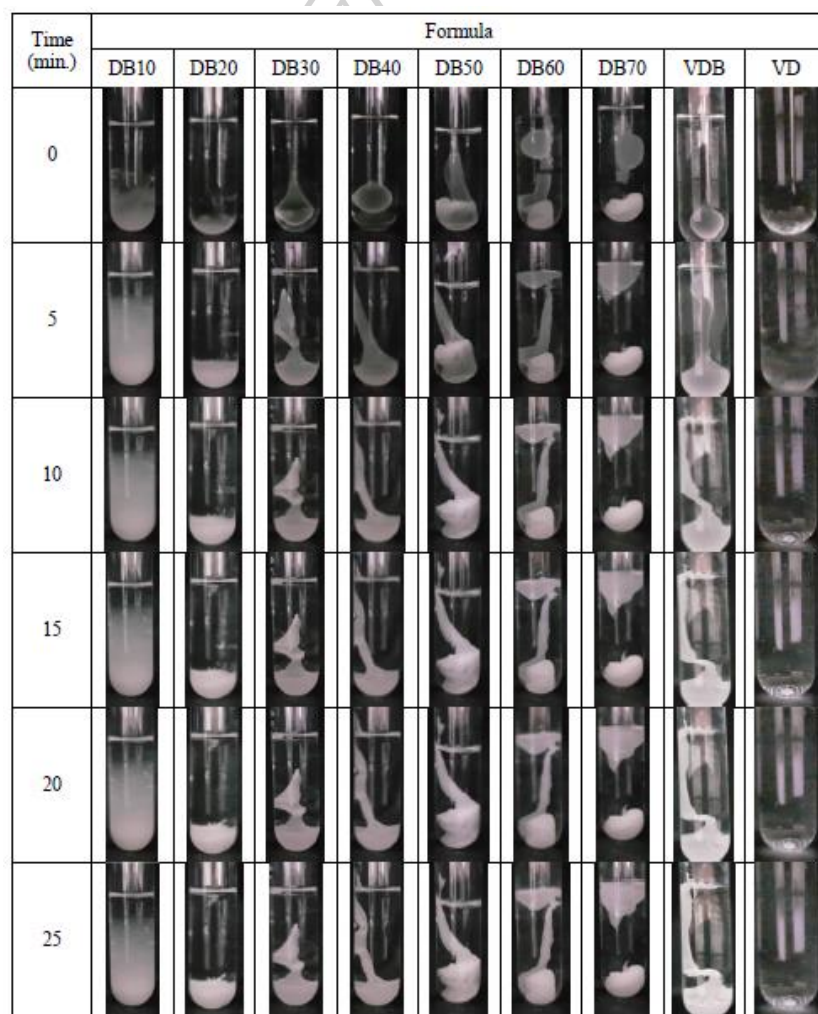


Figure 32. *In vitro* matrix formation behavior of ISMs through a 27-gauge needle into PBS pH 6.8.

The thickness of the occurred borneol matrix increased over time from phase inversion in the agarose hole (Figure 33). At the initial state, a dense network of the borneol matrix formed around the agarose rim but the formation

slowed down over time due to high tortuosity and low porosity effect of the borneol matrix [188]. The formation of the opaque borneol matrix ring occurred rapidly through phase inversion upon exposure to a buffer from agarose. While there was a gradual presence of borneol matrix formation, the outward diffusion of the solvent suggested that it could escape through the borneol matrix with higher tortuosity and less porosity. The rate of matrix formation demonstrated a significant decrease over time ($p < 0.05$) (Table 11). Comparatively, the rate of matrix formation for DB70 was notably faster than that of DB10 at the same time ($p < 0.01$). Additionally, the rate of matrix formation for DB40 at the initial times (1 and 5 min.) was significantly faster than those for DB10 ($p < 0.05$) (Table 11). The thickness of the matrix depended on the borneol concentration, leading to an increase in the rate of matrix formation with a higher borneol concentration. Importantly, vancomycin HCl had almost no impact on the rate of matrix formation. When comparing DB40 and VDB, their rates of matrix formation were not significantly different ($p < 0.05$) at 1, 5, 10, and 30 min (Table 11). The borneol network formation at the agarose surface front hindered phase inversion, resulting in a denser matrix with higher tortuosity and less porosity [42]. Consequently, the rate of transformation decreased with time. However, evidence of a longer distance of matrix formation over time was apparent due to the ongoing exchange of solvent and water, leading to continuous growth of the borneol matrix, similar to the case of the lauric acid ISM system [216].



Figure 33. Matrix formation behavior of different formulations after contact with agarose gel containing PBS pH 6.8 in an artificial crevicular pocket under a stereo microscope at (magnification of 4 $\times$).

Table 11. Rate of matrix formation at different times of formulations upon contact with agarose gel containing PBS pH 6.8, with n = 3.

Formula	Matrix formation rate at various time (mean $\pm$ SD.) (mm./min)			
	1 min	5 min	15 min	30 min
DB10	0.556 $\pm$ 0.137 ^{a,c}	0.149 $\pm$ 0.028 ^{*,b,d}	0.070 $\pm$ 0.016 ^{*,e}	0.042 $\pm$ 0.011 ^{*,f}
DB20	0.698 $\pm$ 0.106	0.170 $\pm$ 0.022 [*]	0.076 $\pm$ 0.009 [*]	0.049 $\pm$ 0.006 [*]
DB30	0.722 $\pm$ 0.167	0.173 $\pm$ 0.031 [*]	0.075 $\pm$ 0.008 [*]	0.045 $\pm$ 0.004 [*]
DB40	0.730 $\pm$ 0.165 ^a	0.183 $\pm$ 0.035 ^{*,b}	0.081 $\pm$ 0.014 [*]	0.044 $\pm$ 0.008 [*]
DB50	0.786 $\pm$ 0.134	0.194 $\pm$ 0.032 [*]	0.089 $\pm$ 0.016 [*]	0.050 $\pm$ 0.008 [*]
DB60	0.802 $\pm$ 0.106	0.203 $\pm$ 0.028 [*]	0.094 $\pm$ 0.017 [*]	0.052 $\pm$ 0.010 [*]
DB70	1.087 $\pm$ 0.220 ^c	0.256 $\pm$ 0.039 ^{*,d}	0.099 $\pm$ 0.010 ^{*,e}	0.055 $\pm$ 0.006 ^{*,f}
VDB	0.680 $\pm$ 0.162	0.164 $\pm$ 0.033 [*]	0.079 $\pm$ 0.012 [*]	0.044 $\pm$ 0.007 [*]

* represent $p < 0.01$ compared with matrix formation rate at 1 min of each formulation.

^a and ^b represent $p < 0.05$. ^{c, d, e} and ^f represent $p < 0.01$.

2.2.6. Interfacial phenomena of matrix formation

The matrix formation behavior at the interface between the formulation and the aqueous fluid was observed using agarose gel as a mimetic crevicular fluid. This experimental setup aimed to simulate the phenomenon occurring at the interface of the formulation, facing the target site containing biological fluid. The agarose platform served as a simulated biological site where ISM were administered and transformed into a matrix [129, 130]. At the interface, the appearance of a crystal matrix was observed (Figure 34). The matrix formation from borneol crystals occurred progressively, and its rate increased with a higher borneol concentration. Theoretically, there exists energy at the interface between any immiscible objects, promoting nucleation and subsequent crystallization through a thermodynamic driving force. The addition of vancomycin HCl marginally slowed the formation of the borneol crystal matrix compared to that of DB40, and the crystal matrix was absent in the case of DMSO and VD due to the absence of borneol matrix (Figure 34).

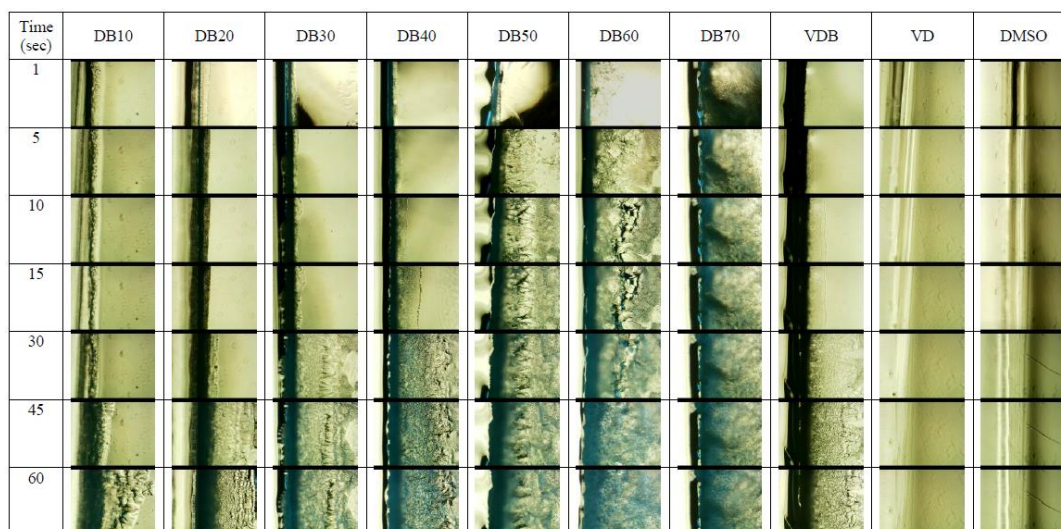


Figure 34. Interfacial phenomena of matrix formation at the interface between formulation and PBS pH 6.8 under an inverted stereomicroscope (magnification of 40×)

2.2.7. *In vitro* drug release

DMSO was considered the proper solvent for Borneol-ISMs due to its apparent low viscosity, which facilitates injectability and safety in humans, given the low toxicity of DMSO (LD₅₀: 2.5-8.9 g/kg) [83]. The low borneol concentrations of ISMs, such as DB10 and DB20, did not form a dense matrix. Conversely, the formulations with high borneol concentrations, such as DB50, DB60, and DB70, showed rapid matrix formation (Figures 32-34); thus, these formulations may transform into solid-like matrices before filling into the crevicular pocket, and their shapes might not fit in the pocket. Hence, formulations, as mentioned above were excluded, whereas DB40 was selected to incorporate an antimicrobial drug as VDB for testing of *in vitro* drug release. Vancomycin HCl was released from VDB completely for 14 days while vancomycin HCl from VD exhibited the fastest-burst drug release above 90% at 24 h (Figure 35); thus, VDB showed a sustainable drug release. Commercial products such as Atridox[®] containing 10% DH using 33.03% poly(D,L-lactide) or PLA as a matrix material controlled the drug release for 7 days. This commercial product was injected into the crevicular pocket until the formulation reached the top of the gingival margin, and the amount of formulation depended on the individual pocket size of patients [33]. For our previous publications the dialysis tube method was applied for drug release test, the apparent faster drug release was obtained compared to using cup or membrane-less method owing to the barrier effect from membrane retarding the rate of phase inversion [63]. Therefore, this experiment applied the cup or membrane-less method to simulate the drug administration into crevicular pocket since the formulation could expose the environmental fluid suddenly promoting the matrix formation for modulating the vancomycin HCl release. Moreover, many researchers have applied the cup

method to study drug release. DH was released from different polymeric-based ISMs containing 40% Eudragit RS, 30% bleached shellac, 40% beta-cyclodextrin and 20% Ethocel in 16 h, 2 days, 7 days and 2.5 days, respectively, by using the cup method [63, 172, 230, 231]. In these experiments, a porcelain cup (10 x 12 mm) containing a sample was immersed in 100 mL of PBS (pH 6.8) at 37 °C. The experimental details and conditions in this study were comparable to those mentioned above and in other studies, ensuring that the results of vancomycin HCl release are consistent. Additionally, vancomycin HCl release was compared with other polymeric systems, such as thermosensitive methoxy polyethylene glycol-co-poly (lactic acid-co-aromatic anhydride) hydrogel, β -tricalcium phosphates-FeAg nanocomposites, and oxidized hyaluronic acid/adipic acid dihydrazine hydrogel, with release durations of 10, 14, and 3 days, respectively [123, 232, 233]. Consequently, VDB exhibited a slower vancomycin HCl release compared to polymeric ISM, providing sustained local drug release in the crevicular pocket, thereby improving patient compliance through reduced medication frequency. The matrix from VDB, after immersion in PBS pH 6.8, settled in the cup throughout the two-week experiment. Furthermore, the density of VDB was greater than that of the aqueous phase, leading to the formulation settling in the cup. Additionally, the results of matrix formation after injection (Figure 32) confirmed that VDB remained submerged over time.

For the treatment of periodontitis, local drug delivery systems like Atridox[®] are administered following the cleaning of the periodontal pocket with an irrigating solution. The use of an irrigating solution, such as chlorhexidine, aids in reducing plaque bacteria before the application of a prolonged drug delivery system [234]. In the case of vancomycin HCl, it is encapsulated in the borneol matrix through phase inversion, thereby slowing down the release of vancomycin HCl for a period of one week or more. The release is rapid initially at the surface of the matrix, followed by a gradual release over time due to the longer diffusion path length. A similar initial burst release of vancomycin HCl was observed in the lauric acid-ISM system, attributed to the rapid DMSO out of the gel. The sustained release of vancomycin HCl was achieved through the higher tortuosity and lower porosity of the remaining lauric acid wax matrix after extensive crystallization of this fatty acid [128]. The insoluble borneol matrix could also reduce the penetration of DMSO from the matrix to the medium and retarded the diffusion of water into the matrix [235]. The transformation from the borneol solution into the insoluble matrix with a shell retarded drug diffusion [188]. The long path length with high tortuosity and low porosity of the borneol matrix over time was the main mechanism of the borneol matrix for decreasing the release rate of the drug [236]. The other insoluble matrix materials, such as Eudragit RS, PLGA, HPMC, bleached shellac, beta-cyclodextrin and lauric acid have been used as matrix materials for prolonged drug release in the crevicular pocket [128, 229, 230]. In contemporary drug delivery systems, borneol has garnered attention for its role as a component. Borneol-modified solid lipid

nanoparticles have been shown to enhance the permeation of certain drugs into the brain [44]. Borneol, when combined with polyethylene glycol (PEG) modification on bovine serum albumin nanoparticles, holds promise as a vehicle for delivering Itraconazole with a focus on brain targeting [237]. Additionally, nano-micelles loaded with doxorubicin exhibit potential in improving therapeutic outcomes for glioblastoma, emerging as a promising candidate for nanodrug application in glioblastoma therapy [238]. Moreover, borneol has biodegradable, biocompatible and non-toxic properties approved by the FDA [50]. It is also more cost-effective compared to poly(lactic-co-glycolic acid) (PLGA), commonly used as a matrix in commercial products. Consequently, borneol presents an intriguing option as a matrix-forming component for ISM.

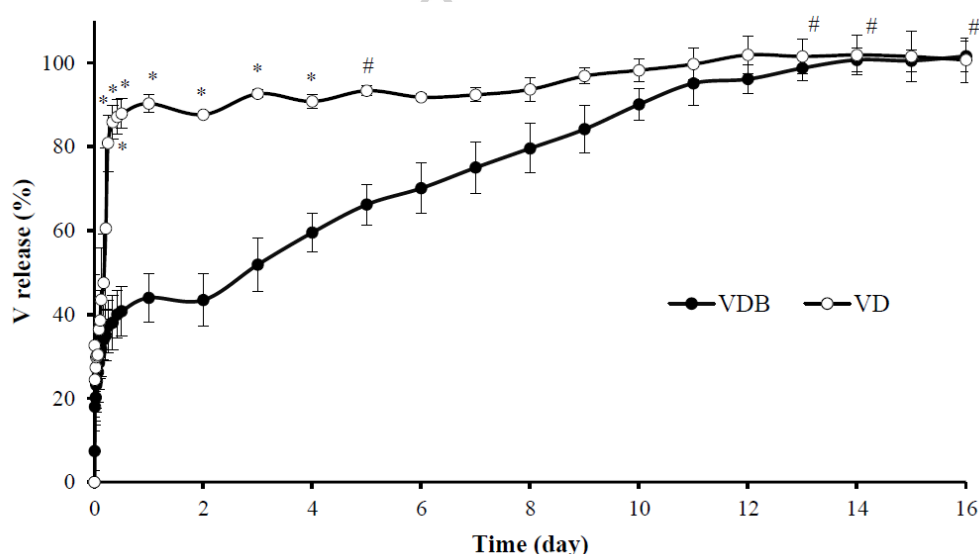


Figure 35. Release profile of vancomycin HCl from 40% borneol ISM (VDB) and borneol-free formulation (VD) in PBS pH 6.8, determined by the porcelain cup method. Results are presented as mean $\pm$ SD, with $n = 3$. *,# represent $p < 0.01$ and $p < 0.05$ compared with VDB.

The vancomycin HCl release profile from VDB was analyzed using zero-order ($r^2 = 0.8949$), first-order ($r^2 = 0.9098$), and Higuchi's equations ($r^2 = 0.9665$). The best fit was observed with Higuchi's equation, suggesting that the drug release from VDB was proportional to the square root of time. This implies a slower release over time, primarily governed by diffusion control, attributed to the hydrophobic borneol matrix. Additionally, the release profile aligns well with the Krosmeier-Peppas equation ($r^2 = 0.9961$), where the exponent release value (n) of VDB was close to 0.45. This indicates that the drug release mechanism follows Fickian diffusion, with a much greater influence than chain relaxation [239]. Fickian diffusion mechanism is typically mentioned in a sustained drug delivery system containing matrix component that can entrap the drug and control the drug release [235]. Practically, the drug diffusion was provoked by high velocity of solvent diffusion to the interior of the matrix and low velocity of

matrix relaxation [236]. The higher release was found in initial phase and slower over the time due to saturated drug concentration with longer path-length for drug diffusion into environmental fluid [235, 239]. The vancomycin HCl release was prolonged after an initial period via crystallization over time after borneol changed into a solid matrix with higher tortuosity and/or lower porosity.

2.2.8. Antimicrobial activities

The inhibition zone diameters of VDB formulations against six microbes, which are periopathogens found in dental plaque or the periodontal pocket, are presented in Table 12. Periodontitis is an infectious disease resulting from polymicrobial infection around the tooth surface, gingival margin, and subgingival environment. Microbial overgrowth in the crevicular pocket subsequently promotes periodontal inflammation, leading to attachment loss and bone loss [240]. Numerous studies have highlighted that *P. gingivalis* is a common periodontic microbe associated with the initiation and progression of periodontal destruction [241]. It can invade the gingival tissues of patients suffering from chronic and aggressive periodontitis. On the other hand, certain bacterial species in the periodontal environment, part of the commensal flora such as *Actinomyces*, certain *Streptococcus*, and *Staphylococcus* spp., can damage periodontal structures and trigger opportunistic infections when the periodontal ecosystem is disturbed [240]. Furthermore, these opportunistic and nosocomial infections may occur in severe periodontitis patients requiring hospitalization, especially those in need of surgical therapy. Meanwhile, MRSA is a well-known opportunistic pathogen; over 50% of ICU infections in the USA caused by *S. aureus* involve methicillin-resistant organisms [242, 243]. Additionally, *Candida* spp. are implicated in periodontal diseases. Although *Candida* has not been classified as a primary periodontal microbe, it plays a subsidiary role and possibly acts in synergy with major microbes [244]. Refractory periodontitis has been related to an infection with oral flora such as *C. albicans* [245]. Furthermore, *S. aureus* could be isolated from the periodontal pockets of patients with aggressive periodontitis and *E. coli* was considered as a microorganism usual in patients with periodontitis [246]. Therefore, these five microorganisms including *P. gingivalis*, *S. aureus*, MRSA, *E. coli* and *C. albicans* were selected for the antimicrobial investigation of our developed formulations.

Table 12. Inhibition zone diameter of borneol ISM and related formulations against *S. aureus*, MRSA, *P. gingivalis*, *C. albicans* and *E. coli*, with n = 3.

Microbes	Inhibition zone diameter (mean $\pm$ SD.) (mm.)					
	DB20	DB40	DB60	VDB	VD	DMSO
<i>S. aureus</i> ATCC 25923	14.33 $\pm$ 1.15	15.67 $\pm$ 0.58 ^a	13.67 $\pm$ 0.58	20.33 $\pm$ 0.58 ^{a,f}	21.33 $\pm$ 0.58 ^g	NC
<i>S. aureus</i> ATCC 6923	14.33 $\pm$ 0.58	15.67 $\pm$ 0.58 ^b	13.67 $\pm$ 0.58	20.67 $\pm$ 1.15 ^b	22.00 $\pm$ 1.00 ^h	NC
MRSA ATCC 43300	16.00 $\pm$ 1.00	16.00 $\pm$ 1.00 ^c	14.00 $\pm$ 1.00	23.00 $\pm$ 1.00 ^{c,f}	24.33 $\pm$ 1.00 ^{g,h}	NC
<i>P. gingivalis</i> ATCC 33277	NC	NC	NC	23.83 $\pm$ 0.29	26.00 $\pm$ 1.00	NC
<i>C. albicans</i> ATCC 17100	10.33 $\pm$ 0.58	9.67 $\pm$ 1.15	8.33 $\pm$ 0.58	11.33 $\pm$ 0.58	12.33 $\pm$ 1.15	10.33 $\pm$ 0.58
<i>E. coli</i> ATCC 8739	NC	NC	NC	10.67 $\pm$ 0.29 ^d	12.67 $\pm$ 0.58 ^{e,d}	9.67 $\pm$ 0.58 ^e

The superscripts a, b, c, e, and g represent $p < 0.01$; the superscripts d, f, and h represent $p < 0.05$; NC = the clear zone not found.

The inhibition zone diameters of DB20, DB40, DB60, VDB, and VD against various microorganisms are detailed in Table 12. Notably, VDB demonstrated outstanding antimicrobial activity against all *S. aureus* strains, including the MRSA strain (*S. aureus* ATCC 43300), with a considerably large clear zone diameter. Evidently, inhibition against all *S. aureus* and MRSA strains was observed in all formulations, except for DMSO. The presence of an inhibition zone in the borneol-containing formulation suggested antibacterial activity for this compound [247, 248]. The chiral carbons within the bicyclic stereochemical structure of borneol were found to possess anti-adhesion properties against bacterial cells or proteins, interfering with bacterial colonization. Consequently, bacteria might be repulsed, preventing their growth on agar [249]. Intriguingly, VDB exhibited inhibition of growth for all *S. aureus* and MRSA strains, with significantly larger inhibition zones than DB40 ($p < 0.05$). Vancomycin HCl, known for inhibiting cell wall synthesis in susceptible microorganisms, prevents the polymerization of the phosphodisaccharide-pentapeptide lipid complex during the second stage of cell wall synthesis [125]. The synergistic effect of borneol and vancomycin HCl contributed to the substantial inhibition zone observed in VDB. Moreover, the ISM with vancomycin HCl effectively inhibited the *P. gingivalis* strain (Table 12). The susceptibility of *P. gingivalis* to vancomycin HCl was 15.7 $\mu\text{g/mL}$ [132]. Borneol exhibited antifungal activity against *C. albicans* by promoting the generation of lipid peroxides and increasing the content of ergosterol [250]. In addition, DMSO exhibited antimicrobial activities against *E. coli* and *C. albicans*. The growth rate and P-galactosidase formation of *E. coli* were inhibited from DMSO effect, and over 90% inhibition of *E. coli* occurred in the presence of 12% DMSO [251]. Moreover, DMSO influenced the growth of *C. albicans* by inhibiting the germination of arthrospores. This effect increased in a concentration-dependent

manner, whereas 10% DMSO exhibited almost completely inhibited of *C. albicans* growth [251]. Comparatively, the antifungal activity against *C. albicans* followed the order DB20 > DB40 > DB60. The gradual reduction in the antifungal activity of the ISM was attributed to the retardation of DMSO diffusion from the transformed larger borneol matrix. Consequently, the inhibition zone against *C. albicans* can be attributed to the antifungal effects of both DMSO and borneol, whereas the inhibition against *E. coli* can be primarily ascribed to DMSO. Furthermore, vancomycin HCl inhibits *E. coli* by altering the integrity of the outer membrane and inhibiting peptidoglycan biosynthesis, thereby preventing cell wall synthesis [252]. Consequently, the inhibition zones against *E. coli* by VD and VDB were larger than that by DMSO alone. Typically, the inhibition zone obtained from agar diffusion is influenced by the diffusion characteristics of the antimicrobial agent. The borneol matrix impeded the diffusion of vancomycin HCl on agar; thus, the sustained release of vancomycin HCl from VDB resulted in a smaller inhibition zone compared to VD.

Presently, periodontal disease is characterized by polymicrobial infections that vary in their resistance to antibiotics, and certain bacteria have the potential to trigger opportunistic infections [18, 240]. The escalation of bacterial resistance has become a growing concern for health officials [101]. Approximately 50% of *Enterococcus faecalis* strains isolated from individuals with marginal and apical periodontitis were found to be resistant to one or more antimicrobial medications, primarily tetracycline and/or erythromycin [253]. Recent studies on the plasmidome within *E. faecalis*, isolated from periodontal patients in Norway, revealed that the majority of *E. faecalis* strains in periodontitis lesions likely serve as a reservoir for different mobile genetic elements and related antibiotic resistance determinants. In addition, a study involving 400 patients with chronic periodontitis in the USA investigated subgingival biofilm specimens from inflamed deep periodontal pockets, revealing that 74.2% of the patients had subgingival periodontal pathogens resistant to at least one of the antibiotics commonly used in clinical periodontal practice [254]. The lauric acid ISMs with vancomycin HCl has been reported for periodontitis treatment. It revealed an effective antimicrobial activity with prolonged drug release over 6 days [128]. Furthermore, they demonstrated efficient antimicrobial activities against *S. aureus*, *E. coli*, *C. albicans* including MRSA [129]. Moreover, some researchers have discovered that vancomycin HCl minimizes the growth of tooth plaque following periodontal surgery [28]. MICs of vancomycin HCl against *S. aureus*, MRSA and *P. gingivalis* are 1-2, 0.125-1 and 4 µg/mL, respectively [25]. The volume of gingival crevicular fluid in the crevicular pocket of patients with periodontitis is 0.5-1.0 µL [255]. When 1.0 µL of VDB was injected into the crevicular pocket, the amounts of 1% vancomycin HCl released from VDB at 60 min, 7 days and 14 days were 2400, 4400 and 10000 µg/mL, respectively, which are above the MIC against all microbes.

Moreover, periodontitis constitutes a risk factor for respiratory tract infections, and periodontal infection can influence the development of nosocomial pneumonia. Over 50% of *Staphylococcus aureus* infections in ICUs are attributed to methicillin-resistant organisms [243]. Vancomycin HCl or linezolid are currently sensitive to all multidrug-resistant bacteria. Moreover, vancomycin HCl has been the established standard of initially empiric treatment for MRSA in cases of hospital-acquired pneumonia patients [242]. Consequently, vancomycin HCl was chosen as the antimicrobial drug in this study due to its efficacy against MRSA and various pathogens associated with periodontitis. The localized injection of vancomycin HCl into the periodontal pocket could effectively prevent infection and the progression of this disease, particularly for severe patients undergoing oral surgery or hospitalization. However, it is crucial for antibiotic treatment regimens for each patient to be at the discretion of the dentist or physician. Factors such as the prevalence of drug-resistant infections, the local environment, and the risk of resistance should be carefully considered in tailoring treatment plans for individual patients.

In the release study, the sample was injected into a cylindrical porcelain cup to simulate the shape and condition of the crevicular pocket. The results (Figure 35) demonstrated that VDB exhibited sustained drug release for longer than 14 days. In comparison, Atridox[®] is US-FDA approved as a local drug delivery system for periodontitis treatment, containing 10% doxycycline hyclate (DH) dissolved with 33% polylactic acid (PLA) in 57% NMP, and it solidifies to achieve controlled release over 7 days. [135]. The borneol has been approved by the FDA as a food flavoring because of its non-toxicity [50]. It effective penetration enhancer for ocular drug administration with safe [48]. Moreover, it shows an anti-fibrotic activity and use as a penetration enhancer for the treatment of oral submucous fibrosis. Borneol, with concentrations ranging from 18.75 to 150 µg/ml, exhibited no cytotoxicity on primary mice oral fibroblasts. As a result, it was deemed a safe substance for application in oral submucous fibrosis. Moreover, a cytotoxicity study of borneol on human nasal epithelial cell (HNEC) monolayer indicated no cell cytotoxicity within the concentration range of 0-300 µg/ml [256]. Additionally, borneol has the potential to metabolize into camphor, with an LD₅₀ of 1.3 g/kg in mice, representing about 1/3 of the borneol received [44]. Toxic effects are observed after the ingestion of approximately 2 g, while taking small amounts of camphor is considered safe [50]. When the formula is injected into the crevicular pocket, the patient receives approximately 0.40 µg, which is considered a very small quantity. Consequently, this amount is not expected to have a significant impact on the toxicity of metabolization.

In addition, DMSO shows the low toxicity and safety when used less than 0.1 g/day (LD₅₀: 2.5-8.9 g/kg) [83]. In FDA Guidance for Industry (Q3C), DMSO has been classed as a Class 3 solvent and is recommended for use not more than 50 mg/day (equivalent to 5000 ppm or 0.5 percent) [257]. In the usual practice, dentists typically recommend a small amount of the formulation (0.5-1.0 µL) for

injection into the periodontal pocket. Consequently, the amount of DMSO received (approximately 0.6 μg) is considered minimal and does not exceed the recommended daily dose. Additionally, the flow of crevicular fluid helps reduce the concentration of DMSO over time [215]. Furthermore, recent exploration into the safety of DMSO revealed that its use as a penetration enhancer may lead to partial local toxicity only when used in high concentrations. However, the use of low concentrations did not result in any observed toxicity [258]. In dentistry, DMSO has been applied in the oral cavity, including being spread on the dentin of teeth and formulated as a component of oral devices. Reports indicate that it is considered safe and does not cause irritation when used in conjunction with adhesive monomers [257]. Moreover, several medical products utilizing DMSO as a carrier have received approval from the FDA. DMSO was found to have no cytotoxic effects on the pulp tissue repair-related activity of odontoblast-like cells. It did not impact cell viability, adhesion, and the percentage of cell death by necrosis in the range of 0.05 to 1.0 mM [259]. Currently, the FDA has approved DMSO for the treatment of interstitial cystitis, even in pediatric patients. Additionally, it has been reported as a solvent for ISMs [77]. The results of a cell recovery with a toxicity kinetic investigation of DMSO in undamaged articular cartilage revealed that 1 M DMSO did not exert a toxic effect on the chondrocytes [260]. DMSO has been identified as a solvent for various dosage forms, including ISMs, for the treatment of atypical antipsychotics such as risperidone and paliperidone [77]. Another topical solution, comprising 5% idoxuridine in DMSO, has been utilized for treating herpes zoster [261].

As a result, the proposed ISM might function as an intra-crevicular antimicrobial drug delivery system with minimal or no systemic absorption. Furthermore, an apparent lower dosage is required for effective therapy, potentially reducing side effects. DMSO, borneol, and by-products are biocompatible, non-toxic, and harmless. Despite safety data and the uses of DMSO and borneol showing the potential for both compounds in injectable dosage forms, the safety and therapeutic efficacy of the created VDB ISM should be established in a clinical investigation. Additionally, various solvents such as NMP, PYR, and triacetin may be considered as carriers for future antimicrobial agent-loaded ISMs in research.

In this study, a borneol ISM with phase inversion process for crevicular pocket delivery of vancomycin hydrochloride was successfully prepared and developed. Both the drug-free and borneol ISMs with vancomycin HCl in DMSO appeared as homogeneous, mildly alkaline solutions with a pH close to physiological levels. The density of borneol ISM solutions gradually decreased in a narrow range in a borneol concentration-dependent manner. Due to their low viscosity, the borneol ISMs could better facilitate injectability compared to viscous polymer ISMs. The low contact angles of the borneol ISM solutions indicated their favor wettability. The contact angles of the drug-free and vancomycin HCl-loaded highly concentrated borneol ISMs increased after being

in contact with agarose gel or the bulge tissue of porcine due to phase inversion from a solution into a matrix. The denser the matrix formation, the higher the borneol concentration. The phase inversion of borneol-ISM occurred after the fluid formulation contacted the mimetic aqueous crevicular fluid; thereafter, DMSO from the formulation diffused out while water diffused in simultaneously, causing the formation of a solid-like borneol matrix. The matrix comprising borneol crystal formation from phase separation increased with increasing borneol concentration. Moreover, vancomycin HCl had almost no effect on the rate of matrix formation. DMSO proved to be an appropriate solvent for the ISM of borneol because of its low viscosity and high injectability. VDB exhibited sustainable drug release longer than 2 weeks and fit well with Higuchi's equation, indicating diffusion control retarding vancomycin HCl release owing to the hydrophobic borneol matrix. Moreover, VDB exhibited efficient antimicrobial activities against *S. aureus* ATCC 25923, *S. aureus* DMST 6935, *E. coli* ATCC 8739, *C. albicans*, *P. gingivalis* ATCC 33277 and MRSA ATCC 43300. Therefore, VDB is a potentially effective local antimicrobial drug delivery system for periodontitis treatment through crevicular pocket injection.

3. DH-loaded borneol-based ISM using triacetin as additive solvent for periodontitis treatment

3.1. Appearance and basic physical properties

All the prepared ISM and control solutions appeared to be clear homogeneous solutions with amber color. The fundamental physicochemical properties ISM, including density, viscosity, and surface tension, are presented in Table 13. The densities of triacetin, NMP, and DMSO were 1.1472, 1.0283, and 1.0924 g/cm³, respectively. The densities of DH-loaded formulations (N1 or D1) were higher than those of pure solvents (NMP or DMSO) or borneol ISMs due to the low density of borneol (1.01 g/cm³) [183]. In the NMP series, the density of ISM gradually decreased as the proportion of triacetin increased, as observed in the N3 to N8 formulations. This decrease is attributed to the hydrophobic and oily liquid properties of triacetin [262]. Notably, the density of N4 and of N8 was significantly higher ($p < 0.05$) than that of N2. Similarly, the ISM formulations employing DMSO as a solvent presented a significantly higher density ($p < 0.05$) when adding more triacetin. All ISM exhibited higher density values than water, ensuring their ability to sink and become embedded in the crevicular pocket without being lost from the crevicular fluid.

The borneol ISMs exhibited a higher viscous due to the strong hydrophilic interaction between the borneol-solvent and the diminished amount of solvent as reported previously [183]. The viscosity of ISM solutions containing triacetin dissolved in NMP and DMSO was in the range of 5.55-9.67 and 5.92-10.43 cP, respectively. Their viscosities gradually but significantly increased ($p < 0.05$) with the increasing amount of triacetin (Table 13), while the viscosity of triacetin alone was

25.53 cP. ISMs in DMSO had slightly higher viscosity than those in NMP because DMSO is more viscous than NMP. The viscosity of each formulation in this study was <20 cP, indicating modest viscosity for facile injection via a needle when administered into the crevicular pocket [128]. These fundamental physicochemical characteristics were employed to interpret the results of contact angles, surface tension, and injectability, encompassing the phenomenon of phase inversion at the interface of each formulation after exposure to the aqueous phase.

The surface tension of borneol was lower than that of the borneol-free formulation (Table 13), attributable to the steric hindrance effect of borneol in the mixture. Furthermore, the presence of 40% borneol interfered with the intermolecular forces of the solvent and increased the free volume space, as reported previously [183, 263]. However, triacetin had a diminishing effect on the surface tension of the mixture due to the similar surface tension values of triacetin and the solvents (NMP and DMSO).

Table 13. Physical properties of borneol ISM. Results are presented as mean $\pm$ SD, with $n = 3$.

Formulation	Density (g/cm ³)	Viscosity (cP)	Surface tension (mN/m)
Triacetin	1.1472 $\pm$ 0.0002	25.53 $\pm$ 0.52	38.87 $\pm$ 0.55
NMP	1.0283 $\pm$ 0.0004	1.88 $\pm$ 0.03	39.62 $\pm$ 0.02
N1	1.0425 $\pm$ 0.0009	2.59 $\pm$ 0.16	40.33 $\pm$ 0.42
N2	1.0204 $\pm$ 0.0014 ^{a,b}	5.38 $\pm$ 0.09 ^{e,f}	35.18 $\pm$ 0.16
N3	1.0228 $\pm$ 0.0011	5.55 $\pm$ 0.12	34.65 $\pm$ 0.23
N4	1.0268 $\pm$ 0.0017 ^a	6.16 $\pm$ 0.07 ^e	34.61 $\pm$ 0.22
N5	1.0290 $\pm$ 0.0009	6.66 $\pm$ 0.13	34.08 $\pm$ 0.17
N6	1.0340 $\pm$ 0.0014	7.06 $\pm$ 0.06	33.43 $\pm$ 0.07
N7	1.0386 $\pm$ 0.0017	8.84 $\pm$ 0.09	33.29 $\pm$ 0.08
N8	1.0435 $\pm$ 0.0013 ^b	9.67 $\pm$ 0.04 ^f	33.14 $\pm$ 0.09
DMSO	1.0924 $\pm$ 0.0003	2.25 $\pm$ 0.04	42.61 $\pm$ 0.46
D1	1.1090 $\pm$ 0.0003	3.07 $\pm$ 0.08	44.59 $\pm$ 0.36
D2	1.0524 $\pm$ 0.0009 ^{c,d}	5.49 $\pm$ 0.12 ^{g,h}	34.28 $\pm$ 0.95
D3	1.0538 $\pm$ 0.0008	5.92 $\pm$ 0.33	35.49 $\pm$ 0.15
D4	1.0557 $\pm$ 0.0013 ^c	6.30 $\pm$ 0.19 ^g	34.94 $\pm$ 0.38
D5	1.0579 $\pm$ 0.0009	7.19 $\pm$ 0.09	34.23 $\pm$ 0.38
D6	1.0601 $\pm$ 0.0004	8.19 $\pm$ 0.33	34.69 $\pm$ 0.15
D7	1.0626 $\pm$ 0.0011	8.76 $\pm$ 0.11	35.06 $\pm$ 0.25
D8	1.0656 $\pm$ 0.0006 ^d	10.43 $\pm$ 0.62 ^h	34.86 $\pm$ 0.31

The superscripts a, b, c, d, e, f, g, and h represent $p < 0.05$.

3.2. Contact angle and wettability

Wettability is defined as the ability of a fluid to maintain contact with a surface and reflects the nature of the hydrophilic or hydrophobic interaction with the target surface [264]. ISM contact angles on various surfaces were measured in order to study the wettability, as was previously described. Furthermore, matrix formation was also observed from the change of the ISM contact angle on a water-containing surface, such as an agarose gel [183]. Herein, a glass slide, paraffin-coated glass slide, and agarose gel containing water were used as the test surfaces. The contact angles of borneol-based ISMs on the glass slide were lower than those of the solvents (NMP or DMSO) (Figure 36). The interference of borneol molecules decreased the cohesive force of solvent molecules and diminished the interactions between DMSO and the glass slide [183, 263]. By comparison, borneol-based ISMs in DMSO exhibited a higher contact angle than that of borneol-based ISMs in NMP because the high viscosity of DMSO retarded droplet spreading. Furthermore, the reduction in contact angles on the paraffin-coated glass slide in the presence of borneol, indicating a significant hydrophobic interaction between borneol molecules (log P of 2.85) and a hydrophobic surface, such as paraffin [183, 263]. However, the proportion of triacetin in the formulation did not markedly affect the contact angle between the glass slide and paraffin.

The contact angle was examined on an agarose gel to simulate the crevicular pocket and evaluate the behavior of ISM transformation from liquid to solid-like matrix upon contact with aqueous fluid on the agarose gel surface. The contact angle significantly increased in ISM formulations containing borneol but not in the case of triacetin-loaded ISMs (Figure 36). The higher viscosity of borneol-based ISM can hinder the spreading of droplets on surfaces [176]. The increased contact angle reflected the instantaneous phase transition from a solution to the matrix of ISM formulations. No contact angle values exceeded 45°, indicating that the ISM formulation had favorable wettability and was likely to adhere to the gingival surface when administered in the crevicular pocket. Since DMSO has a greater viscosity than NMP, ISMs in DMSO showed larger contact angles than ISMs in NMP.

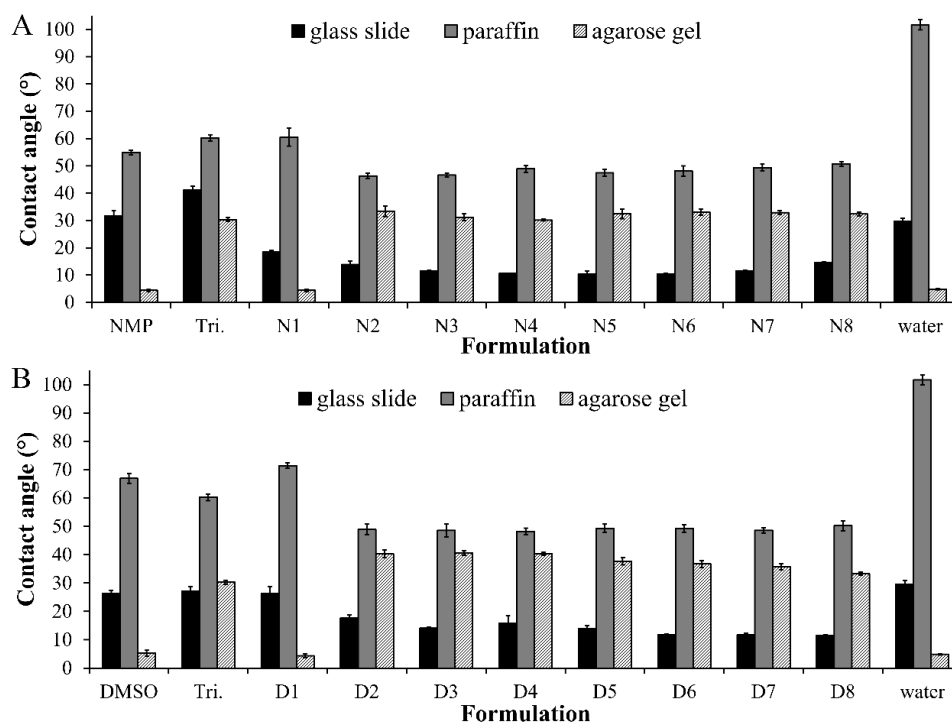


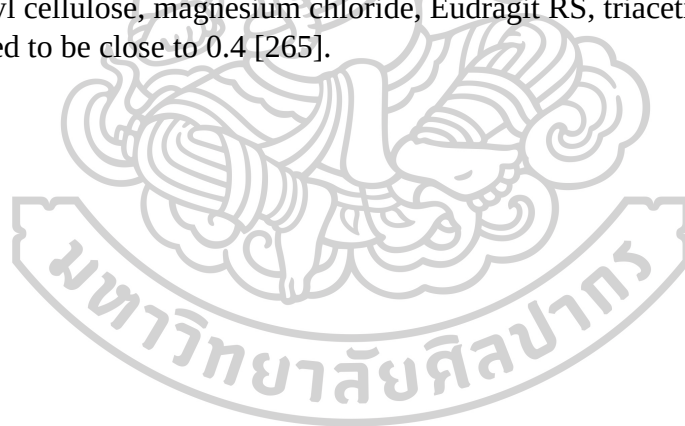
Figure 36. Contact angle of borneol ISM by using NMP (A) or DMSO (B) as solvent, with $n = 3$.

3.3. Injectability and mechanical properties

The injectability of a fluid is a measure of the force and energy needed to inject the fluid *via* the needle from a syringe [63]. The addition of borneol and triacetin enhanced both the maximum force and energy required for injection. Notably, the maximum force and energy of N8 and D8 were significantly higher than those of N2 and D2, respectively ($p < 0.05$). However, minimal energy was required for injections of formulations containing varying triacetin amounts in both the NMP and DMSO series (21.02-33.56 mJ; Figure 37(A) and Figure 37(B)). The energy used for injection of these ISM solutions was comparable to that of polymeric ISMs containing 5%-20% w/w ethyl cellulose (21.73-41.98 mJ), 15%-30% w/w bleach shellac (11.73-27.69 mJ), and 15%-35% w/w Eudragit RS (8.04-23.99 mJ) [219]. The maximal injection force for all ISMs in this investigation was <10 N, signifying the simplicity of injection and acceptability for injection, including reduced discomfort at the injection site [220]. Consequently, all triacetin-mixed ISMs were acceptable and suitable for use as an injectable drug delivery system, providing convenient administration and satisfactory patient comfort/compliance [63, 220].

Mechanical property assessments were conducted to evaluate the characteristics of the resulting matrix after phase transformation. Each formulation was introduced into a hole in an agarose gel and left for complete phase inversion and matrix formation. The robustness of the matrix is indicated by its maximum compressive resistance, with the proportions of remaining force and maximum force describing additional matrix properties [181]. Both maximum penetration force and

remaining force decreased with an increasing triacetin ratio, suggesting reduced matrix sturdiness (Figure 37(C)). Consequently, a high proportion of triacetin in the formulation might be unsuitable for ISM application in crevicular pocket drug delivery due to low matrix firmness. Additionally, other factors, such as the rate and morphology of matrix formation, should be considered. The adhesion force of each formulation remained consistently low (Figure 37(D)), indicating that the presence of triacetin did not significantly affect the adhesive force of borneol-based ISM. In comparison, the maximum adhesion force was similar to or slightly lower than that of resin-based ISMs containing 35% w/w propolis, 35% w/w benzoin, or 35% w/w rosin (0.24, 0.14, and 0.04 N, respectively) [181]. The ratio of remaining force to maximum penetration forces was calculated to quantify deformation properties (elastic and plastic deformation characteristics); a high ratio (close to 1.00) indicates high elasticity, while a low value reflects high plasticity [265]. In this case, the ratio for all formulations ranged from 0.12 to 0.37, suggesting that all obtained matrices exhibited plastic deformation properties (Figure 37(D)), making them persistent and suitable for desirable deformation within the shape and size of crevicular pockets [265]. The ratio of other polymer ISMs, such as poly (D,L-lactic-co-glycolic acid) mixed with a plasticizer (acetyltributyl citrate or dibutyl sebacate), was less than 0.15, demonstrating favorable plastic deformability. Additionally, the ratio of the commercial product Parocline[®] (a dental gel containing minocycline (2%), hydroxyethyl cellulose, magnesium chloride, Eudragit RS, triacetin, and glycerol), has been reported to be close to 0.4 [265].



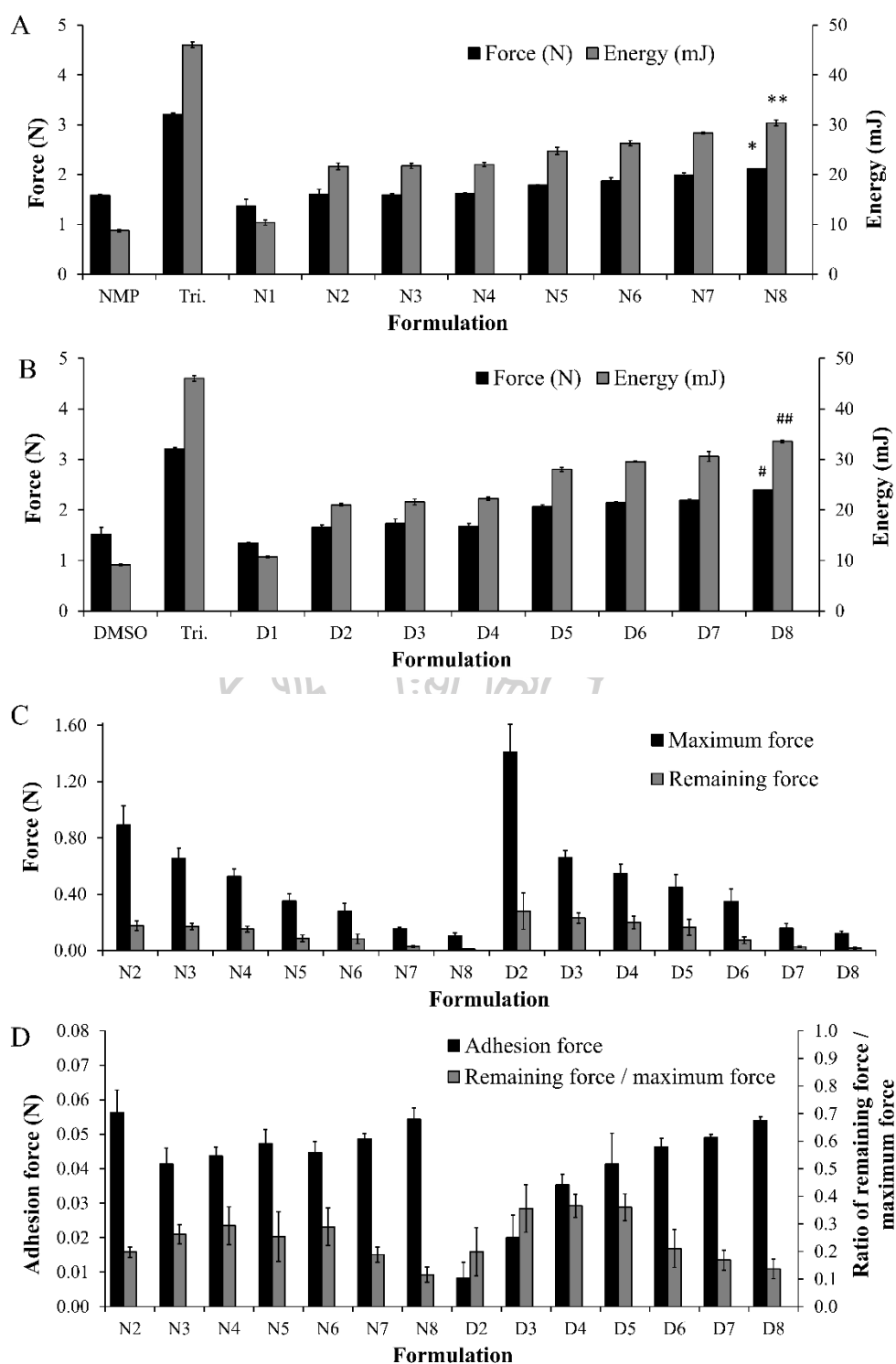


Figure 37. Force and energy for expelling syringes of borneol ISM using NMP (**A**) or DMSO (**B**) as solvent. (**C**) maximum and remaining force, (**D**) adhesion force and mechanical properties of borneol matrix, with $n = 3$. Tri represent triacetin. *** $p < 0.05$, compared with N2, #,## $p < 0.05$ compared with D2.

3.4. Matrix formation behavior at the macroscopic level

To study matrix formation behavior and formation rates, the stereomicroscope was used to detect the macroscopic matrix change of ISM. The water from the agarose instantly diffused into the formulation once the formulation was injected into the agarose hole, changing the borneol solution into an opaque white matrix as a result of phase inversion of the borneol. The NMP in the formulation diffused into the agarose because of its affinity with water, and this process occurred as a phase inversion with the mutual invasion of the water phase into the ISM, thereby inducing phase transformation of ISM. The matrix formation started at the interface between the ISM and the agarose gel. The mass and thickness of the matrix continuously increased over time from the interface to the center of the agarose hole as shown in Figure 38(A) and Figure 38(B). The thickness of the cloudy matrix increased slowly with time with the increase in ratio of triacetin in the formulation because triacetin retarded the phase inversion of ISM [78, 266]. The borneol matrix-formed ISMs using NMP as solvent were tightly clustered. The matrix from DMSO series possessed a loosely clustered appearance, which was observed in the center of the agarose hole 30 min after the formulation was exposed to the aqueous phase of the agarose gel. Moreover, the matrices from both series of ISMs were crystal-like in appearance because of the borneol phase inversion [267]. High matrix firmness contributes to controlled drug release because of entrapment of drug in the matrix, whereas the absence of the sufficient matrix firmness may not hold the drug in the matrix [267]. Therefore, formulations employing NMP as a solvent have favorable characteristics that support their developed into ISM formulations for periodontitis treatment and were evaluated in further testing.

The effect of triacetin incorporation on matrix formation in ISMs using NMP as a solvent was evaluated in matrix formation studies. Initially, a liquid ISM sample was introduced into a test tube containing PBS (pH 6.8) to simulate matrix formation when injecting the formulation into crevicular fluid. The obtained matrix formation is illustrated in Figure 39. The ISM solution rapidly transformed into a solid-like matrix upon initial contact with PBS (pH 6.8) and progressively converted into a cloudy gel-like matrix *via* phase inversion, as the poor solubility of borneol [183, 269]. This phase inversion phenomena occurred due to the interaction between NMP in ISM and aqueous from the environment [183, 270]. While an increased amount of triacetin was expected to retard drug release, ISM with higher triacetin content tended to undergo slower transformation compared to formulations with lower amounts. Specifically, N5, N6, and N8 failed to transform into a matrix within 25 minutes, instead showing a soft gel-like matrix. This delayed matrix formation may impede the efficiency of drug entrapment in the borneol matrix and potentially lead to a failure of extended drug release [183, 267]. In contrast, ISMs with low triacetin loading, such as N3, underwent rapid phase inversion into a matrix, potentially limiting control over drug release. Conversely, an acceptable matrix transformation occurred with N4 due to appropriate triacetin loading, allowing for extended drug release. Therefore, N4 was selected for further investigations.

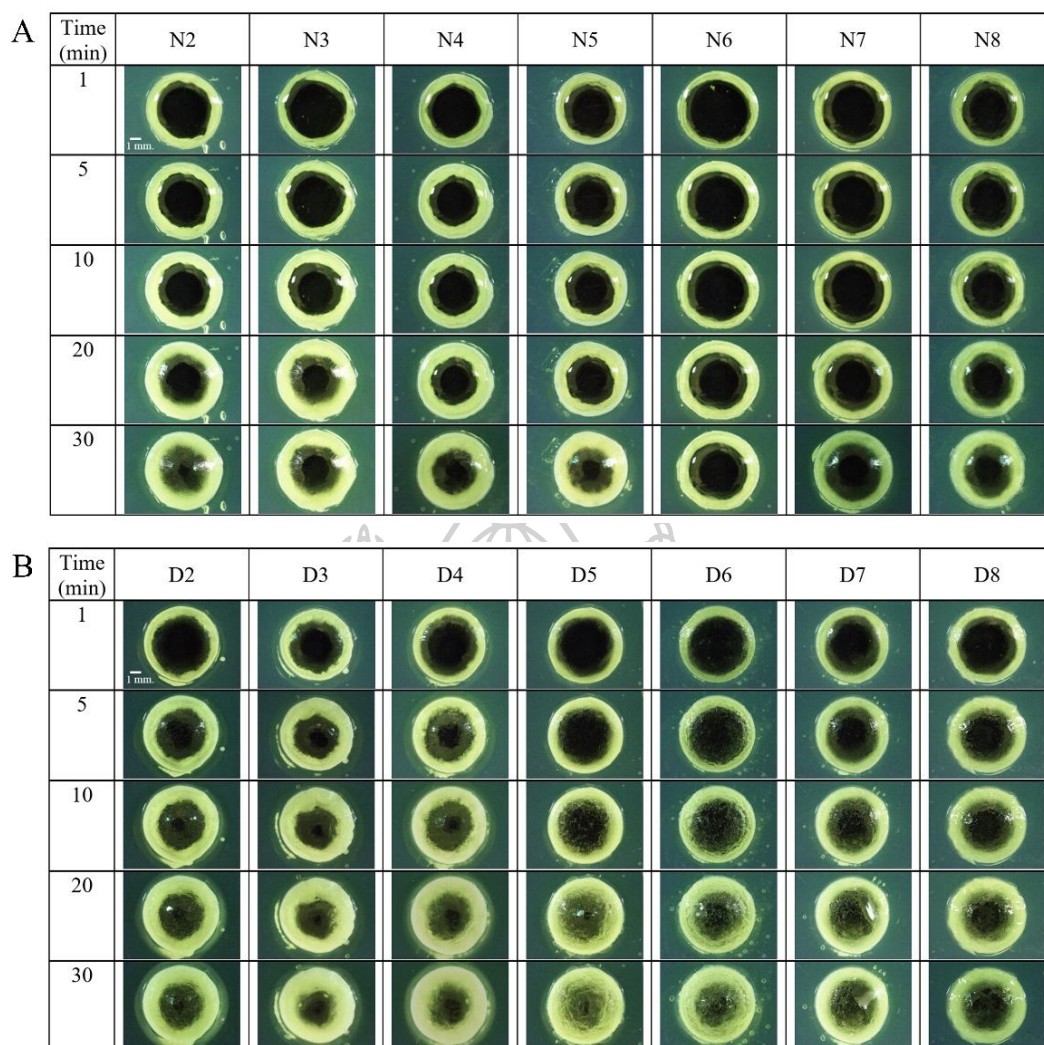


Figure 38. Matrix formation behavior in an artificial crevicular pocket of ISMs using NMP (**A**) or DMSO (**B**) as solvent under a stereo microscope at 4× magnification.

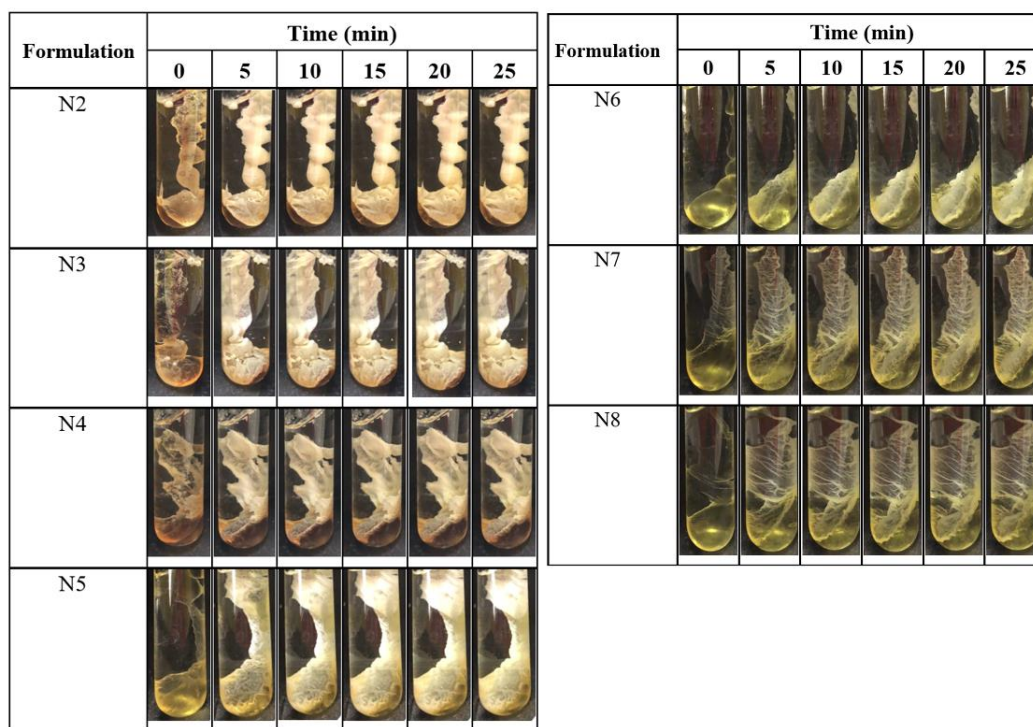


Figure 39. *In vitro* matrix formation behavior of ISMs through a 27-gauge needle into PBS pH 6.8.

3.5. Interfacial phenomena of matrix formation

Upon contact with the agarose matrix rim, the ISM formulation interface was observed microscopically using a stereomicroscope to better understand the creation of the borneol matrix, as shown in Figure 40(A). At the interface, a matrix developed and grew with time, particularly into the formulation part (right side). The matrix formation initiated with the onset of borneol phase separation. Normally, when the prepared ISM solution comes into contact with aqueous fluid in the crevicular pocket, the solvent from the ISM diffuses into the environment, and water molecules diffuse into the formulation, a process referred to as phase inversion. The continuous formation of the cloudy white borneol matrix occurred due to the phase separation of borneol. Subsequently, drug molecules were enclosed within the matrix and gradually released over time [178, 271]. In the early phase, the rate of phase inversion typically occurs rapidly and gradually decreases over time as the reaction reaches equilibrium, according to thermodynamic theory [129, 272]. A higher borneol content was observed near the interface area and gradually decreased with increasing distance from the interface [145]. A black opaque cluster in several parts caused by matrix formation was deposited in many layers in the matrix (Figure 40(A)). This process formed a firm precipitate where light from the stereomicroscope could not pass through. The high triacetin content in the formulation slowed the diffusion of solvents, causing slow matrix formation and a noticeably lower matrix firmness of ISM as shown in the results of N6, N7, and N8. On the contrary, the ISM with low triacetin underwent a transformation into a matrix with high firmness, characterized

by dark clusters observed under the microscope. It was evident that dense matrix clusters tended to form at the interface, while loosely clustered matrices developed further away from the interface area. Furthermore, the matrix of the ISM with highly triacetin showed reduced matrix aggregation, attributed to its interference against borneol matrix formation.

The morphology of the borneol matrix after phase inversion process was observed under an inverted microscope as shown in Figure 40(B). The triacetin-free ISM (N2) formed small grain-shaped borneol crystals within a continuously intricate matrix. However, the presence of triacetin, with its high viscosity, prolonged the induction time needed for matrix growth [273]. In addition, the triacetin-induced hydrophobicity in the system reduced the phase inversion of ISM and a late phase inversion of borneol [274]. Consequently, a borneol matrix with large grain-shaped and translucent crystals appeared in triacetin-loaded ISM formulations. At the interface, the nucleation process occurred because of the high borneol content adjacent to the aqueous phase of PBS. As a result, crystal crystallization and aggregation progressed according to thermodynamics [275, 276]. The nucleation and crystallization processes of these ISMs have been elucidated using ibuprofen and fatty acids as matrix-forming agents [178]. The examination of the topography of borneol matrices through scanning electron microscopy (SEM) was not conducted in this study due to borneol sublimation occurring under vacuum conditions at high voltage [267].

To understand matrix formation at the macroscopic level, this experiment was designed to observe the initiation of matrix formation. The distinctive borneol transformation observed at the microscopic level aids in explaining the matrix formation of the formulation after contact with the aqueous phase. The continuous and progressive growth of the matrix observed at the macroscopic level corresponds with the results in Section 3.4. Furthermore, the morphology of the formed matrix is valuable for elucidating the rate and pattern of drug release for each ISM, which will be discussed in the next section.

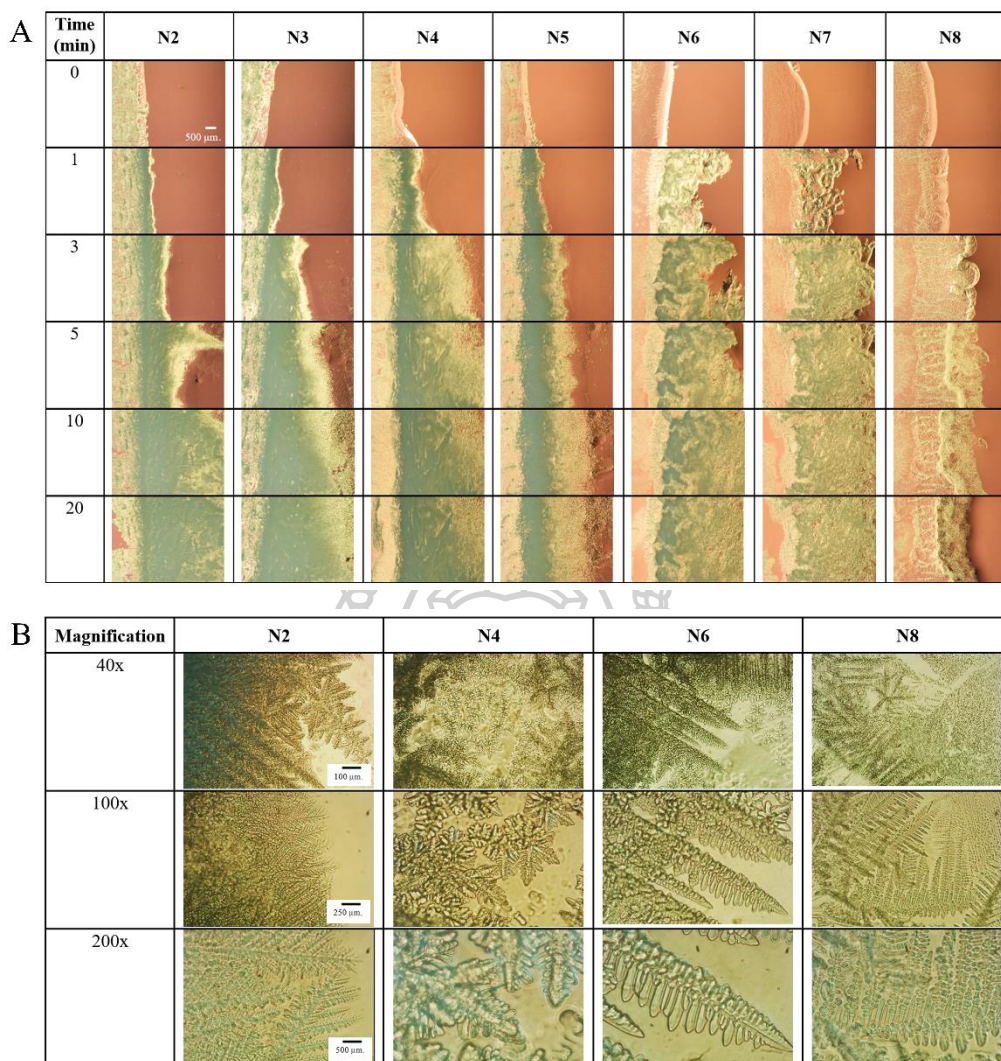


Figure 40. (A) Interfacial phenomena of matrix formation at the interface between formulation and PBS pH 6.8 under a stereomicroscope (magnification of 8×) and (B) the morphology of borneol under an inverted microscope (magnification of 40×, 100×, and 200×).

3.6. Water-induced matrix formation sensitivity

In order to determine the water sensitivity or aqueous tolerance of ISM, water titration was performed to calculate the amount of water required to induce the phase inversion of borneol [178]. These results demonstrated the water sensitivity of ISM and ease of phase inversion. A low water loading demonstrated that even a minimal amount of water could induce phase inversion. In this experiment, the percent water capacities of N2, N4, N6, and N8 were $18.87\% \pm 0.73\%$, $20.18\% \pm 0.77\%$, $22.99\% \pm 0.32\%$, and $26.17\% \pm 0.40\%$ v/v, respectively. This value increased with the increase in the triacetin amount in the formulation because of the high hydrophobicity and viscosity of triacetin [216]. Typically, increased viscosity poses challenges in matrix formation. For instance, the ISM solution based on bleached shellac, using PYR as a solvent, exhibits high water resistance or capacity owing to the elevated viscosity of

PYR [216]. Elevated viscosity in an ISM solution can impede phase inversion and decrease the rate of matrix formation [128]. Hydrophobic solvents such as triacetin are recognized to produce a slow-forming phase inversion because of the limited water solubility (around 7%) [277]. Triacetin has a low water affinity [278], and the ISM solution containing triacetin was more viscous [262], which diminished the phase inversion rate and reduced the borneol phase inversion [216]. Hence, an increased amount of water was needed to initiate borneol matrix formation, indicating a lower susceptibility to water induction. Additionally, the reduced quantity of hydrophilic solvent, such as NMP in the formulation, decreased the overall hydrophobicity. In comparison, triacetin (log P value of 0.25) exhibited greater hydrophobicity than NMP (log P value of -0.38) [208, 277] while borneol acted as a nonpolar substance (log P value of 2.85) [269]. Consequently, borneol displayed similarities in hydrophobicity to triacetin rather than NMP due to their comparable polarities. Therefore, N8 had the highest water capacity among the formulations. In hydrophobic ibuprofen-based ISM dissolved in DMSO, a lower water capacity was observed compared to that using NMP as the solvent because the log P of DMSO (-1.35) is lower than that of NMP (-0.38). This indicates that easier ibuprofen phase inversion occurs with DMSO than with NMP [178]. These findings align with the matrix formation of borneol-based ISMs in both morphology and microscopic changes. Hydrophobicity, log P value, and viscosity were identified as crucial factors influencing the sensitivity of water-induced matrix formation [178, 216]. While there is no specified optimum value for water capacity in ISM, a high value may result in slow matrix formation, potentially leading to burst drug release. Conversely, low values may pose challenges during preformulation, causing matrix formation by air humidity before ISM injection into the crevicular pocket or too rapid matrix formation before the formulation fits the crevicular pocket geometry [183].

3.7. *In vitro* drug release

One possible way to sustain drug release is to include a hydrophobic component, such as triacetin, in the formulation [78]. Nevertheless, it is crucial to assess the appropriate triacetin content to ensure the correct proportion necessary for extended drug release. The duration of matrix formation, at both microscopic and macroscopic levels, increased with higher triacetin content, as supported by the data from the sensitivity of water-induced matrix formation. Consequently, formulations N4 with low triacetin content (5%w/w) and N8 with high triacetin content (25% w/w) were selected for testing, along with N2 as the original formulation with borneol-based ISM without triacetin. The control group consisted of DH solution in NMP (N1). This study employed the porcelain cup or membrane-less method, where the sample was added to a small porcelain cup and placed into 100 mL of PBS buffer, to simulate drug administration into the crevicular pocket [183]. This method resembled the conditions in the periodontal pocket, as crevicular fluid from the environment directly contacts the ISM formulation, leading to immediate matrix formation upon exposure to the release medium [231]. The release patterns of DH from the test ISMs in PBS pH 6.8 using this method are illustrated in Figure 41(A). In the first 6 hours,

approximately 14.93% of DH was released from N8, N2, and N4. In contrast, N1, lacking both borneol and triacetin, rapidly released up to 51.20% of DH due to the absence of matrix formation, which could entrap the drug [178, 183]. The release of DH continued from N1, reaching 90% within 1 day. N2 transformed into a gel-like matrix upon contact with the release medium, and its release reached 80% after 2.5 days. The hydrophobic borneol matrix facilitated prolonged DH release over time [183]. Other hydrophobic matrices, such as 25% Eudragit L, 15% Eudragit RS, 30% blended shellac, and 40% ibuprofen, could sustain DH release for >7 days [178, 225, 271, 279]. The borneol ISM with vancomycin HCl-incorporated has been shown to act as a potentially effective local antimicrobial drug delivery system for the treatment of periodontitis [183]. A 40% borneol formulation has been successful in controlling drug release; nevertheless, potential novel techniques are being researched for prolonged drug release targets. Typically, the drug is quickly released from its deposit on the matrix surface at the initial state and then gradually liberated over time through a longer diffusion route [183]. The long path length with high tortuosity and low porosity of the gradually formed borneol matrix over time was crucial for decreasing the DH release rate [236]. Practically, minimizing burst drug release is crucial for more efficient control of drug release from drug delivery systems, including injectable ISM systems. The incorporation of a hydrophobic substance into the ISM system is a useful technique for reducing the hydrophilicity of the system and addressing this issue [274]. In mixed solvent systems, a hydrophilic solvent (e.g., NMP) promotes rapid phase inversion, leading to the swift diffusion of the solvent and sudden solid-like matrix formation. Conversely, a hydrophobic solvent (e.g., triacetin) slows down the phase inversion, limiting solvent diffusion and, consequently, drug release [78, 280]. Consequently, the addition of 5% triacetin in ISM was introduced to modify drug release. The DH release was subsequently extended, as evidenced by the release profile of N4, which transformed into a solid-like matrix within 2 h. Surprisingly, the addition of high triacetin levels, up to 25% in ISM, induced delayed matrix formation due to the potentially sluggish phase inversion caused by the hydrophobic characteristics [78, 280]. Therefore, the matrix formation was difficult to achieve and drug retention in the matrix was not practicable [280]. As a result, the release profile of N8 was faster than that of N2 but still slower than that of N1, making it impractical for controlled drug release. Triacetin prolonged drug release due to the hydrophobic nature of the polymer; however, the impact is more pronounced with a hydrophobic polymer and less significant with a hydrophilic polymer [280]. For example, using triacetin as a cosolvent in the ISM to reduce the burst release was more effective with the hydrophobic poly(D,L-lactide) matrix and less effective with the relatively hydrophilic Resomer[®] RG 502, poly(D,L-lactide-co-glycolide) matrix [280]. Therefore, the proper concentration of triacetin as a cosolvent for prolonged drug release depends on the type of matrix forming agent [280]. The high content of triacetin, when combined with a hydrophilic solvent, may not ensure the prolonged release of the drug. As a result, N4, which contains 40% borneol and 5% triacetin, proved effective in extending drug release for 10 days. This minimized the burst release of DH and provided continuous control over drug release throughout the

specified period. In comparison, Atridox[®], a commercial ISM containing DH and 33% poly(D,L-lactide) as a matrix former, controls drug release for 1 week after administration into the crevicular pocket [33].

The solvents employed in ISM systems should ideally be safe for injection, biocompatible, and biodegradable [278]. NMP is the most commonly used solvent in ISM systems due to its safety profile and established pharmaceutical use [52]. Recognized as a solubilizing excipient in both parenteral and oral medications, NMP has a well-documented safety record and is frequently utilized in commercial pharmaceutical products for periodontitis treatment, such as Atridox[®], Periocline[®], and Arestil[®] [31, 268, 281]. According to the EU Scientific Committee on Consumer Safety, NMP exhibits low acute toxicity via oral, dermal, and inhalation routes. It falls under Class 2 based on ICH guidelines and is recommended for use in pharmaceutical products at low concentrations [278]. With a median lethal dose exceeding 2 mL/kg, NMP is considered safe for incorporation into ISM. Triacetin, a non-toxic edible fatty acid, holds Generally Recognized as Safe (GRAS) status and has FDA approval as a food additive. Additionally, triacetin is utilized as a parenteral nutrient [262].

Following the completion of drug release experiments, residues from each formulation were extracted from PBS pH 6.8 and excess water was eliminated. The surface morphology of the borneol matrix, transformed into a gel-like matrix and soaked in PBS for 11 days, was investigated, as depicted in Figure 41(B). The cup containing N1 showed no remnants due to the absence of borneol in the formulation. In contrast, spongy solid matrices with an amber hue were discovered in the cups of N2, N4, and N8, representing the remains of water-insoluble borneol matrices. At high triacetin content, the slow phase inversion hindered matrix formation, making it infrequent. Consequently, in N8, the matrix predominantly encircled the interior of the cup, as observed in cross-sectional images, but did not form in the central region. The addition of triacetin seemed to enhance matrix porosity [280]. The cross-sectional images of poly(D,L-lactide) matrices in DMSO/triacetin mixed solvent and their cross-sectional morphologies as a function of time have been investigated previously [280]. The cross-section of the matrix from formulations utilizing DMSO as the sole solvent displayed denser structures compared to matrices from solvents containing triacetin. Furthermore, a more porous structure was evident, and this effect became more pronounced with higher concentrations of triacetin in the solvent system [280]. By comparison, the matrix from N4 was more porous than that of N2. Normally, the efficiency of controlling drug release from ISM corresponds to the topography and is usually investigated with SEM [178]. The examination of topography under SEM was not achieved in this investigation [267] due to the sublimation of borneol. Consequently, stereomicroscope observation was applied in this work.

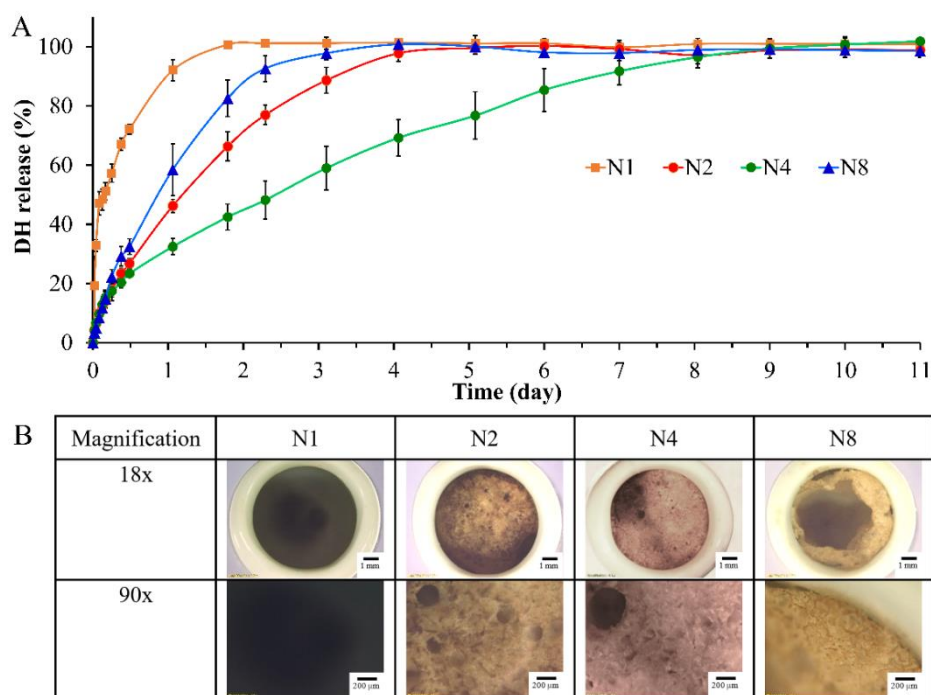


Figure 41. (A) Release profile of DH from difference borneol ISM and related formulation in PBS pH 6.8, determined by the porcelain cup method. Results are presented as mean $\pm$ SD, with $n = 3$. **(B)** The matrix of their formulation after completed DH release in a porcelain cup under a stereomicroscope (magnification of $2.6\times$, and $13.0\times$).

3.8. Drug release kinetics

The estimated parameters derived from fitting different mathematical equations to the drug release profiles are presented in Table 14. Various mathematical models, including zero-order, first-order, Higuchi's, Korsmeyer–Peppas's, Hixon's, Hofenberg's, and Peppas–Sahlin's equations, were employed in this study. The release profile of DH from N1 exhibited a good fit with the Korsmeyer–Peppas model ($r^2 = 0.9956$) and the Peppas–Sahlin model ($r^2 = 0.9979$). This indicated that the drug release was predominantly controlled by diffusion, and the rate of diffusion correlated with the drug concentration in the formulation at that specific time. This release kinetic was characterized as the Fickian diffusion model, with a diffusivity value reflected by the n parameter from the Korsmeyer–Peppas model, which was 0.318 (less than 0.45) [239]. Furthermore, the k_1 value derived from the Peppas–Sahlin equation remained higher than the k_2 value ($k_1 = 8.630$ and $k_2 = -0.048$). This finding supported the hypothesis that the release mechanism was more prominently controlled by Fickian diffusion rather than Case II relaxation [239]. Similarly, for N2, the release profile aligned well with the Korsmeyer–Peppas model ($r^2 = 0.9984$) and the Peppas–Sahlin model ($r^2 = 0.9984$). The release kinetics of N2 were characterized as non-Fickian or anomalous transport, given the n value of 0.629 from the Korsmeyer–Peppas model. This indicated that the drug-release mechanism was influenced by both diffusion and swelling of the matrix. The diffusion and swelling rates were

comparable. The k value from the Peppas–Sahlin model was used to describe the mechanism of diffusion and swelling in detail. In this case, k_2 was more dominant than k_1 ($k_1 = -0.157$ and $k_2 = 0.518$), and the swelling or erosion mechanism from the borneol matrix was subsequently more prominent than the diffusion mechanism. The release profile of N4, containing 5% triacetin and 40% borneol, also conformed to the Korsmeyer–Peppas model ($r^2 = 0.9974$), with an n value of 0.518. The Peppas–Sahlin model for N4 ($r^2 = 0.9987$) indicated that k_1 was more dominant than k_2 ($k_1 = 1.291$ and $k_2 = 0.026$). Consequently, the addition of a hydrophobic substance (e.g., triacetin) to the ISM resulted in a matrix with the Fickian diffusion mechanism for drug release. Furthermore, the release profile of N8 matched the Korsmeyer–Peppas model ($r^2 = 0.9985$) and Peppas–Sahlin models ($r^2 = 0.9986$). However, the release kinetics were non-Fickian or anomalous transport with an n value of 0.679. In comparison, the diffusion release mechanism of N8 appeared less pronounced than that of N4 because the k_1 value of N8 was lower than that of N4. Nonetheless, the release mechanism of N8, influenced by erosion, was significant due to the increased k_2 value compared to that of N4. However, when considering both k_1 and k_2 values, the dominant drug-release mechanism of N8 was diffusion.

Table 14. Degree of goodness-of-fit and estimated parameters from curve fittings to mathematical equations of DH release profiles of different ISM formulations in PBS pH 6.8, with $n = 3$.

Formula	Modeling	Criteria for Model Selection			Kinetic Parameters		
		r^2	AIC	MSC			
N1	Zero order	0.9480	32.8196	2.3843	$k_0 = 0.058$		
	First order	0.9831	24.9566	3.5076	$k_1 = 0.001$		
	Higuchi	0.9799	26.1750	3.3335	$k_H = 2.578$		
	Korsmeyer–Peppas	0.9956	17.5847	4.5607	$k_{KP} = 9.011$	$n = 0.318$	
	Hixson–Crowell	0.9833	33.9840	3.5898	$k_{HC} = 0.0000$		
	Hopfenberg	0.9921	30.0182	4.0855	$k_{HB} = 0.0000$	$n = 185.258$	
	Peppas–Sahlin	0.9979	21.2822	5.1775	$k_1 = 8.630$	$k_2 = -0.048$	$m = 0.332$
N2	Zero order	0.9111	90.8974	2.1756	$k_0 = 0.023$		
	First order	0.9889	63.8739	4.2543	$k_1 = 0.000$		
	Higuchi	0.9762	73.7490	3.4947	$k_H = 1.253$		
	Korsmeyer–Peppas	0.9984	40.7684	6.0316	$k_{KP} = 0.461$	$n = 0.629$	
	Hixson–Crowell	0.9812	70.7012	3.7291	$k_{HC} = 0.000$		
	Hopfenberg	0.9889	65.8890	4.0993	$k_{HB} = 0.000$	$n = 653.884$	
	Peppas–Sahlin	0.9984	42.7074	5.8825	$k_1 = -0.157$	$k_2 = 0.518$	$m = 0.309$
N4	Zero order	0.8408	121.9651	1.6132	$k_0 = 0.011$		
	First order	0.9471	104.3304	2.7154	$k_1 = 0.000$		
	Higuchi	0.9969	58.8696	5.5567	$k_H = 0.889$		
	Korsmeyer–Peppas	0.9974	57.9719	5.6128	$k_{KP} = 0.761$	$n = 0.518$	
	Hixson–Crowell	0.9288	109.0962	2.4175	$k_{HC} = 0.000$		
	Hopfenberg	0.9471	106.3399	2.5898	$k_{HB} = 0.000$	$n = 1295.786$	
	Peppas–Sahlin	0.9987	48.5514	6.2015	$k_1 = 1.291$	$k_2 = 0.026$	$m = 0.399$
N8	Zero order	0.9310	69.2509	2.3960	$k_0 = 0.036$		
	First order	0.9943	41.8565	4.8864	$k_1 = 0.001$		
	Higuchi	0.9411	67.5029	2.5550	$k_H = 1.417$		
	Korsmeyer–Peppas	0.9985	10.5336	5.7222	$k_{KP} = 0.399$	$n = 0.679$	
	Hixson–Crowell	0.9901	47.8600	4.3407	$k_{HC} = 0.000$		
	Hopfenberg	0.9943	43.7404	4.7152	$k_{HB} = 0.000$	$n = 26.098$	
	Peppas–Sahlin	0.9986	12.3015	5.3686	$k_1 = 0.652$	$k_2 = 0.191$	$m = 0.376$

Models that fit each formulation are represented by the high r^2 , which is the **bold**.

3.9. Antimicrobial activities

Periodontitis, categorized as an oral health disease, can be caused by various pathogens, leading to microbial proliferation in the crevicular pocket [240]. This proliferation results in periodontal inflammation, attachment loss, and bone loss around the teeth. *A. actinomycetemcomitans* and *P. gingivalis* are commonly associated with periodontitis [5]. In cases where the periodontal environment is disrupted, commensal bacteria like *Actinomyces*, *Streptococcus*, and *Staphylococcus* spp. may invade, causing opportunistic infections [240]. *S. aureus* and *E. coli* were chosen to represent Gram-positive and Gram-negative bacteria, respectively, to assess antibacterial activity [246]. *Candida* spp., including *C. albicans*, *C. krusei*, *C. lusitaniae*, and *C. tropicalis*, were included due to their potential role in periodontitis [245]. Opportunistic infections, particularly hospital-acquired ones, are a significant concern for patients with severe periodontitis. MRSA, a well-known opportunistic pathogen, was selected for microbial testing [243].

The antimicrobial testing included selected formulations (N1, N2, N4, and N8), with NMP and triacetin serving as control groups. The experiment was conducted using the cup agar diffusion method, as reported previously [183]. The inhibition zone diameters of test formulations against *P. gingivalis*, *A. actinomycetemcomitans*, MRSA, *S. aureus*, *E. coli*, *C. albicans*, *C. krusei*, *C. lusitaniae*, and *C. tropicalis* are summarized in Table 15. Moreover, the inhibition zones of formulations against *A. actinomycetemcomitans* and *C. albicans* are displayed in Figure 42. Notably, the clear zones observed in formulations containing borneol (N2, N4, and N8) were smaller compared to N1, indicating that the borneol matrix impeded DH diffusion into the inoculated agar [183]. Generally, ISMs containing other water-insoluble matrix formations like Eudragit®L and zein commonly result in a reduced size of the clear zone due to their limited penetration of the bioactive agent [271]. Moreover, the addition of triacetin in the formulation reduced DH diffusion, and the drug's movement was influenced by the concentration of triacetin. Higher concentrations of triacetin in the formulation led to a slower drug diffusion due to triacetin's hydrophobic nature. All selected formulations (N1, N2, N4, and N8) demonstrated excellent antimicrobial activities against *P. gingivalis*, and their inhibition effects were significantly superior to those of NMP ($p < 0.05$). This enhanced inhibition was attributed to the marked effectiveness of DH in the formulation against *P. gingivalis*, surpassing the MIC value. Nonetheless, NMP showed effectiveness against *P. gingivalis*, as it could solubilize lipids in the cell membrane, promoting the leakage of microbial cell membranes [42]. Therefore, the combined antimicrobial effects of both NMP and DH contributed to the overall effectiveness of antimicrobial activities. While the antimicrobial effectiveness of N4 slightly decreased due to the addition of borneol and triacetin, the clear zone of N4 remained significantly larger than that of NMP ($p < 0.05$). All DH-loaded formulations exhibited antimicrobial activities against *A. actinomycetemcomitans*, as DH is highly effective against this anaerobic bacteria [282]. NMP also displayed a notable antimicrobial activity against *A. actinomycetemcomitans* as reported

previously [178]; thus, while the inhibition zone diameter of NMP was rather high, this was still lower than that of N1 ($p < 0.05$). The inclusion of borneol led to a reduction in the diameter of the inhibition zone as its matrix impeded the diffusion of solvent and drug molecules. Despite this, the inhibition against *A. actinomycetemcomitans* by N4 was still considered effective and remained satisfactory. DH demonstrated significant antimicrobial effectiveness against MRSA, *S. aureus*, and *E. coli* ($p < 0.05$) when compared with NMP [178]. Moreover, borneol can inhibit bacteria due to the antiadhesive properties of its chiral carbons for bacterial cells, interfering with bacterial colonization [247]. While N4 contained borneol and triacetin, the clear zone was significantly larger than that of NMP ($p < 0.05$), demonstrating effective antimicrobial activity against both Gram-negative and Gram-positive bacteria, including drug-resistant strains. Despite DH not inhibiting yeast, NMP as a solvent in the formulation efficiently inhibited these fungi. Therefore, the inhibition zone against *Candida* spp., including *C. albicans*, *C. krusei*, *C. lusitaniae*, and *C. tropicalis*, between NMP and N1 was not significantly different [178]. While borneol has antifungal activity against *Candida* spp. by promoting the generation of lipid peroxides, the solid-like borneol matrix could retard solvent diffusion on agarose [250]. As mentioned earlier, borneol transformed into a gel-like matrix, slowing the diffusion of NMP and triacetin into inoculated agar. Consequently, the clear zone of borneol-containing ISM appeared smaller than that of N1. However, N4 still demonstrated effective antifungal efficacy due to the antifungal activities of NMP and triacetin [198, 283].

The MICs of DH against *P. gingivalis*, *A. actinomycetemcomitans*, MRSA, *S. aureus*, and *E. coli* are 0.125, 0.21, 8.00, 0.28, and 1.54 mg/L, respectively [247, 282, 284, 285]. Typically, patients with periodontitis have 0.5 to 1.0 μL of gingival crevicular fluid in their crevicular pockets [255]. The formulation (about 1.0 μL) was administered into crevicular pocket, the contents of 5% DH released from N4 at 1 h, 1 day, and 5 days were 2,500, 11,500, and 38,000 mg/L, respectively. These values are significantly higher than the MIC values against all test microbes and demonstrate antimicrobial efficacy from the first hour of administration. In conclusion, borneol ISM with DH and triacetin-loaded exhibited effective antimicrobial activity for the treatment of periodontitis.

Table 15. Inhibition zone diameter of borneol ISM and related formulations against *P. gingivalis*, *A. actinomycetemcomitans*, MRSA, *S. aureus*, *E. coli*, *C. albicans*, *C. krusei*, *C. lusitaniae*, and *C. tropicalis*, with n = 3.

Microbes	Inhibition zone diameter (mean $\pm$ S.D.) (mm)					
	NMP	Triacetin	N1	N2	N4	N8
<i>P. gingivalis</i> ATCC 33277	15.00 $\pm$ 1.73 ^{a,b}	NC	40.33 $\pm$ 1.53 ^a	35.33 $\pm$ 0.58	34.33 $\pm$ 1.15 ^b	32.67 $\pm$ 0.58
<i>A. actinomycetemcomitans</i> ATCC 29522	46.33 $\pm$ 0.58 ^c	21.33 $\pm$ 0.58	50.00 $\pm$ 1.00 ^c	37.33 $\pm$ 1.15	36.67 $\pm$ 0.58	30.67 $\pm$ 0.58
<i>S. aureus</i> (MRSA) ATCC 43300	17.00 $\pm$ 1.00 ^{d,e}	12.33 $\pm$ 2.52	42.67 $\pm$ 1.15 ^d	39.33 $\pm$ 1.15	38.00 $\pm$ 0.58 ^e	36.67 $\pm$ 1.53
<i>S. aureus</i> ATCC 25923	18.67 $\pm$ 0.58 ^{f,g}	NC	38.67 $\pm$ 0.58 ^f	37.00 $\pm$ 1.00	35.33 $\pm$ 1.15 ^g	33.33 $\pm$ 0.58
<i>E. coli</i> ATCC 8739	22.33 $\pm$ 0.58 ^{h,i}	NC	29.33 $\pm$ 0.58 ^h	28.67 $\pm$ 0.58	27.33 $\pm$ 0.00 ⁱ	25.67 $\pm$ 0.58
<i>C. albicans</i> ATCC 17100	26.00 $\pm$ 1.00	12.00 $\pm$ 1.00	26.33 $\pm$ 0.58	22.33 $\pm$ 1.00	20.00 $\pm$ 0.00	17.33 $\pm$ 1.15
<i>C. krusei</i> TISTR 5259	31.30 $\pm$ 1.20	NC	32.33 $\pm$ 0.58	21.00 $\pm$ 1.00	18.33 $\pm$ 1.53	NC
<i>C. lusitaniae</i> TISTR 5156	38.33 $\pm$ 1.15	12.33 $\pm$ 0.58	38.33 $\pm$ 0.58	35.33 $\pm$ 1.15	34.33 $\pm$ 1.53	26.00 $\pm$ 1.00
<i>C. tropicalis</i> TISTR 5306	32.67 $\pm$ 0.58	NC	31.67 $\pm$ 0.58	29.67 $\pm$ 2.08	25.67 $\pm$ 1.53	18.00 $\pm$ 2.00

The superscripts a-i represents p < 0.05; NC = the clear zone not found.

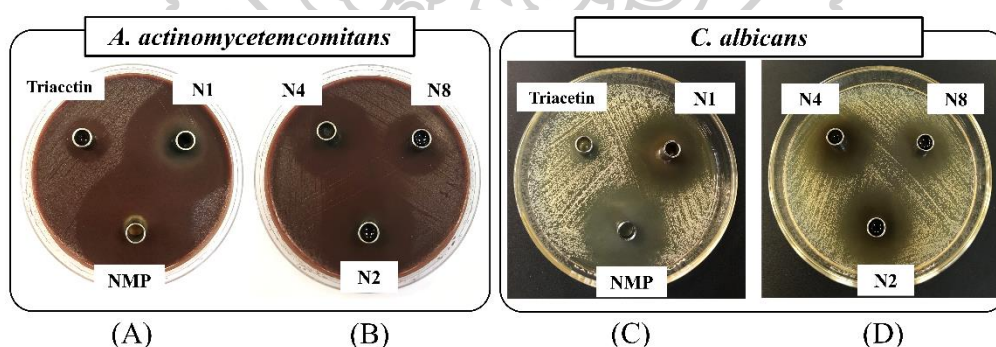


Figure 42. Photographs of agar plate after complete antimicrobial activities testing depicting the inhibition zones of borneol ISM and related solvent against *A. actinomycetemcomitans* (A, B) and *C. albicans* (C, D), with n = 3.

Triacetin was effectively utilized as an additional hydrophobic solvent in borneol ISM with DH-loaded. The prepared ISMs exhibited homogeneous transparent solutions, and their density and viscosity gradually decreased with the addition of triacetin, especially when the viscosity was below 12 cP. While triacetin had a slight impact on the surface tension of ISM, an increase in its proportion elevated the

injection force and energy required for ISM expulsion from the needle. Nonetheless, the force needed remained below 10 N, indicating the ease of administration through injection into the crevicular pocket. Upon contact with the aqueous phase, ISMs underwent a transformation into a gel-like matrix through solvent exchange and phase inversion. The resulting matrix demonstrated favorable wettability, with a low contact angle (less than 45°) and exhibited plastic deformation. NMP was identified as a suitable primary solvent for ISM, as the resulting matrix displayed increased firmness. ISMs with a high triacetin content (25%) tended to slow solvent diffusion, matrix formation, and firmness but displayed lower sensitivity to water. On the other hand, ISM with a low triacetin concentration (2.5%) exhibited overly rapid phase inversion and prolonged DH release. The most optimal ISM, containing 5% triacetin, demonstrated the longest DH release over 10 days, following Fickian diffusion. Additionally, this formulation exhibited effective antimicrobial activities against various microbes, including periodontitis pathogens (*P. gingivalis* and *A. actinomycetemcomitans*). Therefore, DH-loaded 40% borneol-based ISM with 5% triacetin holds promise as a locally administered ISM for periodontitis treatment *via* crevicular pocket injection. In comparison to systemic drug regimens, this developed ISM could provide higher concentrations of antimicrobial drugs at the subgingival site, reducing the risk of drug-resistant microbial populations in non-oral body sites. Furthermore, it offers a less invasive alternative to surgical interventions. However, it is the responsibility of the dentist to carry out the application of ISM on the patient. Despite the availability of safety data regarding the medical applications of borneol and triacetin, it is crucial to conduct additional clinical experiments to assess the efficacy and safety of this developed DH-incorporated borneol ISM. Furthermore, conducting an in-depth exploration of the mechanisms behind matrix formation and phase inversion remains a challenging task, yet it is essential to gain a comprehensive understanding of these phenomena. This understanding will prove valuable in the development of future ISM formulations.

4. Unraveling mechanistic phase inversion of borneol-based ISM

4.1. Utilizing UV-vis imaging to investigate the phase inversion mechanism of ISM

Non-solvent-induced phase inversion-based borneol ISMs have demonstrated success in the preparation and development of treatments for oral cavity diseases, including oropharyngeal candidiasis and periodontitis. The 40% w/w borneol-based ISM has shown significant promise as a clotrimazole-loaded spray for treating oropharyngeal candidiasis. Additionally, it serves as a potentially effective local antimicrobial drug delivery system for periodontitis treatment when administered *via* crevicular pocket injection. Previous research has indicated that an ISM composed of 40% w/w borneol and augmented with 5% w/w triacetin has the potential to serve as a localized drug delivery system for periodontitis treatment, offering extended drug release compared to triacetin-free borneol-based ISM. Interestingly, ISMs formulated

with a low triacetin concentration (5% w/w) have demonstrated a more favorable and sustained release profile than formulations with high triacetin content (25% w/w). Despite the low ratio (5% w/w) of triacetin used as a hydrophobic cosolvent in ISM, this formulation unexpectedly contributes to prolonged drug release. This paradox highlights the need for an in-depth exploration underlying the mechanisms behind phase inversion, necessitating a comprehensive study to elucidate the intricate interplay potentially responsible for this phenomenon. Therefore, this study has selected two different concentrations of triacetin, namely 5% w/w and 25% w/w, to represent the low and high cosolvent content of ISM, respectively.

Traditionally, evaluations of ISMs have been limited to physicochemical tests, analyses of matrix formation morphology, and drug release assessments. However, these tests cannot provide detailed, real-time observations of phase inversion and related phenomena. To address this limitation, the UV-vis imaging technique has been introduced to the field of drug formulation development, enabling a closer examination of matrix formation processes, particularly the phase inversion mechanism. The primary objective of this study is to unravel the intricate mechanisms of phase inversion and facilitate real-time observation and analysis of matrix formation processes. Furthermore, data obtained from this technique contribute to a comprehensive understanding of the interplay between borneol-based matrix formation, solvent diffusion, and drug release, providing predictive insights into ISM behavior. UV-vis imaging was expected to serve as a bridge between the formulation development field and the analytical field, offering a direct visualization of the dynamic processes that were previously challenging to monitor in real-time for formulation development.

To gain a deeper understanding of the role of borneol and triacetin in their effects on drug release control and to elucidate the mechanisms of phase inversion when ISM formulation contacts with an aqueous solution, an UV-vis imaging technique was used in this study for observing the gradual phase inversion phenomena. Moreover, this technique can simultaneously monitor matrix formation and drug diffusion behavior. This observation under three different wavelengths using UV and Vis imaging techniques was able to the concomitant monitoring of drug release, phase inversion, and matrix formation of ISM formulation [156]. Since the occurred matrix, drug, and solvent have different light absorption wavelengths, it is possible to study the changes of these substances simultaneously using different wavelengths of observation. The combination of obtained data from the experimental results of this technique and the previous experiments (sections 3.4, 3.5, and 3.7) will contribute to a better understanding of the relation between the phase inversion, matrix formation, and drug release phenomena from ISM formulation upon contact with the agarose gel. However, this experiment was limited to observations for the first 12 hours after the solute exchange occurred due to the limitations of the experimental equipment and the size of the cuvette used to contain the drug and agarose.

4.1.1. Borneol-matrix formation

For the borneol-based ISM in NMP, the high absorbance at 525 nm was employed to observe the occurred solid-like matrix from the borneol solution at the interface area upon ISM solution contact with the 0.6%w/v agarose gel which mimicked the periodontal membrane. The occurred matrix has an opaque translucent white matrix which can absorbance at the visible wavelength. The absorbance map at 525 nm represents an occurred matrix that was shown in Figure 43(A). In the case of F1 contact with agarose gel, nothing was found on the map because the F1 did not contain the matrix former. The absorbance signal at 525 nm was detected and displayed in Figure 44. A slight absorbance signal (< 0.025 AU) was detected in the presumed interface area at the initial time point (0 h), which is a signal from the agarose boundary. However, this signal has disappeared over time because of the miscibility of NMP with agarose gel. Whereas the matrix from F2 suddenly appeared since the NMP was highly miscible with water [52]. The rapid phase inversion between water from agarose and NMP from ISM occurs in fast matrix formation at the interface while the interiors of ISM still were the clear ISM solution [78]. The thickness and firmness of the matrix from the F2 and F3 gradually increased by time. By comparison, the absorbance of F3 was significantly lower than that of F2 (Figure 44(B) and Figure 44(C)). This lower absorbance indicates a lower matrix strength. In addition, the thicker of an occurred matrix of F3 was lower than that of F2 (Figure 43(A)). The little amount of triacetin incorporated in ISM resulted in interference in a matrix formation process which led to a reduction in the strength of the matrix. This is a small change in terms of matrix formation. The rate of matrix formation and the morphology of the matrix occur both affect the rate of phase inversion and controlled drug release, which will be discussed in the next section. In the case of F4, the thin matrix appeared (Figure 43(A)) and the absorbance signal was detected around 0.1 AU, which was less than F2 and F3. The occurred matrix was loose and may not be able to entrap the drug to control its release. This result confirmed the above results as mentioned in section 3.4 and 3.5. In terms of matrix formation, firmly matrix can support the drug retention capacity for controlling the drug release. The orderliness and strength of the matrix usually led to the formation of a thick matrix [267]. When a drug was loaded in the strength matrix, the drug was trapped in the matrix system and released slowly over time. Conversely, the loose matrix might reduce the strong matrix formation and could not control the drug release [267]. Therefore, the appearance and morphology of the occurred matrix was crucial factor used to determine and predict drug release. The appropriate proportion of triacetin will result in the formation of the matrix properly which leads to effectively delaying the release of the drug.

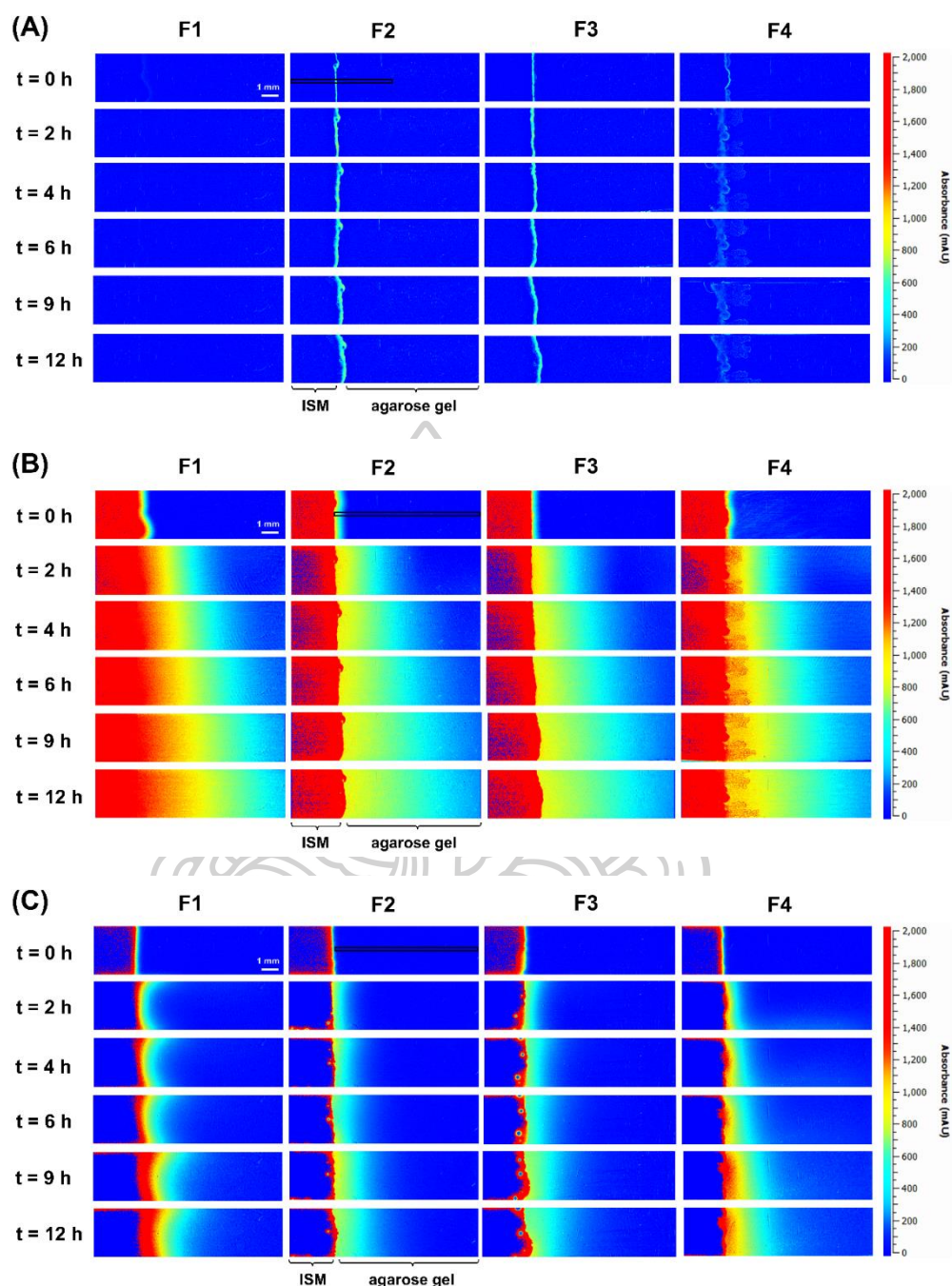


Figure 43. Absorbance maps of (A) borneol matrix formation, (B) NMP diffusion, and (C) DH diffusion from drug-loaded ISM in contact with 0.6% (w/v) agarose gel as measured by UV imaging at 280 nm, 214 nm, and Vis imaging at 525 nm, respectively. The black horizontal bars in (A) of a 0.3×6.0 mm.² and bars in (B and C) of a 0.3×8.0 mm.² area were used to produce the concentration-time and absorbance-distance profiles in the agarose gel displayed in Figure 44, 45, and 47.

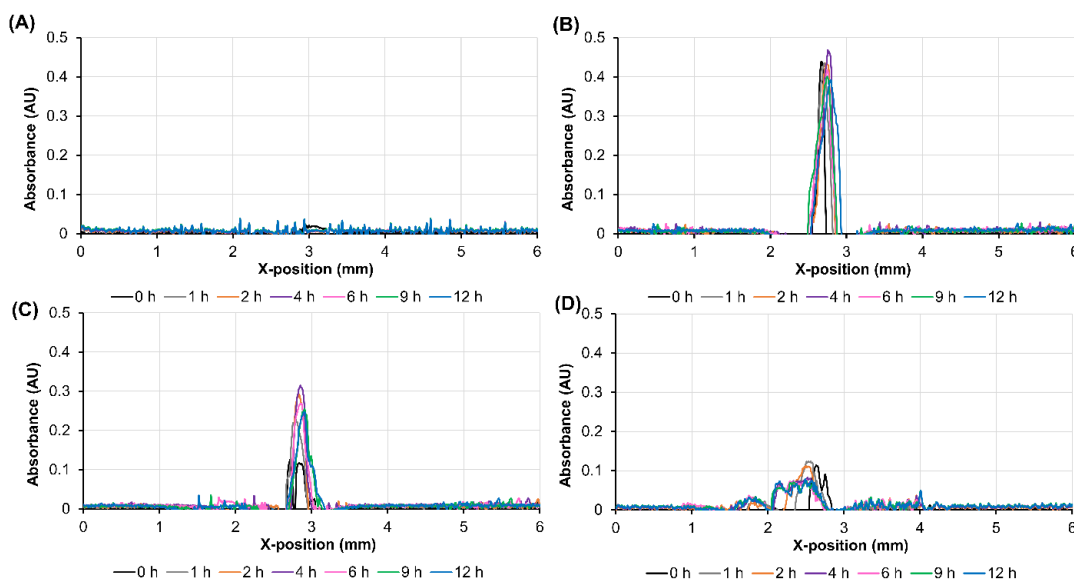


Figure 44. Absorbance-distance profiles for formulation (A) F1, (B) F2, (C) F3, and (D) F4 at 525 nm. The profiles were obtained from the image area corresponding to the black horizontal bar in Figure 43 at the selected time points.

4.1.2. Solvent diffusion

Typically, the phase inversion occurred upon ISM contact with aqueous solvent. It was an initial mechanism of the ISM that leads to matrix formation and controls the drug release as consequently [42]. The diffusion of the two types of solvents moved in opposite directions: the organic solvent from the prepared ISM diffuses into the environment (e.g., crevicular pocket in periodontal or agarose gel) while the water molecules diffuse into the formulation [40, 65]. To visualize the diffusion of NMP into an agarose gel, techniques such as UV-vis imaging, staining or fluorescent labeling can be employed. These methods can help track the movement of NMP within the gel and provide visual evidence of the diffusion process [152, 286]. To address this mechanism and find the relation between phase inversion and drug release, the solvent diffusion was observed in first 12 hours under UV-vis imaging technique. This is one of three experiments to determine the relationship between solvent diffusion, matrix formation, and drug diffusion. The wavelength of 214 nm was chosen to observed mixture solvent of ISM including NMP and triacetin in each ISM formulation because it was the wavelength essentially employed to observed the presence of solvents [152]. To prevent interference effects at 214 nm from the absorbance signal of DH, the ISM formulation without DH (according to the preparation procedure described) was selected for testing in this study. For our preliminary studies, the pure of triacetin and NMP were separately tested on agarose gel. The diffusion of NMP in agarose gel was predominant observed, whereas the detected signal of triacetin was lower in agarose gel at the same time point. This phenomenon was possible due to the water affinity of NMP and hydrophobicity of triacetin [78, 287]. Agarose gel was

semisolid in gel state while ISM was non-aqueous solution initially; therefore, NMP could move more easily to the side of agarose gel than triacetin.

Upon ISM contact with agarose, the solvent suddenly diffuses into the agarose gel and gradually slows over time. The absorbance map of solvent on the agarose gel was presented as color mapping and shown in Figure 43(B). Different colors displayed on the map represent different solvent absorbances at each area on agarose gel where the red was the region with high solvent concentration while the concentration cascades down to near zero with blue areas expressed. The concentration along the black horizontal bar in Figure 43(B) at each time point was measured and normalized by the initial solvent concentration of each formulation. Because the amounts and ratios of solvents in each formulation were different, the molar concentration profiles were normalized by their respective initial solvent concentrations. These solvent-normalized concentration-distance profiles are displayed in Figure 45. Moreover, rate of solvent diffusion in agarose gel was calculated and exhibited in Table 16(A).

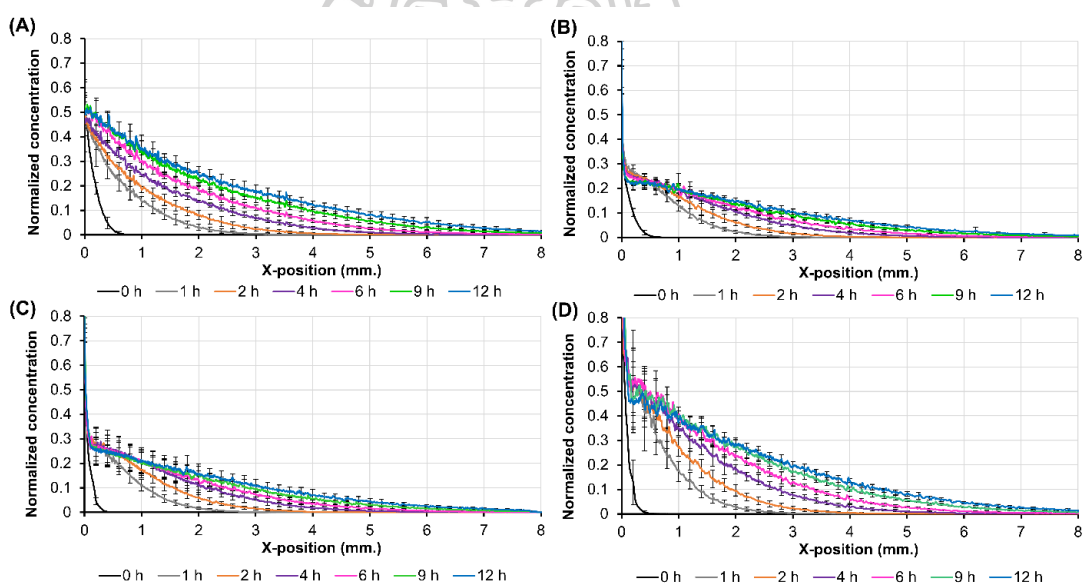


Figure 45. Solvent concentration-distance profiles for formulation (A) F1, (B) F2, (C) F3, and (D) F4 at 214 nm. The profiles were obtained from the image area corresponding to the black horizontal bar in Figure 43 at the selected time points.

Table 16. Rate of solvent (A) and DH (B) diffusion from different formulations at different time points, with $n = 3$.

(A)

Time (hour)	Rate of solvent diffusion (normalized molar/hour)			
	F1	F2	F3	F4
1	0.036 ± 0.002	0.030 ± 0.006	0.030 ± 0.009	0.048 ± 0.011
3	0.028 ± 0.007	0.022 ± 0.005	0.026 ± 0.007	0.043 ± 0.002
5	0.023 ± 0.007	0.010 ± 0.001	0.012 ± 0.005	0.028 ± 0.003
7.5	0.020 ± 0.006	0.005 ± 0.006	0.009 ± 0.002	0.021 ± 0.004
10.5	0.005 ± 0.001	0.003 ± 0.001	0.001 ± 0.001	0.001 ± 0.001

Data were calculated from the concentration that was measured at the position of $x = 2$ mm from the interface line.

(B)

Time (hour)	Rate of DH diffusion (mg/mL/hour)			
	F1	F2	F3	F4
1	0.27 ± 0.03	0.25 ± 0.04	0.22 ± 0.01	0.27 ± 0.02
3	0.18 ± 0.03	0.13 ± 0.05	0.13 ± 0.02	0.10 ± 0.01
5	0.13 ± 0.03	0.04 ± 0.02	0.06 ± 0.02	0.20 ± 0.03
7.5	0.11 ± 0.02	0.03 ± 0.01	0.03 ± 0.02	0.09 ± 0.00
10.5	0.08 ± 0.01	0.00 ± 0.01	0.00 ± 0.00	0.00 ± 0.02

Data were calculated from the concentration that was measured at the position of $x=1$ mm from the interface line.

Initially, the rate of solvent diffusion was highest and minimized over time. The measured solvent concentration at interface of F1 was around 0.5, while an initial concentration of both F2 and F3 had of only 60% (~ 0.3) of the initial concentration of F1 because the 40%w/w borneol in ISM changed to solid-like matrix. The matrix was suddenly occurred as discussed in the previous experiment (Section 4.1.1); thus, the diffusion of solvent was partial blocked by this matrix and the phase inversion process was retarded [225]. Besides, the diffusivities of F2 and F3 were less than those of F1 at all time points throughout the 12-hour experimental period. By comparison between F2 and F3, the small amount of triacetin incorporation in ISM did not appear a significant effect on the rate of solvent diffusion. Thus, the concentration-distance profiles of F2 and F3 were almost the same and their diffusivities of both formulation were not significantly different. Nevertheless, the initial concentration of F4 was highest (~ 0.8). Its rate was higher than other formulations. Given the high ratio of triacetin in F4, this phenomenon may arise from three synergist mechanisms. First, NMP can easily migrate from the ISM side to the agarose gel containing water owing to a water miscible and high-water affinity of NMP ($\log P = -0.38$), [208, 287]. In contrast, triacetin exhibited limited water solubility, approximately

7% [78]. Only a few triacetin molecules migrated to the agarose side, as seen in preliminary results. Most triacetin molecules remained on the ISM side. In contrast, NMP exhibited notably greater and faster migration toward the agarose, resulting in the highest solvent movement rate in F4. The solvent (NMP) in F1 moved to the agarose side due to its inherent hydrophilic properties, independent of other substances. The matrix in F4 did not significantly impede solvent molecule movement. Although the borneol matrix formed upon contact with water, it formed slowly, and the resulting matrix was gel-like and less compact [280]. The incompletely formed matrix and loosened agglomerate of crystalline matrix, which are shown in the interface area of F4 in Figure 43(B), were some of the factors resulting in uncontrolled phase inversion retardation. For these reasons, triacetin remained within the ISM formulation, simultaneously facilitating the rapid movement of NMP molecules into the agarose gel. As a result, the formed matrix was less firm. Consequently, the measured concentration and rate of solvent diffusion of this formulation were higher than those of other formulations.

The concentration-time profiles, obtained at 1 mm distances from the interface and shown in Figure 46(A), revealed a rapid initial increase followed by a slow decline in all formulations. The normalized concentration of F2 and F3 reached 0.2 and remained constant for 4 hours. In contrast, the concentration in F1 continuously increased, reaching twice the level of F2 and F3. This was due to the absence of matrix formation in the ISM, allowing continuous solvent diffusion. Although F4 contained a matrix former, the addition of a high ratio of triacetin hindered matrix formation. The hydrophobic nature of triacetin and the high-water affinity of NMP contributed to the faster expulsion of solvent from the ISM to the agarose gel [78, 287]. Therefore, the detected concentration of solvent at $x = 1$ quickly increased. However, the concentration slightly dropped after 9 hours. This was probably because the matrix formed slowly from the borneol, and it began to block the diffusion of the solvent. In addition, the increased solvent diffusion distance and the depletion of solvent in the ISM also contributed to the decrease in solvent concentration [156]. The concentration-time profile at $x = 2$ mm (Figure 46(B)) had the same pattern as the profile at $x = 1$ mm, that was, the appeared concentrations of F4 were greater than those of F1, F2 and F3. By comparison with the profile at $x = 1$ mm, this profile had a lower value and reached constancy after 9 hours, except for F1, which tended to increase over time due to the higher NMP concentrations in its preparation than in other formulations and the absence of borneol, which slowed the diffusion of the solvent.

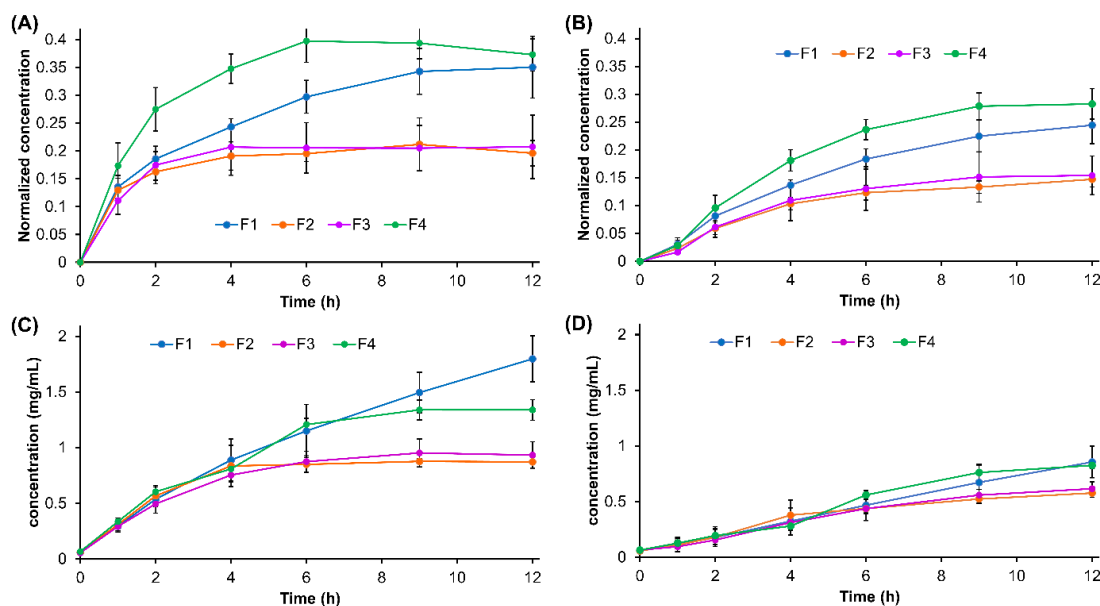


Figure 46. Solvent concentration-time profiles (A, B) and DH concentration-time profiles (C, D) obtained at (A, C) 1 mm. and (B, D) 2 mm. from the interface line. The interfaces line between ISM and agarose gel were defined as $x = 0$ mm.

In conclusion, the matrix-forming borneol played a significant role in the retardation of solvent diffusion, whereas the addition of small amounts of triacetin did not affect the phase inversion and the rate of phase inversion within the first 12 h. However, the addition of excessive triacetin up to 25%w/w seemed to affect matrix formation and led to uncontrolled phase inversion. The occurred loosely formed agglomerate with no dense matrix possibly led to failure to retard the phase inversion and causing an inefficient for entrapping the drug. Furthermore, the properties of NMP and triacetin affect to rapid pushing of solvent to the agarose gel. These can lead to failure to control the release of the drug, which was a main purpose in ISM design. A better understanding of matrix formation and phase inversion process was thus a crucial foundational knowledge for elucidating the drug release mechanism of ISM, which will be discussed in the next section.

4.1.3. DH diffusion

Typically, the main goal in designing and developing ISM formulations was to create a self-matrix formulation to control drug release. The process of drug release controlled by the ISM system starts with phase inversion upon ISM contact with an aqueous solution. Subsequently, phase inversion occurred, and the solution was transformed into a solid-like matrix to entrap the drug and control drug release. In this section, the effects of borneol and triacetin in ISM on drug diffusion from the ISM system were observed and described. Besides, data from phase inversion and matrix formation behaviors in the previous study (section 4.1.1 and 4.1.2) were also included for support in this discussion.

The wavelength of 280 nm was chosen because it was close to the maximum absorbance of the DH spectrum and was the available wavelength of the instrument. However, preliminary studies had shown that other substances in ISM (borneol, triacetin, and NMP) do not interfere with the DH absorbance in agarose gel area at the 280 nm. From the experiment, the sample was added to the cuvette and in contact with agarose gel which mimicked the periodontal membrane. The behavior of DH diffusion through the agarose was processed as absorbance maps which are shown in Figure 43(C). After ISM contact with agarose gel, the diffusion of DH simultaneously occurs with increasing distance in agarose gel over time. The highest concentration of DH was found at the interface area between the formulation and agarose gel and its concentration was descended with distance from the interface. Besides, the concentration along the black horizontal bar in Figure 43(C) at each time point was measured from the interface and constructed as concentration-distance profiles as shown in Figure 47.

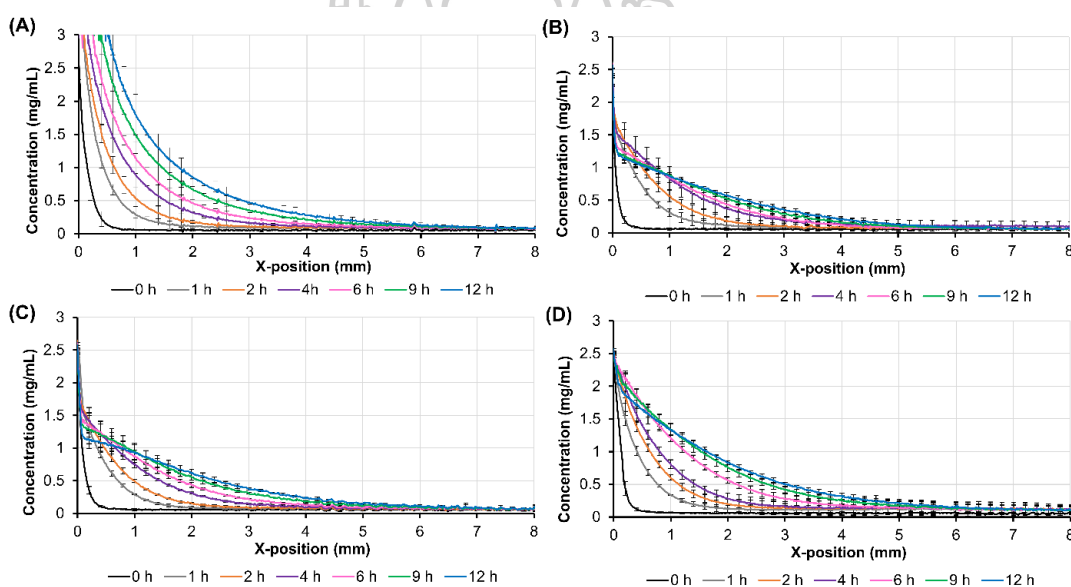


Figure 47. DH concentration-distance profiles for formulation (A) F1, (B) F2, (C) F3, and (D) F4 at 280 nm. The profiles were obtained from the image area corresponding to the black horizontal bar in Figure 43 at the selected time points.

In the case of the borneol-free formulation (F1), which represents the control group, the concentration measured near the interface was approximately 4 mg/mL, the maximum value detectable by our instrument. Initially, the original concentration in the F1 preparation was 5 mg/mL. The concentration of DH decreased progressively with increasing distance from the interface. Furthermore, calculations were conducted to ascertain the rate of DH diffusion at various time points, and these findings have been documented in Table 16(B). It is worth highlighting that the rate of DH diffusion was high in early state upon ISM contact with agarose due to the different concentration of DH between ISM and agarose gel. However, as time advanced, the measured concentration of DH

experienced a gradual decline, primarily due to the diminishing concentration gradient at the interface and the simultaneous increase in diffusion distance [156].

Regarding F2, the concentration around the interface initiated at 2 mg/mL, which was half of the initial concentration in F1. This occurrence was attributed to the rapid formation of the borneol matrix, a crucial barrier that significantly retarded the diffusion of DH into the agarose. The diffusion retardation of DH was like the diffusion retardation of solvents (as discussed in Section 4.1.2), both of which were caused by the formation of the borneol matrix. In comparison, the DH diffusion rate of F2 was significantly lower than that of F1 throughout the duration of the experiment. Meanwhile, the concentration-distance profile pattern of F3 resembled that of F2, like the NMP concentration-distance profile as mentioned earlier.

Although the results from the in vitro drug release testing (as discussed in Section 3.7) indicated that F3 exhibited a longer release duration compared to F2, the results of DH diffusion from formulas F2 and F3, as observed through UV-vis imaging, revealed similar diffusion rates (see Table 16) and patterns (see Figure 47(B) and Figure 47(C)). The limitations of the equipment in the UV-vis imaging experiment and the observation period (12 hours) resulted in a similar pattern of DH concentration-distance profiles for both F2 and F3. Therefore, the incorporation of triacetin (5% w/w) into the ISM formulation probably did not significantly affect the diffusion of DH during the first 12 hours. However, this experiment had some limitations, such as the use of a small, closed cuvette and the observation period limited to 12 hours, which resulted in no significant difference between F2 and F3 [156, 288]. The experiment of long-term study and adapted sizes of cuvette for UV-vis imaging experiment should be designed and investigated in the future to clearly distinguish DH diffusion patterns from both formulations.

Surprisingly, the initial DH concentration at the interface was around 2.5 mg/mL, higher than in F2 and F3. The measured concentration gradually declined with increasing distance from the interface. However, this pattern was distinctly different compared to F2 and F3, possibly due to the high amount of triacetin as a component. Triacetin was commonly used as a solvent in ISM preparation to facilitate a slow-forming phase inversion [278]. One of its noteworthy characteristics was its high hydrophobicity, coupled with low water solubility (less than 7%). Given these properties, the addition of triacetin in ISM preparation could lead to two phenomena: blocking of phase inversion and interference with the matrix formation process [277]. In terms of the first assumption, the high amount of hydrophobic triacetin added to ISM blocked the diffusion of DH, which was a highly water-soluble molecule [78, 289]. As a result, DH should have had difficulty diffusing through the interface to the agarose gel, resulting in slower DH diffusion. However, the results of the experiment did not align with this expectation.

On the other hand, the addition of a high amount of triacetin interfered with the process of matrix formation. For this reason, the formation of the borneol matrix was slow, resulting in a matrix that appeared soft, weak, and less firm, as previously discussed in Section 3.7. The DH contained in the ISM was not effectively entrapped by the borneol matrix because the matrix formed slowly and was not strong. Therefore, its profile exhibited a burst release and faster diffusion into the agarose gel than anticipated [280]. It appeared that the incorporation of triacetin into ISM caused two opposing effects. On one hand, it resulted in a controlled and extended release of the drug. On the other hand, in some cases, it led to a lack of control over drug release. Both of these phenomena depended on the amount of triacetin added and the matrix-forming agent used in ISM [280]. From the research of Liu and Venkatraman [280], the addition of too much triacetin affected the lack of control over drug release, while adding too little triacetin resulted in poor drug release efficiency. Therefore, the appropriate amount of triacetin needed to be carefully designed and developed for the ISM formulation to contribute significantly to drug retention in the matrix and prolong its release duration. Based on these results, it became evident that adding 25% w/w triacetin to the borneol-based ISM led to incomplete matrix formation with a gel-soft matrix (Figure 39) and ineffective control over drug diffusion as shown in Figure 41(A). Consequently, the amount of DH diffusion from F4 was higher than from F2 and F3 but less than from F1. Moreover, the DH diffusion rate of F4 was close to that of F1. This value fluctuated during the first 10 hours due to incomplete matrix formation, resulting in variations in the controlled release of the drug. However, the diffusion rate became close to 0 mg/ml/h after 10 hours because the matrix had become firm and had fully formed, as discussed in Section 3.7.

In addition, the DH concentration-time profiles obtained at 1- and 2-mm distances from the interface were observed and shown in Figure 46(C) and Figure 46(D), respectively. The observed concentration at $x = 1$ of F1 continuously increased over the 12-hour experiment, in line with the free diffusion of the drug into the agarose gel. In contrast, the concentrations of both F2 and F3 increased slightly less than F1 and plateaued after 9 hours at approximately 0.95 mg/mL. This suggested that the formed borneol matrix efficiently retarded DH diffusion. Initially, F4 exhibited the same increasing pattern in drug concentration as the other ISMs, with the concentration reaching and remaining constant at 1.3 mg/mL. However, due to the slow formation of the borneol matrix in F4 resulting from the addition of triacetin to the formulation, the measured F4 concentration was greater than that of F2 and F3. When observing the DH concentration at $x = 2$, it became evident that the measured concentration increased at a slower rate compared to the concentration at $x = 1$, primarily because the observation point was farther from the interface. The concentration showed a tendency to keep rising and did not reach saturation. Notably, the concentration of F1 and F4 appeared to be slightly higher than that of F2 and F3.

In conclusion, borneol played a crucial role in regulating DH release. The incorporation of a low amount of triacetin into ISM did not significantly affect the retardation of drug release during the initial 12 hours. However, a more extended study using UV-vis imaging may provide further insights. Interestingly, the addition of a high amount of triacetin led to rapid release, primarily due to the interference of triacetin in matrix formation. This highlights the hydrophobic nature of triacetin, resulting in less controlled drug release from F4. Furthermore, the diffusion patterns of DH in each formulation closely resembled the NMP diffusion pattern, as discussed earlier. Therefore, the relationship between drug and solvent diffusion into agarose gel will be further explored in the next section. The outcomes of this study will contribute to predicting the diffusion rate of other drugs in ISM applications and aid in designing controlled-release drugs in the future.

4.1.4. Cumulative DH diffusion

Typically, the ISM formulations were designed and expected to transform into the solid-like matrix upon contact aqueous solution and prolong the drug release [183]. Triacetin was used as an additive solvent in these ISM formulation with the expectation of prolonging drug release. The proper proportions of triacetin were investigated. In this study, drug release, solvent diffusion, and matrix formation in the ISM formulation were concurrently monitored to better understand the effects of borneol and triacetin on controlled release. The discussion of these phenomena was explained as mentioned above.

To confirm these results, the amounts of both DH and solvent in the agarose gel part were investigated from the absorbance map at various time points. The cumulative amounts of DH and solvent were plotted at different times and are shown in Figure 48(A) and Figure 48(B), respectively. The amount of DH measured in the agarose side increased gradually, with a rapid rate of increment in the early stages. Interestingly, the amount of DH diffusion from F1 and F4 was significantly greater than that from F2 and F3. The amount of solvent diffusion from F2 and F3 was also the lowest. F1 exhibited more solvent diffusion than F2 and F3, while F4 had a significantly greater diffusion than other formulations. The fact that F4 had significantly greater solvent diffusion than F1, while the DH diffusion of F1 and F4 was not significantly different, was unexpected and most likely depended on the size of diffusion molecules, the formed matrix, and the additional substances.

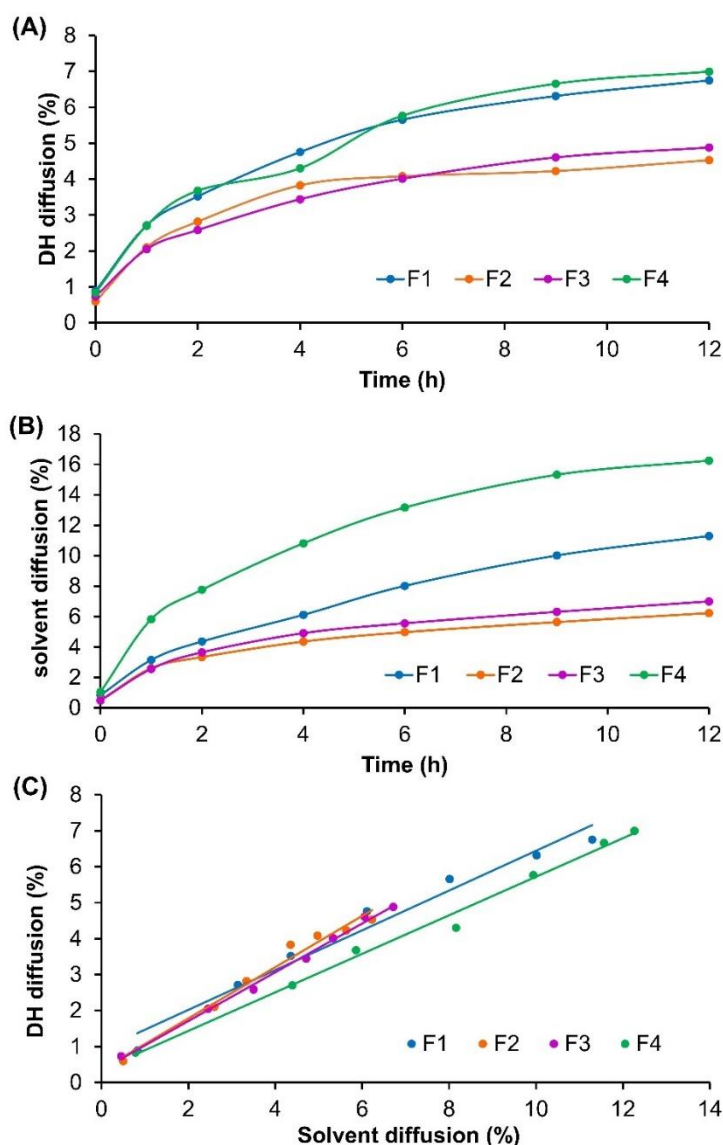


Figure 48. (A) Cumulative release of DH and (B) cumulative solvent diffusion from ISMs in the 0.6% w/v agarose gel over time. (C) relationship between DH and solvent diffusion in agarose gel.

Normally, small molecule was able to diffuse faster than larger molecules. The size of NMP molecule (molecular weight of 99.13) was smaller than DH (molecular weight of 512.94). Agarose gels typically have a porous structure, allowing small molecules like NMP to diffuse more easily compared to larger molecules [152]. In addition, the high-water affinity of NMP caused solvent to migrate into agarose gel faster than DH. The forming matrix of borneol behaves like a large barrier retard the diffusion of DH but was less effective against NMP due to their small NMP size. However, the slow transformation of matrix may ineffectively regulate drug release. Additionally, the hydrophobicity of triacetin had the effect of retarding DH; on the other hand, it pushed the NMP to move out from the ISM.

Based on the results of the above experiments on the diffusion behavior of the drug and the solvent, it was worth mentioning that both diffusions into the agarose gel tended to follow similar patterns. The results from the solvent diffusion experiments reflected the phase inversion phenomenon, while the drug diffusion results represented the ISM drug release phenomenon. This leads to the question of whether phase inversion and drug release are related. The solvent diffusion and drug diffusion of each formulation were plotted as a percentage (Figure 48(C)), and it was discovered that there is a linear relationship between the percentage of drug diffusion and solvent diffusion, with an R-squared value greater than 0.97 for borneol-based ISM.

When calculating the correlation trendlines in each formulation, it was found that the slope values of F1, F2, F3, and F4 were 0.5525, 0.7125, 0.6780, and 0.5357 respectively. The values from firmly matrix formulations (F2 and F3) were rather higher than those from non-firm or borneol-free matrix formulations (F1 and F4). It is worth mentioning that the morphology of the occurred matrix affected the behaviors of drug and solvent diffusion. In this study, different matrix morphologies had a minor effect on drug and solvent diffusion because phase inversion influenced drug release in all prepared ISM formulations. However, it could be hypothesized that the firmness and morphology of the occurred matrix might result in a higher ratio of drug to solvent diffusion. Generally, the matrix retards both solvent and drug diffusion, but in the case of a highly tight or firm matrix, the drug may be released where the diffusion of solvent is not necessarily very high. This was only a preliminary assumption based on the results of this experiment. The relationship between the formed matrix, drug, and solvent release based on the above assumptions deserved further study in the future. Lastly, this implied that the rate of phase inversion was one of the crucial factors controlling drug release. Knowledge of phase inversion processes could be useful for predicting and designing drug release behavior.

In this study, the UV-vis imaging technique was utilized to concomitantly monitor the matrix formation, phase inversion process, and drug release. The solvent diffusion and drug diffusion into the agarose gel were clearly presented in the absorbance map, which was represented by color gradations. Additionally, the emergence of the matrix was presented to determine the correlation between matrix characteristics and the effectiveness of drug release control. From this study, it was found that the occurrence of the matrix led to the retardation of both phase inversion and drug release. In addition, the correlation between phase inversion and drug release was further investigated, and it was found that an increase in phase inversion led to increased drug release. It may be concluded that these three variables, namely matrix formation, phase inversion, and drug release, are related and may be exploited to design or predict the effectiveness of novel ISMs. However, this experiment was limited in terms of duration (12 h), covering only the initial state, as well as the limitation of the diffusion cuvette size [156,

288]. Therefore, long term study and augmentation of agarose gel area were challenging to develop.

4.2. Computer dynamics simulation for understanding mechanistic phase inversion

The UV-vis imaging techniques from previous research have unveiled the phase inversion mechanism of ISM, together with real-time simultaneous visualization of drug release and matrix formation. This accomplishment sheds new light on the phase inversion mechanism. However, UV-vis imaging techniques have only revealed the mechanism of this change on a scale of minutes to hours, but a full understanding of the phase inversion mechanism will require in-depth on molecular scale studies at initial phase of phase inversion phenomena. The current study used MD simulation to better understand the phase inversion mechanism underlying this ISM and its macro/microscopic properties. Such an experiment was used and discussed in conjunction with the multiple results from previous study, including the matrix formation and *in-vitro* drug release results as discuss in section 3.4-3.5 and 3.7. Through the use of computational analysis and MD simulation, it was possible to determine the fundamental process to understanding phase inversion mechanism and its relevance mechanisms.

4.2.1. Development of computational dynamics modeling for borneol-based ISM

The aim of this study was to investigate the phase inversion mechanism of borneol ISM with doxycycline dissolved in triacetin and NMP (F1-F4), specifically focusing on the initial moments after contact with water. The models of F1-F4 were rearranged, simulated, and analyzed. Figures 49(A)-(D) illustrate that the components in the model boxes mixed evenly within 200 ns of simulation time, indicating that all formulations exhibited homogenous formulation. This aligns with experimental results showing that doxycycline dissolved and mixed well with other components in all formulations. Furthermore, Figure 49(E) demonstrates that F1 equilibrated after 30 ns and maintained at a level of 73 Å, while F2-F4 reached equilibrium after 125 ns and remained constant at 70, 68, and 55 Å, respectively. It is notable that F2-F4 took longer to equilibrate than F1, possibly due to the interference of borneol molecules in the system. Moreover, the root-mean-square deviation of atomic positions (RMSD) of F4 at equilibrium was much lower than those of F1-F3 due to the higher amount of triacetin and the higher viscosity of F4.

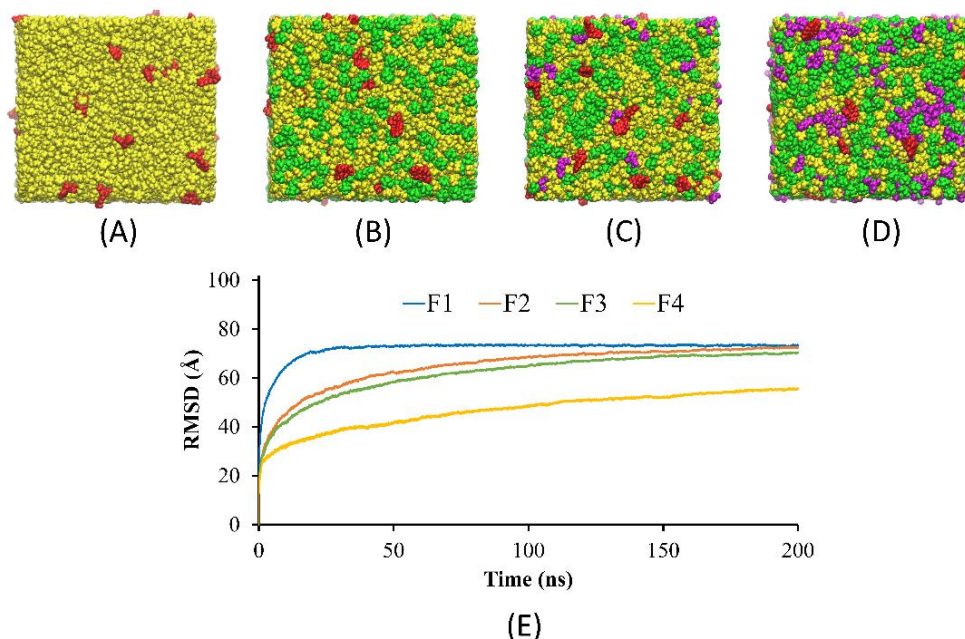


Figure 49. Molecular modeling of (A) F1, (B) F2, (C) F3, and (D) F4 after 200 ns running in MD simulation (red, green, violet, and yellow molecules represent doxycycline, borneol, triacetin, and NMP molecules, respectively), and (E) the RMSD of F1-F4.

4.2.2. MD simulation of borneol-based ISM upon contact with water

To observe the phase inversion of borneol ISM during the diffusion of water, dynamic simulations of F1-F4 with water were conducted for 200 ns. Snapshots at different time points (from 0 to 200 ns) of F1-F4 after contact with water were displayed in Figure 50, and their RMSD values were shown in Figure 51(A). For the borneol-free formulation (F1), water penetration occurred rapidly within 5 ns, and NMP mixed well with water. The RMSD curve of F1 with water reached equilibrium at about 35 ns and remained at 78 Å (Figure 51(A)). The absolute phase inversion, during which the drug freely migrated, occurred suddenly for F1 after contact with water due to the lack of a matrix former in the formulation. In the case of F2, water mixed well with NMP within 15 ns, and its RMSD became constant at 78 Å after 90 ns. Similarly, water in the system of F3 mixed well with NMP within 20 ns, and F3 equilibrated at 78 Å after 125 ns. The phase inversion of F4 occurred within 100 ns, and the RMSD of F4 with water increased slowly, reaching a steady value of 55 Å in about 180 ns. It is evident that F4 with water required the highest simulation time to reach equilibrium, followed by F3, F2, and F1, implying that borneol and triacetin could slow down the phase inversion of the ISM formulation. The rate of water infiltration decreased with the increased quantity of triacetin. Moreover, borneol, employed as a matrix former in this study, clearly demonstrated a slower matrix formation, resulting in the retardation of migration between water and doxycycline molecules, enabling controlled drug release. Additionally, triacetin, a

hydrophobic substance used as an additive solvent, contributed to interrupting the phase inversion, aligning with the results obtained from previous experiments (sections 3.4-3.5 and 3.7).

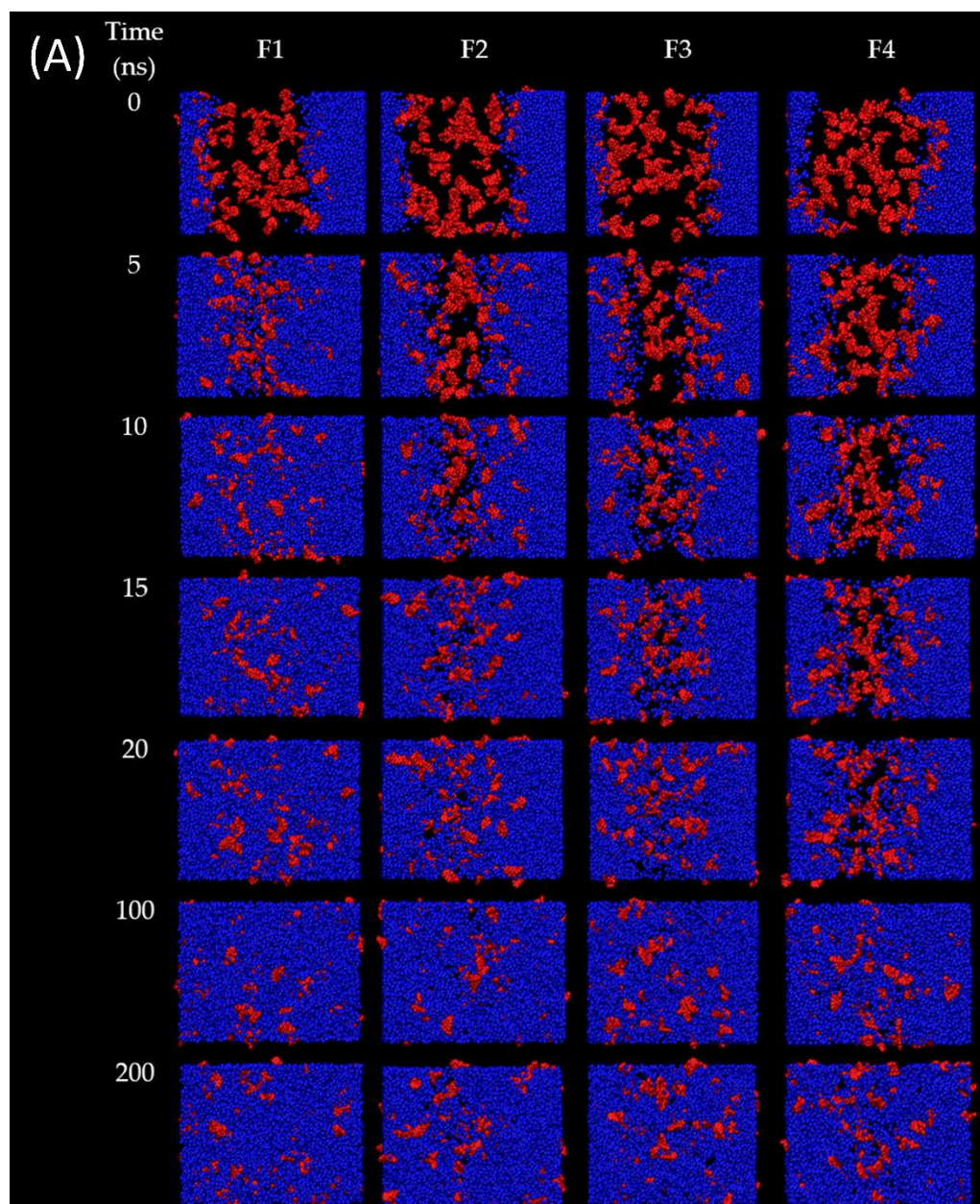


Figure 50. MD of phase inversion mechanism focusses on: **(A)** doxycycline and water; **(B)** doxycycline, borneol, and triacetin; **(C)** NMP and water from MD simulation of formulation box upon contact with water box (red, green, violet, and orange molecules represent doxycycline, borneol, triacetin, and NMP molecules, respectively)

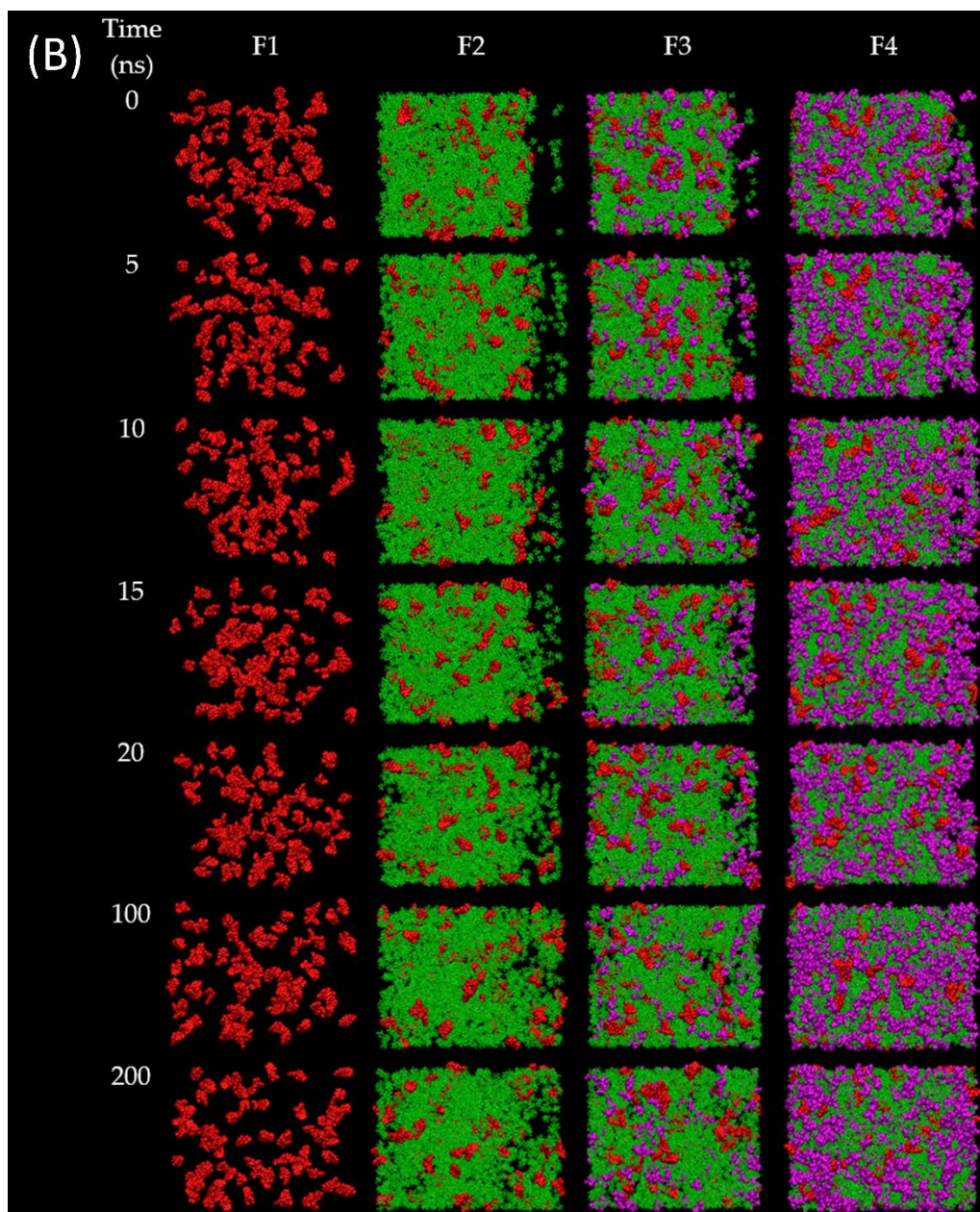


Figure 50. MD of phase inversion mechanism focusses on: **(A)** doxycycline and water; **(B)** doxycycline, borneol, and triacetin; **(C)** NMP and water from MD simulation of formulation box upon contact with water box (red, green, violet, and orange molecules represent doxycycline, borneol, triacetin, and NMP molecules, respectively) (continued)

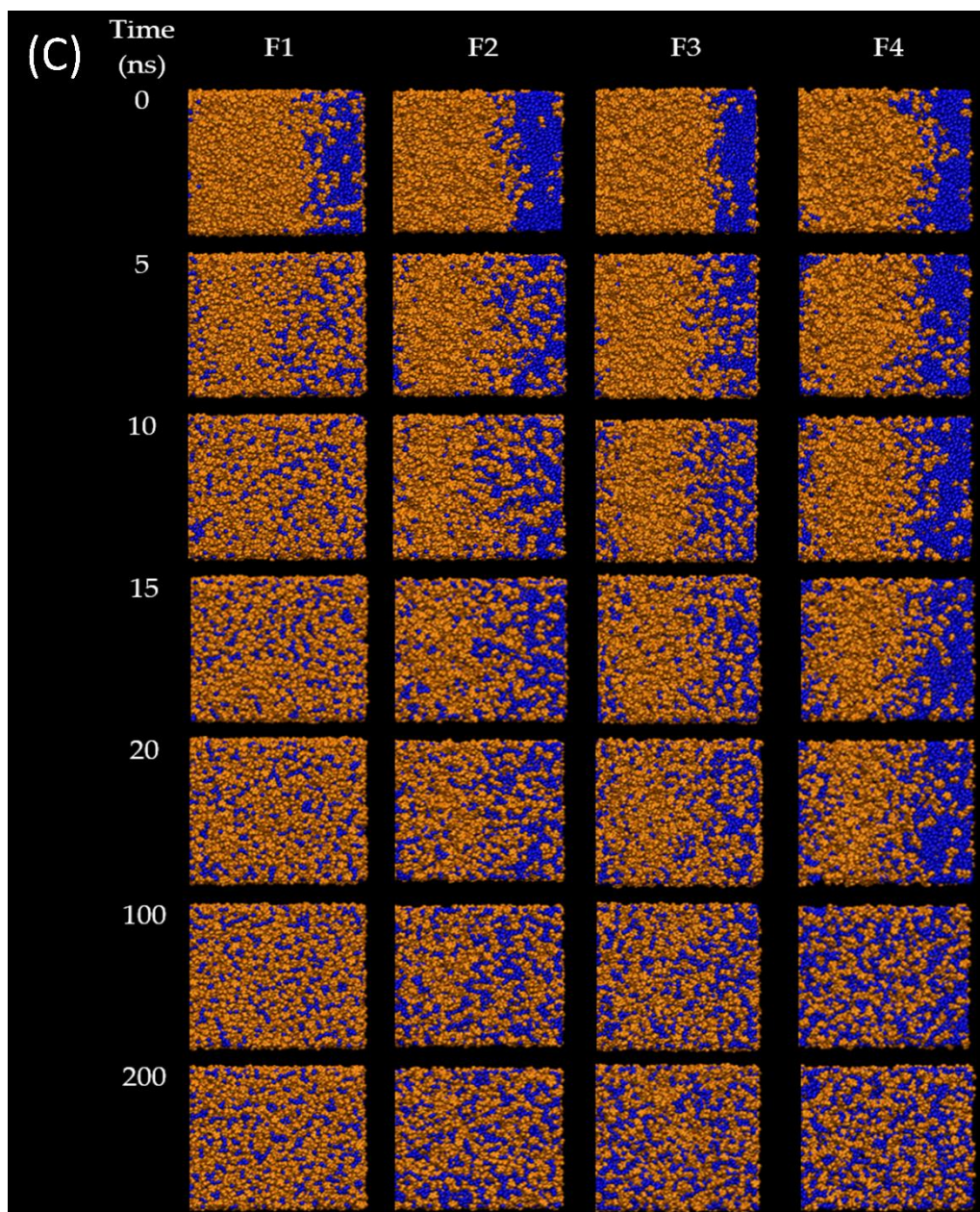


Figure 50. MD of phase inversion mechanism focusses on: **(A)** doxycycline and water; **(B)** doxycycline, borneol, and triacetin; **(C)** NMP and water from MD simulation of formulation box upon contact with water box (red, green, violet, and orange molecules represent doxycycline, borneol, triacetin, and NMP molecules, respectively) (continued)

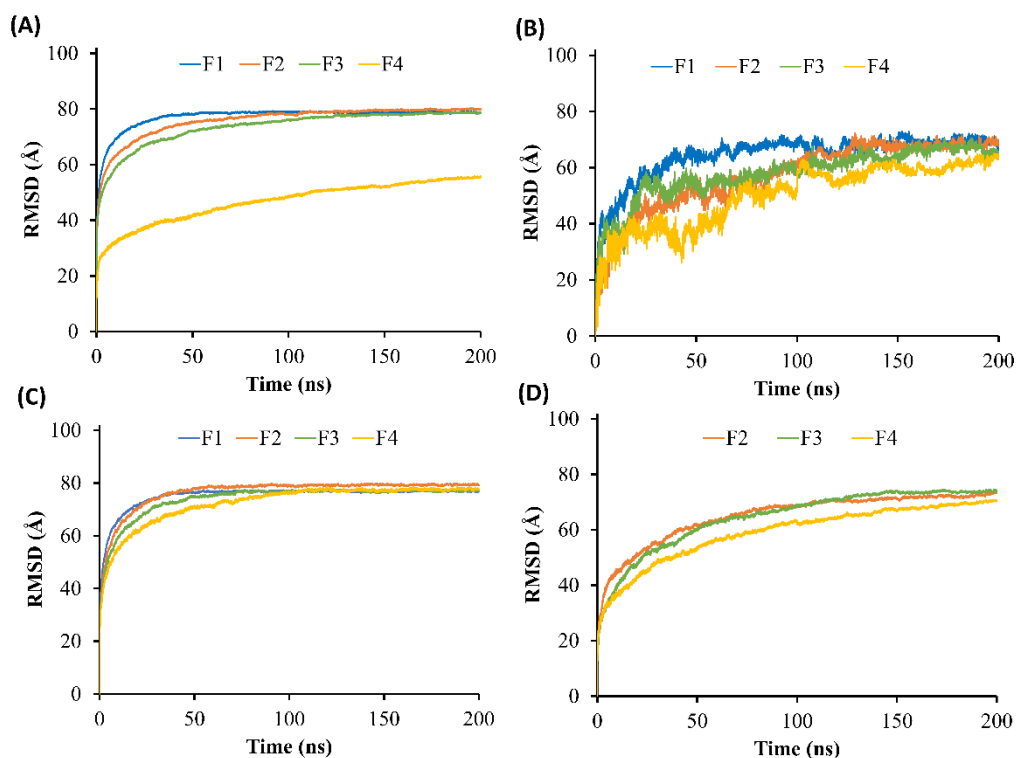


Figure 51. (A) The RMSD of all compartments of F1-F4, (B) RMSD of doxycycline conformation, (C) RMSD of NMP conformation, and (D) RMSD of borneol conformation relative to the starting point of formulation box contact with water.

4.2.3. Atomic position change and diffusion rate of each substance in borneol-based ISM

The RMSD of doxycycline in the simulation of F1-F4 models after contact with water was analyzed to observe the topography change over time. Figure 51(B) illustrates the RMSD curves of doxycycline in each system. In the case of F4, the RMSD curve increased and fluctuated during the first 125 ns, stabilizing at 60 Å after 160 ns. Other ISM (F1-F3) with water exhibited higher RMSD compared to F4 at the same observed time. Although doxycycline in F1-F3 with water models reached equilibrium at a similar value of 69 Å, their duration times to reach equilibrium were different. Doxycycline in F1 with water reached equilibrium in only 60 ns, followed by F2 and F3 (125 and 155 ns, respectively). A low and slowly increasing value of the RMSD reflects a tiny positional change of molecules from the starting point compared to a high value of the RMSD. This indicates that the addition of borneol and triacetin in the ISM influenced the duration to reach equilibrium of doxycycline, demonstrating their direct effect on drug retardation. Additionally, the proportion of triacetin incorporated in ISM also affected the positional change of doxycycline molecules.

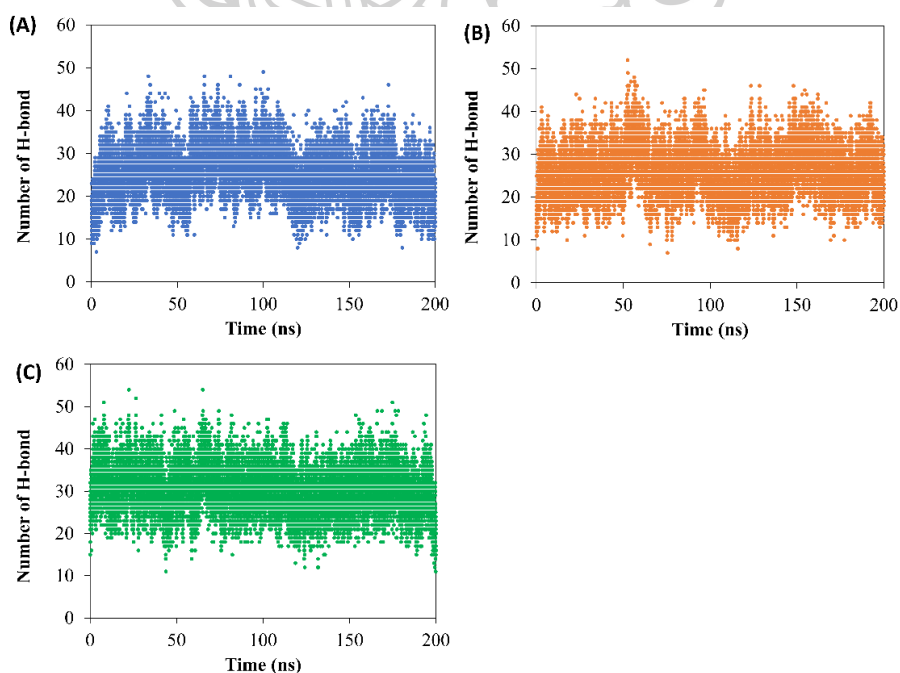
Normally, water molecules permeate the formulation upon its contact with the crevicular fluid in the crevicular pocket. Simultaneously, the organic solvent within the formulation diffuses out into the environment, a phenomenon recognized as solvent exchange [63, 183]. Hence, it was crucial to examine the movement of NMP molecules, the organic solvents in this study, to validate and comprehend the phase inversion phenomena. The RMSD analysis of NMP conformation is depicted in Figure 51(C). The curve for F4 reached equilibrium at 77 Å after 110 ns, while those of F2 and F3 stabilized after 60 and 75 ns, respectively. Borneol and triacetin not only slowed down the movement of the drug but also impacted the solvent's mobility within the formulation. Comparing the RMSD of doxycycline and NMP conformation revealed that the NMP molecule moved faster than doxycycline, leading to a quicker equilibrium for NMP (Figure 51(C)) due to its smaller molecular size. Although the RMSD of doxycycline and NMP conformation remained constant within 200 ns, the RMSD of borneol conformation (Figure 51(D)) exhibited a slight increase even after 200 ns. From the findings of both the current and previous experiments, it can be inferred that this phenomenon was attributed to borneol gradually forming a matrix. Some borneol molecules continued to move, gradually induced by water for matrix transformation. Particularly, the F4 formulation containing 25% triacetin showed a slowdown in solvent and water exchange, resulting in the slowest phase transformation of borneol, as depicted in Figure 51(D). Consequently, the matrix transformation of the F4 formulation occurred gradually. Moreover, the diffusion constants of relevant molecules during the initial stages of matrix formation within nanoseconds were analyzed and presented in Table 17 to substantiate the aforementioned hypothesis. It was observed that the addition of borneol and triacetin reduced the diffusion constant of doxycycline and affected the amount of water/NMP migration (Figures 50(A) and 50(C)). Additionally, the diffusion constant of NMP decreased with the addition of borneol and/or triacetin. In comparison to F1, the inhibition of borneol significantly decreased the diffusion constant of both NMP and doxycycline in F2. The addition of triacetin to F3 also lowered the diffusion constant of both NMP and doxycycline. While the addition of a hydrophobic solvent like triacetin had the effect of retarding the phase inversion, excessive triacetin addition could result in incomplete or delayed matrix formation, as it interfered with the phase inversion process. Consequently, the matrix formed slowly (as discussed in sections 3.4-3.5 and 3.7) and could not impede the solvent exchange process effectively. Therefore, the diffusion constant of NMP in F4 was slightly higher than that in F3. Furthermore, the diffusion constant of borneol decreased with the increasing amount of triacetin due to the interference of triacetin's hydrophobic nature. These results were consistent with the MD and RMSD outcomes. Additionally, the diffusion coefficient aligned with the matrix formation and drug release results, as shown in sections 3.4-3.5 and 3.7.

Table 17. Diffusion constant of each substance in formulation box contact with water.

Substance	Diffusion Constant (m ² /s)			
	F1	F2	F3	F4
Doxycycline	0.52	0.32	0.24	0.18
Borneol	-	0.60	0.51	0.41
Triacetin	-	-	0.63	0.61
NMP	2.24	1.72	1.40	1.43

4.2.4. Bond strength of borneol matrix in ISM for drug entrapment

Over the 200 ns simulation period, the VMD software was employed to assess the number of hydrogen bonds (H-bonds) formed between doxycycline and borneol molecules in F2-F4 after contact with water. The analysis considered H-bonds with a distance between the acceptor and donor atoms less than 3.5 Å at an angle less than 60° [290, 291]. Notably, the H-bond formation between borneol and doxycycline (Figure 52) in F4 exhibited the highest trend value in the range of 20-40, surpassing those of the other two formulations that showed H-bond values in the range of 10-30. This suggests that in F4, borneol had not yet self-aggregated, allowing for greater availability for H-bond formation with doxycycline compared to the other two formulations. This indirectly supports the notion that the borneol matrix of F2 and F3 was better formed than that of F4. These simulations provide additional confirmation of the previous results regarding matrix formation from both macro and microscopic level studies [128-130, 145].

**Figure 52.** Change in number of hydrogen bonds between doxycycline and borneol molecule of (A) F2; (B) F3; and (C) F4 after formulation box contact with water.

According to the simulation results (MD), the addition of borneol and/or triacetin in ISM had an effect on the retardation of both doxycycline and NMP migration. The results of RMSD of NMP (Figure 51(C)), RMSD of borneol (Figure 51(D)) and the decrease of diffusion constant of borneol (Table 17.) upon the addition of borneol and/or triacetin suggested that matrices self-assembly of F2 and F3 were faster than that of F4. In addition, the change in number of hydrogen bonds between doxycycline and borneol molecule of F2-F4 indicated that the formation of borneol matrix of F2 and F3 was better than that of F4. All results from MD simulation, self-formation ability, interfacial network formation and *in vitro* release of ISMs support each other and also conclude that F3 containing both borneol and 5% triacetin could be the promising *in situ* forming system for drug delivery system due to the proper duration of self-assembly and its outstanding drug release prolongation.

These findings align with the macroscopic-level experiments conducted, including observations on matrix formation after injection, morphology of the matrix, and *in vitro* drug release. The augmentation of triacetin led to fewer topographical changes during self-assembly. Therefore, computer simulations provide valuable insights into the potential outcomes of various experiments. Consequently, the *in-situ* formation process at both macro and micro levels has been elucidated. Initially, when water and the system interacted, a mixed water/solvent region formed due to the penetration of water into the system and solvent into the water phase. Subsequently, the initial nucleation of matrix-forming agents, such as borneol, occurred in the high-water-ratio area, where the presence of hydrophobic liquid, such as triacetin, influenced the water ratio and diminished water diffusion. This led to different nucleation origins and matrix growth rates. At the molecular level, the movement of drug and NMP depended on the triacetin ratio, as both sought to move out from the matrix region. Borneol mass growth continued, resulting in complex/non-complex interior structures of matrices depending on the triacetin ratio and the number of nucleation origins. The drug integrated itself into the borneol matrix structure, leading to burst release for several reasons: release from the drug situated on the external site of the matrix, the aggressive movement of solvent carrying the drug, and the high-porosity and less tortuous structure of the matrix. Molecular-level simulations serve as a tool to validate the matrix formation phenomenon and elucidate the *in-situ* formation mechanism at the initial stages.

CHAPTER 5:

CONCLUSION

Given its safety, biocompatibility, biodegradability, and low aqueous solubility, borneol can serve as a matrix forming agent of non-solvent induced phase inversion-based ISM. The success of this research had resulted in the developed clotrimazole-loaded 40% w/w borneol-based ISM spray for oropharyngeal candidiasis treatment. This ISM had proper viscous enabled spraying, rapidly transformed to matrix, and effectively antifungal and antibacterial properties. The progressive development of borneol-based ISM signified an expanding scope of applications, with a particular focus on its utilization in the management of periodontitis. The VDB was a potentially effective local phase inversion-based ISM and 40% w/w borneol was indicated as a proper concentration of ISM. Whilst, DMSO proved to be an appropriate solvent for the vancomycin HCl-loaded borneol ISM because of its high injectability and lowest crystallization change of borneol precipitate from DMSO. VDB showed a sustainable drug release with a diffusion-controlled release mechanism. Moreover, it exhibited strong antimicrobial activities against various periodontal microbes. In this research, the novel contact angle testing with ISM applied to various oral porcine tissues was developed and revealed that the phase inversion was occurred upon ISM contact with porcine tissue. Additionally, the formulation development of doxycycline-incorporated 40% w/w borneol-based ISM was studied. NMP was considered as a proper main solvent whereas triacetin was applied as an additive hydrophobic solvent of this formulation to extend the drug release profile. A 5% triacetin concentration was deemed suitable for ISM, as it transformed into a solid-like matrix within 10 minutes, allowing for the longest DH release over 10 days with Fickian diffusion and demonstrating effective antimicrobial properties. Surprisingly, the incorporation of 25% triacetin in ISM resulted in a loss of control over drug release, prompting the need for further in-depth studies of the phase inversion mechanism. The use of UV-vis imaging has revealed the simultaneous occurrence of phase inversion, drug release, and matrix formation. Borneol played a significant role in retarding both solvent diffusion and DH release. The resulting matrix further influenced phase inversion and drug release. Besides, MD was also revealing the phase inversion at initial phase on a millisecond scale. The MD information can be used to explain matrix formation in terms of nucleation of matrix, drug and solvent movement leading to phase inversion phenomena and formation of a matrix for drug entrapment. Mechanistic underpinning formation of phase inversion including UV-vis imaging and MD technique was expected to serve as a bridge between the formulation development field and the analytical field, offering a direct visualization of the dynamic processes that were previously challenging to monitor. A comprehensive understanding of the mechanistic phase inversion and its associated factors can assist in predicting drug release patterns and provide essential insights for the future development of ISM formulations.

Suggestion

This research focuses on the development and preparation of borneol-based ISM, along with the evaluation of its physicochemical properties, borneol matrix morphology, drug release efficiency, and antimicrobial activity. However, it is important to note that the safety data profile of the developed ISM formulation has not undergone rigorous testing. Furthermore, all the results obtained from this research are derived from *in vitro* studies, allowing for controlled and well-planned experimentation. To enhance the accuracy and align the results more closely with real-world scenarios, future investigations should expand the scope to encompass toxicity testing, compatibility with human tissue, and *in vivo* testing. Additionally, comparing the prepared ISM with existing commercial ISM products should be considered in future studies. Regarding the UV-vis imaging experiment, a long-term investigation would contribute to a deeper understanding of the phase inversion phenomenon. Ideally, this observation should not be limited to just the initial phase but extend throughout the entire drug release period. However, the development of long-term release testing using the UV-vis imaging technique is extremely challenging due to limitations related to cuvette size, instrument CMOS size, and the potential for drug degradation when exposed to UV radiation over an extended period. On the other hand, MD simulation have their own limitations in terms of simulation timescale and computational resources. Currently, the application of MD to study phase inversion is limited to observing the initial phase on a millisecond scale. Conducting studies at longer time scales would result in significantly increased computer processing times. Nevertheless, if the opportunity arises for longer-term studies in the future, it has the potential to significantly enhance our understanding of the phase inversion phenomenon. These observations emphasize the lack of absolute perfection in any experiment, yet the unwavering commitment to improvement is the driving force that steadily advances the said experiment closer to the concept of perfection.

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APPENDICES

Appendix 1. Calibration curve of clotrimazole, vancomycin, and DH for *in vitro* drug release analysis

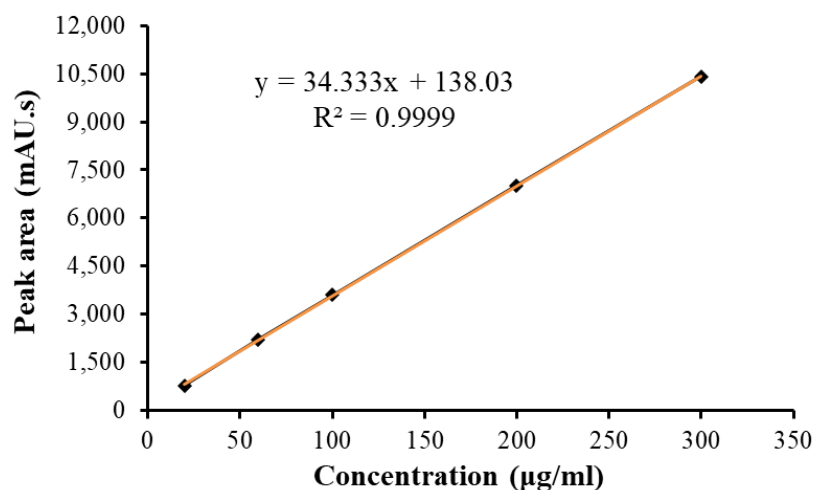


Figure 53. Calibration curve of clotrimazole in PBS pH 6.8 measured by HPLC with C18 Column; mobile phase is methanol : water = 90:10; retention time of 2.4 min and detected under wavelength of 229 nm, with $n = 3$. Curve fitting with linear equation show in orange line.

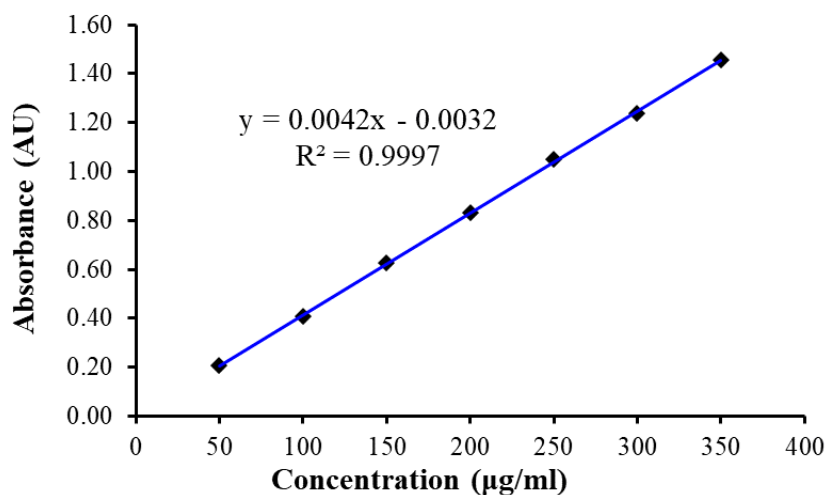


Figure 54. Calibration curve of vancomycin HCl in PBS pH 6.8 measured by UV-vis spectroscopy under wavelength of 280 nm, with $n = 3$. Curve fitting with linear equation show in blue line.

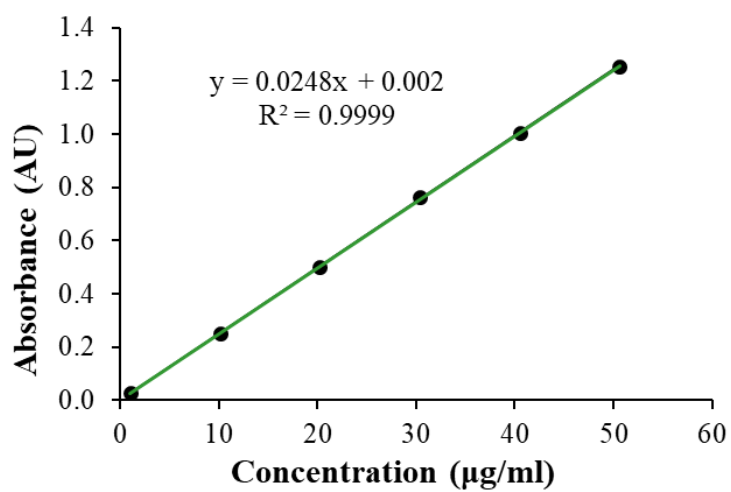
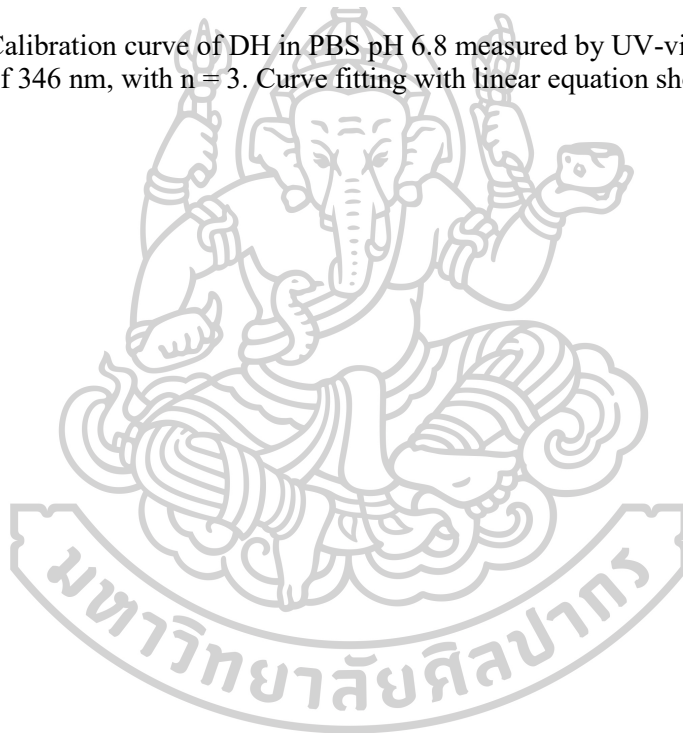


Figure 55. Calibration curve of DH in PBS pH 6.8 measured by UV-vis spectroscopy under wavelength of 346 nm, with $n = 3$. Curve fitting with linear equation show in green line.



Appendix 2. Calibration curve of DH and NMP for UV-vis imaging analysis

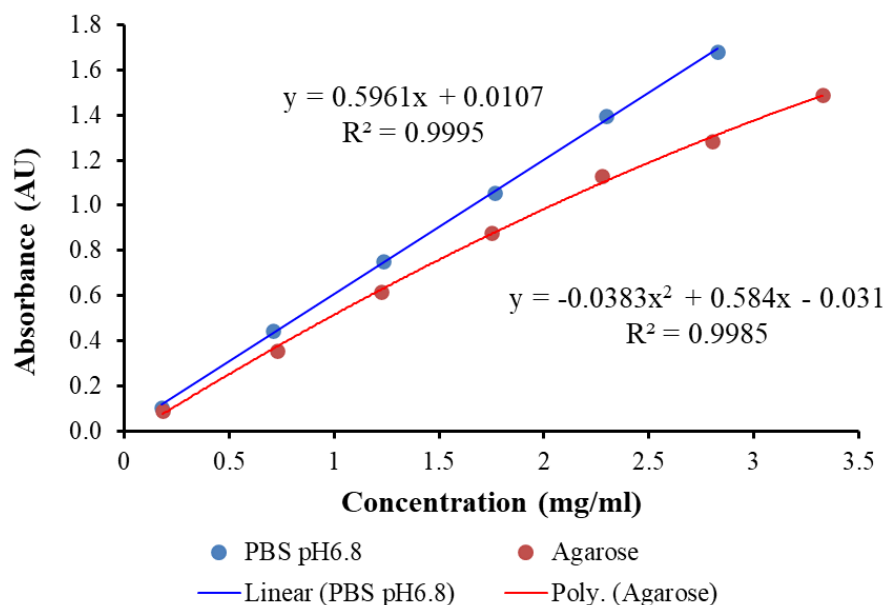


Figure 56. Calibration curve of DH in PBS pH 6.8 (**blue dot**) and agarose gel comprising PBS pH 6.8 (**red dot**) measured by D200 imaging system under wavelength of 280 nm, with $n = 3$. Curve fitting with linear equation from the absorbance data of DH in PBS pH 6.8 (**blue line**), curve fitting with polynomial equation (2nd order) from the data of DH in agarose gel comprising PBS pH 6.8 (**red line**).

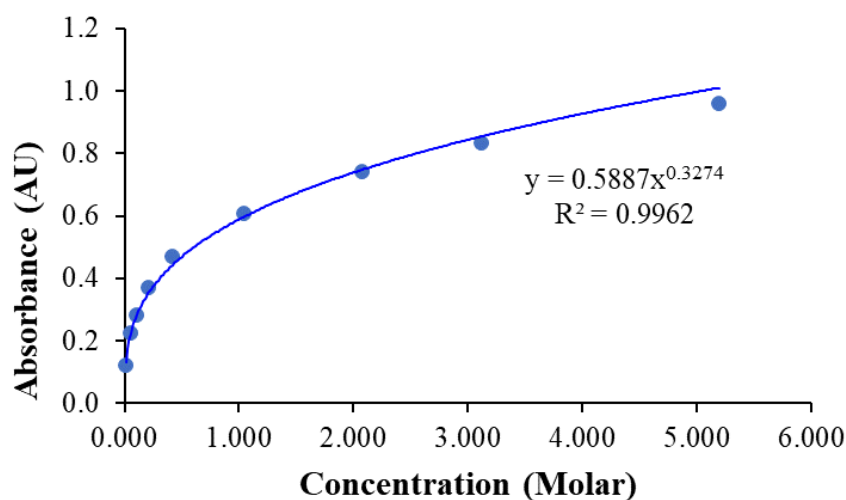


Figure 57. Calibration curve for UV-vis imaging analysis of NMP in agarose gel comprising PBS pH 6.8 measured by D200 imaging system under wavelength of 214 nm, with $n = 3$. Curve fitting with exponential equation from the absorbance data of DH in agarose gel comprising PBS pH 6.8.

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AWARD RECEIVED

2019; Best poster presentation award “Gold medal” in title: Matrix forming behavior of doxycycline hyclate-loaded beta-cyclodextrin in situ forming matrix and microparticle 2019. The international conference and exhibition on pharmaceutical sciences and technology 2019 (PST 2019), 18-19 June 2019, The Ambassador hotel, Bangkok, Thailand.

2018; Outstanding poster presentation award 2018 (together with Takron Chantadee, Nutdanai Lertsuphotvanit and Pongsathorn Issarayungyuen) in title

Emerging role of xanthan gum in triethyl citrate or glycerin-loaded porous natural rubber film. 34th International annual meeting in pharmaceutical sciences and 2nd cuFphS- RIKEN cDB Symposium "Advances in cellular and molecular biology", 8 March 2018, Arnoma hotel, Bangkok, Thailand.

2018; Best poster award (materials research) in title: Doxycycline hyclate in situ forming microparticles using beta cyclodextrin as matrix former for periodontitis treatment 2018. International conference on materials research and innovation (ICMARI) 2018, 17 December 2018, Emerald hotel, Bangkok, Thailand.

2017; Runner-up: Poster Presentation Senior Project. in title: Doxycycline hyclate-in situ forming microparticles using beta-cyclodextrin as matrix former for periodontitis treatment. Senior project contest 2017. Faculty of Pharmacy, Silpakorn University.

2016; Award for outstanding student activity: Head of quality assurance of Pharmacy student association. Pra-Chandra-Phet award 2016. Division of student affair, Silpakorn University.

