CHAPTER 1
INTRODUCTION

1.1 Statement and significance of the research problem

In situ forming gel systems are particularly attractive for the delivery of drugs into the periodontal pocket for periodontitis treatment. This administration can be easily and rapidly carried out, without pain, by using the proper needle and syringe. For solvent exchange-induced *in situ* forming gel**,** the aqueous insoluble polymer and drug are initially dissolved in a water-miscible organic solvent with low toxicity, such as *N*-methyl-2-pyrrolidone (NMP) or dimethyl sulfoxide (DMSO). *In situ* forming gel is the sol form of polymer that transforms into gel form after administration into the specific site of body (Jigar, 2011). There are many advantages of the *in situ* forming system over the conventional formula. The *in situ* forming gel is easy to administer by local delivery, reduce dose and frequency of administration, prolong action, improve patient conpliance and comfort (Nimal *et al*., 2010).

In situ forming microparticle (ISM) system is injectable emulsion which internal phase contains drug and polymer solution and continuous phase has oil and stabilizer. *In situ* forming microparticle (ISM) system is an injectable emulsion which the internal phase contains drug dissolved in polymer solution, and the continuous phase contains oil comprising stabilizer. Two phases were mixed by two syringe connector before administration (Voigt et al., 2012). After injection, the inner polymer phase was changed to hard and formed *in situ* microparticle. In this research, the *in situ* forming gel was used as internal phase of *in situ* forming microparticle.

The antimicrobial agents are used to treat infection caused by bacteria by systemic or local used but the systemic antibacterial medicament causes the tendency of side effect and drug resistant (Slots and Rams, 1990; Schwach *et al*., 2000). On the other hand the local antibiotic agents decrease the side effect and drug resistant (Hoppentocht *et al*., 2013). Teeth are the significant organ for chewing food, talking and beauty. Diseases such as caries, gingivitis and periodontitis causes of lose the

tooth. Gingivitis is a non-destructive periodontal disease which occurred by bacterial invasion. In the absence of treatment, gingitivitis may progress to periodontitis, which is a destructive form of periodontal disease. Periodontitis is a set of inflammatory disease. The periodontium and the supporting collagen are degenerated and then forming a 'periodontal pocket' (Iqbal *et al*., 2008). Periodontitis may be progressive loss of the teeth. The periodontal pocket is gap between the gingival and tooth and the gap has ideal conditions for the proliferation of microbes. The gap is normally deep between 1-2 mm, while the pocket of periodontitis usually exceeds 5 mm (Friedman and Steinberg, 1990). Periodontitis treatment begins with the removal biofilm form tooth surface. The bacterial can be eliminated by scaling and root planning or antibiotic agents. The treatment with systemic and local antibiotic has been recommended for the periodontitis. The systemic antimicrobial agent has been used such as chlorhexidine, tetracyclines, doxycycline, metronidazole, clindamycin, ofloxacin, ciprofloxacin and moxifloxacin. However the use of systemic antibiotic tended to associate with higher side effect. Therefore, the local antibiotic has been developed for periodontitis treatment which intra-pocket drug delivery system promotes the high drug concentration in the gingival cervicular fluid, lowering the side effect improvement of the drug efficacy and enhancement of patient compliance (Jain *et al*., 2008). The local drug delivery systems are such as fiber, strip, gel and film.

Solvent exchange-induced *in situ* forming gel was the system containing gelling agent dissolved in specific solvent. Gelling agent has been used in *in situ* forming gel such as PLA (Polson *et al.,* 1997) and PLGA (Maze *et al*., 1996). In this study, cholesterol and bleached shellac were used as gelling agent for *in situ* forming gel and *in situ* forming microparticle. Cholesterol is sterol and essential structure component of animal cell membrane that is importance within cells, cholesterol also serves as a precursor for the biosynthesis of steroid hormones, bile acids, and vitamin D (Hanukoglu, 1992). Shellac is the product of natural material lac which is secreted by the small parasitic insect *Kerria Lacca*. Shellac is non-toxic and physiologically harmless (Annina, 2010). This polymer has not been reported to be used in the *in situ*

forming gel or ISM. The solvents used in this research are *N*-methyl-2-pyrrolidone (NMP), dimethyl sulfoxide (DMSO), 2-pyrrolidone and eutectic material. These solvents were low toxicity and physiologically harmless. The system was loaded with two drugs individually including metronidazole and doxycycline hyclate. When the system was injected into the aqueous phase, the solvent diffused into the environment and the water diffused into the system, leading water insoluble polymer precipitation and gel formation.

The aim of this study was to develop the solvent exchange-induced *in situ* forming gel and microparticle of antimicrobial agents for periodontitis treatment. The *in situ* gel formulations was containing with gelling agent (bleached shellac and cholesterol), drugs (doxycycline hyclate and metronidazole), solvent (NMP, DMSO, 2-pyrrolidone and eutectic), additive (benzyl benzoate). The *in situ* forming microparticle was containing with gelling agent (bleached shellac), drug (doxycycline hyclate), solvent (NMP, DMSO, 2-pyrrolidone and eutectic), external phase (olive oil) and stabilizer (GMS). The prepared *in situ* systems were prepared and investigated the properties such as appearance, viscosity, rheology, syringeability, gel formation, drug release, degradation and antimicrobial activity.

1.2 Objective of this research

1) To prepare and evaluate *in situ* forming gels prepared from cholesterol and bleached shellac for periodontitis treatment

2) To evaluate the effect of drug types on physicochemical properties of *in situ* forming gels prepared from cholesterol and bleached shellac.

3) To evaluate the effect of benzyl benzoate on physicochemical properties of *in situ* forming gels prepared from cholesterol

4) To prepare and evaluate *in situ* forming microparticle prepared from bleached shellac for periodontitis treatment

5) To evaluate the effect of solvents, amount of emulsifier and phase ratio on physicochemical properties of *in situ* forming microparticle prepared from bleached shellac

1.3 The research hypothesis

1) *In situ* systems prepared from these gelling agent (bleached shellac and cholesterol) could form gel and control the releasing of drug

2) Benzyl benzoate affected the physicochemical properties of *in situ* forming gel prepared from cholesterol

3) Type of solvents affected the physicochemical properties of *in situ* forming gel and *in situ* forming microparticle prepared from bleached shellac

4) Amount of emulsifier affected the physicochemical properties of *in situ* forming microparticle prepared from bleached shellac

5) Phase ratio of emusion affected the physicochemical properties of *in situ* forming microparticle prepared from bleached shellac

CHAPTER 2
LITERATURE REVIEWS

2.1 Periodontal diseases

2.1.1 Introduction to periodontitis

The periodontal pocket is gap between the gingival and tooth and the gap has proper conditions for the proliferation of microbes in periodontitis. The treatment with systemic and local antibiotic has been recommended for the periodontitis. However the use of systemic antibiotic tended to associate with higher side effect. Therefore, the local antibiotic has been developed for periodontitis treatment which intra-pocket drug delivery system promotes the high drug concentration in the gingival cervicular fluid, lower side effect, improvement drug efficacy and enhancement of patient compliance. Periodontal disease affects periodontium (alveolar bone, periodontal ligament, dental cementum and gingiva) (Listgarten *et al*., 1987; Vyas *et al*., 2005) by accumulation of bacteria. More than 700 species of bacteria in the oral carvity (Cobb, 2008) that are the primary cause and initiate damage directly or indirectly by triggering host-mediated responses that lead to self-injury (Vyas *et al*., 2005). The important periodontal pathogens are *Porphyromonas gingivalis, Actinobacillus actinomycetemcomitans, Fusobacterium nucleatum, Bacteroides forsythus* and *Treponema denticola* (Slot, 2002; Jain *et* al., 2008). State of disease has traditionally been divided into two categories such as gingivitis and periodontitis (Page and Schroeder 1976). Gingivitis is a non-destructive periodontal disease but in the absence of treatment, gingitivitis may progress to periodontitis, which is a destructive form of periodontal disease. Periodontitis is a set of inflammatory disease influencing the periodontium and the supporting collagen. The periodontium is degenerated and then forming a ‘periodontal pocket’ (Iqbal *et al*., 2008). Periodontitis may be progressive loss of the teeth. The periodontal pocket is gap between the gingival and tooth and the gap has ideal conditions for the proliferation of microbes. The gap is normally deep between 1-2 mm, while the pocket of periodontitis usually exceeds 5 mm (Friedman and Steinberg, 1990).

2.1.2 Treatment

For dentistry, the treatment of periodontal disease begins with the removal biofilm form tooth surface. The bacterial can eliminate by scaling and root planning or antibiotic. The bacterial can be eliminated by scaling and root planning or antibiotic agents. The treatment with systemic and local antibiotic has been recommended for the periodontitis. The systemic antimicrobial agent has been used such as chlorhexidine, tetracyclines, doxycycline, metronidazole, clindamycin, ofloxacin, ciprofloxacin and moxifloxacin. However the use of systemic antibiotic tended to associate with higher side effect. Therefore, the local antibiotic has been developed for periodontitis treatment which intra-pocket drug delivery system promotes the high drug concentration in the gingival cervicular fluid, lower side effect, improvement drug efficacy and enhancement of patient compliance (Jain *et al*., 2008). The local drug delivery systems are such as fiber, strip, gel and film.

2.2 *In situ* forming gel systems

Practically, the injectable *in situ* forming gels are particularly attractive for the delivery of drugs into the periodontal pocket for periodontitis treatment because they are in sol form before administration into the body and gradually alter to a gel form or solid-like depot. The treatment of periodontitis by these intra-pocket antibacterial delivery systems is interesting due to the prospects of maintaining effective high levels of drug in the gingival crevicular fluid for a prolonged period of time to produce the desirable clinical benefits. *In situ* forming gel has been used in pharmaceutical field as drug delivery for dermal, nasal, ocular, oral, buccal, vaginal, rectal, implant and periodontal pocket (Kulkarni *et al*., 2012). The *in situ* forming gel is solution of polymer that transform into gel after administration into the body (Jigar, 2011). There are many advantages of the *in situ* forming system over the conventional formula. The *in situ* forming gel is easy for administration, local delivery, reducing dose and frequency of administration, prolongation of action, improvement of patient compliance and comfort (Nimal *et al*., 2010). *In situ* gel forming systems have been classified based on mechanism of gelation such as

physiological stimuli (temperature and pH), physical changes in biomaterials (solvent exchange and swelling) and chemical reactions (enzymatic, chemical and photo-initiated) (Nimal *et al*., 2010). Solvent exchange mechanism is polymer precipitation mechanism which the system composes of a water insoluble polymer dissolving in biocompatible solvent such as *N*-methyl-2-pyrrolidone (NMP) or dimethyl sulfoxide (DMSO). When this system is injected into the aqueous, the solvent diffuses into the environment and the water diffuses into the system, leading water insoluble polymer precipitates and formation into gel is occurred.

Nowadays, *in situ* forming gel was wildly used in periodontitis treatment in the research. The *in situ* forming gel with poloxamer, gellan gum and carbopol as gelling agent was contained chlorhexidine HCl as antimicrobial. The system was transformed into gel by temperature change and all system could sustain drug release for 6 h (Garala *et al*., 2013). The *in situ* forming gel with poloxamer as thermoreversible gelling agent in combination with hydroxypropylmethylcellulose as a muco-adhesive agent and containing articaine HCl as local anasthetic was use to relive pain for periodontitis. The system was studied about gelation temperature, spreadability, viscosity, mucoadhesive force strength, *in vitro* drug release, permeation study and stability test. From the result of this research the system had potential for use in periodontal pockets and could sustain drug release for 7 h (Kulkarni *et al*., 2012). The *in situ* forming gel loading moxifloxacin with gallen gum and sodium alginate based on the concept of ion activated system. The system was studied about in vitro gelling capacity, spreadability, release study, viscosity, gel strength and microbiological studies. The system was simple and easy to use, easy insertion of gel formation in to periodontal pocket and could prolong release at periodontal pocket (Kunche *et al*., 2012). The *in situ* forming gel with [poly (lactide) (PLA) and poly (lactide-co-glycolide) (PLGA)] as gelling agent containing secnidazole and doxyxycline hydrochloride could be used in treatment of periodontitis by direct periodontal intrapocket administration. The system was transformed into gel by solvent exchange (Gad *et al*., 2008).

The *in situ* forming gel system has been used as commercial product such as Atrdox®. Atridox is the *in situ* forming gel system that is administrated into the

periodontal pocket. It contains poly(DLlactide) as polymer, *N*-methyl-2-pyrrolidone as solvent and 10% doxyxycline hyclate and used for treatment of periodontal disease.

2.3 *In situ forming microparticle*

In situ forming microparticle system is injectable emulsion which internal phase contains drug and polymer solution and continuous phase has oil and stabilizer (Voigt *et al*., 2012). The internal phase of this system consists of active ingredient and polymer (such as poly(D,L-lactide-co-glycolide), poly(D,L-lactide)) dissolved in a biocompatible solvent (such as *N*-methyl-2-pyrrolidone, dimethyl sulfoxide) (Rungseevijitprapa and Bodmeier, 2009). Two phases were mixed by two syringe connector before administration (Voigt *et al*., 2012). After injection, the inner polymer phase hardens and forms to *in situ* microparticle.

Nowadays, there are many researches about *in situ* forming microparticle. The *in situ* forming microparticle with dissolving poly (lactide-co-glycolide) in NMP as internal phase and peanut oil with 2%w/w span 80 as external phase was used as injectable implant. The system was studied about viscosity, rheology and injectability compared with *in situ* forming gel. In the comparison the *in situ* forming microparticle was more easily injectable with smaller needle size thus expected to be less painful and give better patient comfort (Rungseevijitprapa and Bodmeier, 2009). The *in situ* forming microparticle with dissolving poly(D,L-lactide-co-glycolide) and poly(D,L-lactide) in NMP loading leuprolide acetate as internal phase and peanut oil containing 2%w/w span 80 and 2-2.5%w/w aluminum monosterate as external phase was used as injectable implant. The result showed the initial release was decreased when the polymer concentration was increased whereas the viscosity and amount of external phase was increased when decreased the drug loading (Luan and Bodmeier, 2006). The *in situ* forming microparticle with dissolving poly (lactide-co-glycolide) in DMSO as internal phase and vegetable oil with 5%w/w stabilizer as external phase was used as injectable implant. The goal of this study was to obtain physically stable non-aqueous *in situ* forming microparticle emulsions. From the research, the *in situ* forming microparticle emulsion containing 5%w/w glycerol monosterate as stabilizer was stable for at least 12 h (Voigt *et al*., 2012).

The *in situ* forming microparticle showed advantages over the *in situ* forming gel such as decreasing cytotoxicity, more reproducible, minimizing burst release and better injectability because the drug and solvent did not directly contact with cell and the external phase (oil) performed as lubricant (Luan and Bodmeier, 2006).

2.4 Formulation parameters

2.4.1 Gelling agent

2.4.1.1 Cholesterol

Cholesterol is sterol and essential structure component of animal cell membranes that is importance within cells. Cholesterol also serves as a precursor for the biosynthesis of steroid hormones, bile acids, and vitamin D. Cholesterol is thus considered within the class of lipid molecules and synthesized by animal, all cells in vertebrates the liver typically produces greater amounts than other cells. Cholesterol can be extracted from lanolin (lanolin is obtained by purifying wool grease, the waxy substance which coats sheep's wool). Cholesterol from lanolin has boiling point of 360^{o}C, melting point of 147-149^{o}C and density of 1.067 g/mL at 25^{o}C. Recent research uses cholesterol from lanolin in delivery antiacne drug. The cholesterol form lanolin and phosphatidylcoline from soybean were used as lipid phase in liposome. The loposome was prepared by modified ethanol injection method and rhodomyrtone was used as active ingredient (Chorachoo *et al*., 2013). Moreover, cholesterol has widely used in drug delivery system. Fibroblast growth factor fragment-conjugated cholesterol-block-poly (ethylene glycol) polymer was used as delivery system for targeting to the FGFR-overexpressing tumor cells. Micell containing paclitaxel was prepared by the polymer and was evaluated for drug release, cytotoxicity, intracellilar distribution, cellular binding and uptake study. The cholesterol-block-poly (ethylene glycol) was selected because it was biocompatible and biodegradable polymer (Cai *et al*., 2011). The cholesterol was also used in liposome. Liposome suspension was prepared using cholesterol, lecitin, vitamin E and palmitate which were dropped into *n*-trimethyl chitosan. After that the coated liposome were dispersed in poloxamer 407 solution which the obtained system was

used for ocular drug delivery system to prolong the ocular retention time and improve the bioavailability (He *et al*., 2013).

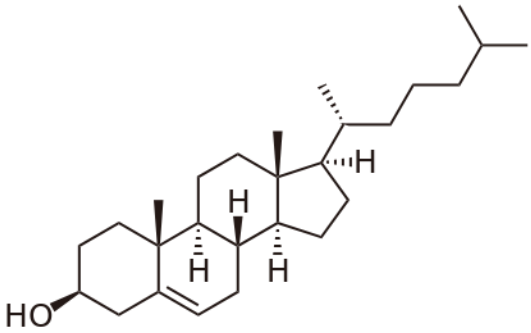


Figure 1 Chemical structure of cholesterol, $C_{27}H_{46}O$

2.4.1.2 Bleached shellac

Bleached shellac is the product of the natural material lac which is secreted by the small parasitic insect *Kerria Lacca*. Bleached shellac is non-toxic and physiologically harmless. Bleached shellac was used in pharmaceutical product such as coated tablet and pellet for control drug release and moisture barrier due to bleached shellac was low water vapor and oxygen permeability (Roda *et al*., 2007). Bleached shellac is gained by dissolution of seedlac in aqueous alkali solution followed by treatment with sodium hypochlorite. Shellac is then precipitated by addition of sulphuric acid. Solutions of shellac are almost colorless. Shellac is water insoluble but soluble in ethanol, methanol and partially soluble in ether, ethyl acetate and chloroform. Shellac can aging by self esterification. The effect of esterification is the loss solubility, decreasing acid value and enhancement of glass transition temperature. Bleached shellac should be storaged in temperature below 27^{o}C, protected from light and incorporated of antioxidant for prevention aging (Annina, 2010).

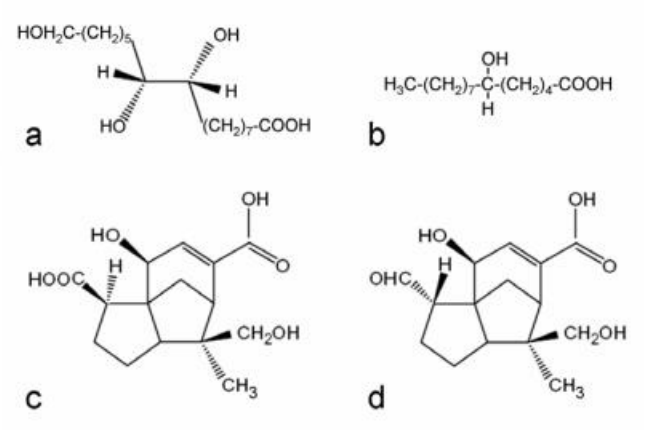


Figure 2 Main components of shellac a) aleuritic acid b) butolic acid c) shellolic acid d) jalaric acid

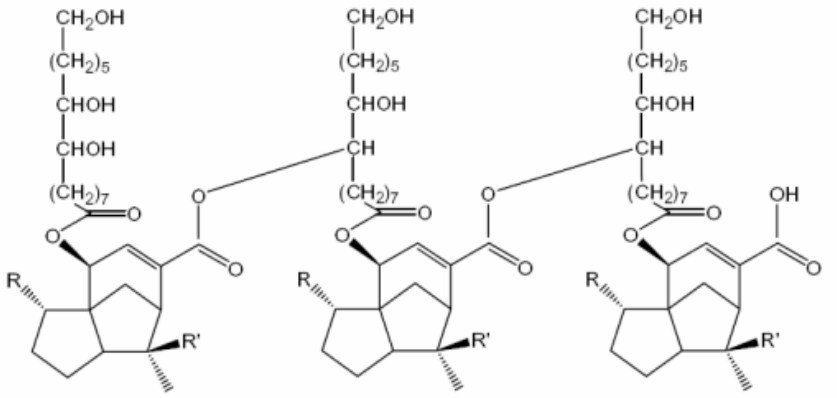


Figure 3 Chemical structures of shellac (Limmatvapirat *et al*., 2007)

2.4.2 Solvent

2.4.2.1 (*N*-methyl-2-pyrrolidone)

N-methyl-2-pyrrolidone (NMP) is a liquid also called by several other names such as 1-methyl-2-pyrrolidone or *M*-pyrrole (C_2H_9NO) is a chemical compound with 5-membered lactam structure. NMP has boiling point at 202-204^{o}C and melting point of -24^{o}C. NMP is a colorless to slightly yellow liquid, with a faint amine odor. It is miscible with water and conventional organic solvents such as ethyl acetate, chloroform and benzene. It has low volatility, low flammability and low toxicity (LD_{50} 3914 mg/kg in rat) (Jouyban *et al*., 2010). NMP is a biodegradable solvent therefore it is a suitable solvent in many different fields. The

chemical structure of NMP is shown in Figure 4. It has non-polar carbons, which can weaken the hydrogen-bonded structure of water, thus allowing it to act as a cosolvent (Jouyban *et al*., 2010; Liu and Venkatraman, 2012). The presence of a large planar nonpolar region can lead to hydrophobic interactions between NMP and drugs (Sanghvi, 2008). NMP is wildly used in pharmaceutical industry (Jouyban *et al*., 2010) that is a solubilizing excipient (Strickley, 2004), penetration enhancer (Godavarthy *et al*., 2009; Rachakonda *et al*., 2008). NMP was widely used in *in situ* forming gel system. There are many preparations such as the *in situ* forming gel system containing PLGA as polymer dissloved in NMP. The system transformed into gel by solvent exchange and was used as injectable implant (Kempe *et al*., 2008). Moreover, NMP was used in other pharmaceutical products such as transdermal delivery system as penetration enhancer (Seki *et al*., 1991).

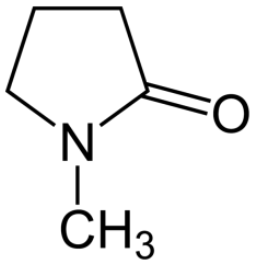


Figure 4 Chemical structure of *N*-Methyl-2-Pyrrolidone (NMP), C_5H_9NO

2.4.2.2 Dimethyl sulfoxide (DMSO)

Dimethyl sulfoxide (DMSO) is an organosulfer compound with the formula $(CH_3)_2SO$. DMSO has boiling point at 189^oC, melting point 19^oC. DMSO is a colorless liquid in room temperature. It is miscible with water and organic solvents. It has low toxicity for human administration (LD_{50} 14,500 mg/kg in rat)(Brobyn, 2012). The chemical structure of DMSO is shown in Figure 5. DMSO is use in pharmaceutical such as topically to decrease pain, speed the healing of wounds, burns, muscle and skeleton injuries moreover intravenous DMSO is used to lower

abnormally high blood pressure in the brain and treat bladder infections. In addition DMSO is use in pharmaceutical research that is a solvent in *in situ* forming implant (Parent. *et al*., 2013). And DMSO was used as solvent of *in situ* forming microparticle. The *in situ* forming microparticle with dissolving poly (lactide-co-glycolide) in DMSO as internal phase and vegetable oil with 5%w/w stabilizer as external phase was used as injectable implant. The goal of this study was to obtain the physically stable non-aqueous *in situ* forming microparticle emulsions. From the research, the *in situ* forming microparticle emulsion containing 5% glycerol monosterate as stabilizer was stable for 12 h (Voigt *et al*., 2012). Moreover, DMSO was used in other dosage forms such as transdermal delivery system as a penetration enhancer owing to its interaction with stratum corneum lipids (Anigbogu *et al*., 1995).

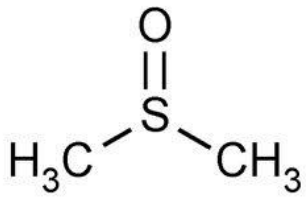


Figure 5 Chemical structure of Dimethyl sulfoxide (DMSO), C_2H_6OS

2.4.2.3 2- Pyrrolidone

2-pyrrolidone is a colorless liquid organic compound with a faint amine odor. This chemical compound is a five-membered lactam. The chemical structure of 2-pyrrolidone is shown in Figure 6. 2-Pyrrolidone has boiling point at 245^{o}C and melting point -25^{o}C. It is miscible with a wide variety of other solvents such as water, ethanol, diethyl ether, chloroform, benzene, ethyl acetate and carbon disulfide. It has low toxicity (LD_{50} 3288 mg/kg in rat), no teratogenic effect, no carcinogenicity, low acute toxicity in mammals with oral and slight irritation at the injection sites. 2-Pyrrolidone is used in pharmaceutical research such as solubilizing enhencer (Jain and Yalkowsky, 2007) and penetration enhancer on transdermal drug delivery (Sasaki *et al*., 1988). It has been reported that skin irritation is low for

pyrrolidone derivatives (Sasaki *et al*., 1990). Moreover, 2-pyrrolidone has been used in *in situ* forming gel system (Parent *et al*., 2013).

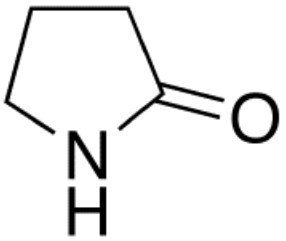


Figure 6 Chemical structure of 2-pyrrolidone, C_4H_7NO

2.4.2.4 Eutectic (menthol and camphor)

Eutectic system is two or more composition mixture that suppresses the melting point of each pure compound (Corvvis and Philippe, 2012). Eutectic is used in pharmaceutical field forincreasing drug solubility (Gohel and Nagori, 2009), absorption and permeation (Stott *et al*., 1998).

Menthol has pharmaceutical action such as local anesthetic, pain reliever, antipyretic, antiflatulent and antimicrobial activities (Bayati, 2009). Menthol at low concentration can decreases cold pain thresholds and enhances pain responses to suprathreshold noxious cold stimuli (Wasner *et al*., 2004). Menthol is a permeation enhancer (Patel *et al*., 2007). Menthol was used as enhancer to improve the skin penetration for film formulation comprising propanolol hydrochloride (Amnuaikit *et al*., 2005). Menthol has low acute toxicity for oral, injection and dermal routes (LD_{50} 3180 mg/kg in rat). Menthol has boiling point at 212^{o}C and melting point of 31-45^{o}C. The chemical structure of menthol is shown in Figure 7.

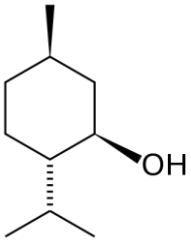


Figure 7 Chemical structure of menthol, $C_{10}H_{20}O$

Camphor has pharmaceutical action such as anesthetic, anti-inflamatory, carminative and antimicrobial activities (Shunying *et al*., 2005) and the

intramuscular injection for treatment of engorgement of the breasts has been mentioned previously (Philpott, 1929). Camphor did not show the mutagenic activity and did not induce the chromosome aberrations. Camphor has low toxicity (LD_{50} 5000 mg/kg in rat). Camphor has boiling point at 204^{o}C and melting point of 175-177^{o}C. The chemical structure of camphor is shown in Figure 8. Camphor could form the liquid eutectic at room temperature with menthol (Tuntarawongsa and Phaechamud, 2012). Camphor and menthol (5:5) was used as vehicle. The eudragit® E PO was dispersed in 1:1 camphor and menthol containing ibuprofen as drug. The system could prolong the drug release longer than 7 days (Tuntarawongsa and Phaechamud, 2012).

Figure 8 Chemical structure of camphor, $C_{10}H_{16}O$

2.4.3 External phase

2.4.3.1 Olive oil

Olive oil has been commonly used in cooking, skin care, ointment, soap and pharmaceuticals. Olive oil is emollient which has anti-inflammatory properties. Moreover olive oil has been used for treat burns, bruises, insect bites, itch and sensitive skin (Price *et al*., 1999). Olive oil has antimicrobial activity against bacteria, fungi and mycoplasma. Besides, olive oil has antioxidant activity from phenolic compound, hydroxytyrosol and oleuropein (Visioli *et al*., 1998; Visioli *et al*., 2000). Olive oil contains oleocanthal that shows a similar pharmacological activity to the non-steroidal anti-inflammatory drug. Though the structure of olive oil and NSAIDs dissimilar both molecules inhibit the same cyclooxygenase enzymes in the prostaglandin-bosynthesis pathway (Gary *et al*., 2005; Yamada *et al*., 2008). The research showed that olive has been used as oil base in emulsion for injection in neonates (Webb *et al*., 2008). The chemical structure of olive oil is shown in Figure 9.

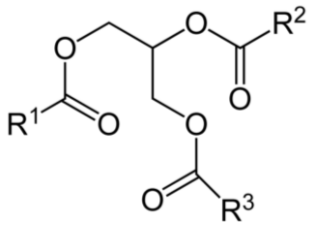


Figure 9 Chemical structure of olive oil, R^1, R^2 and R^3 are alkyl group (20%) or alkenyl group (80%).

2.4.4 Stabilizer

2.4.4.1 Glycerol monosterate

Glycerol monosterate (GMS) is an organic molecule which was colorless, odorless and sweet-testing flaky powder. Glycerol monosterate has been used in food additive, cosmetic and controlled release agent in pharmaceuticals. Glycerol monosterate is a lipophilic non-ionic surfactant with HLB 3.6-4.2. Glycerol monosterate could improve the physical stability and injectability of *in situ* PLGA microparticle forming emulsion. The *in situ* forming microparticle was stable for 12 h when poly (lactide-co-glycolide) was dissloved in DMSO as internal phase and vegetable oil with 5%w/w glycerol monosterate as stabilizer (Voigt *et al*., 2012).

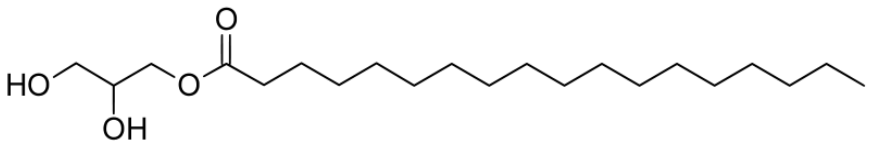


Figure 10 Chemical structure of glycerol monosterate, $C_{21}H_{42}O_4$

2.4.5 Drugs

2.4.5.1 Metronidazole

Metronidazole is a nitroimidazole antibiotic against anaerobic bacteria by a reduction reaction, depriving the organism of required reduction equivalents. The mechanism of action of metronidazole is thought to be due to intermediate or final products of reduction of the nitro-group of metronidazole and the toxic effect of the reduced intermediates binding to DNA leading to loss of helical structure, strand breakage and impairment of DNA function (Freeman *et al*., 1997; Rizzo *et al*., 2010). Metronidazole has been used off label in periodontal therapy such as metronidazole 250 mg 3 times daily plus amoxicillin 375 mg twice daily for 2 week (Berglundh *et al*., 1998). The chemical structure of metronidazole is shown in Figure 11. Metronidazole has a high solubility in water (10.61 mg/mL) (Martino *et* al., 2007). Metronidazole was loaded in composite poly-ε-caprolactone of alginate ring for dental implant. The ring exhibited the sustained release of drug profile longer than 4 weeks. The rings can be designed to fit around the body of any root form dental implants of various diameters, shapes and sizes (Lan *et al*., 2013).

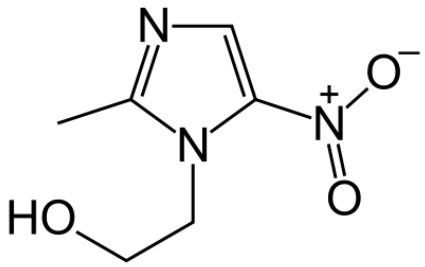


Figure 11 Chemical structure of metronidazole, $C_6H_9N_3O_3$

2.4.5.2 Doxycycline hyclate

Doxycycline hyclate is a broad-spectrum tetracycline antibiotic. It enters through porins in Gram-negative bacteria, and through their lipophilicity in Gram-positive bacteria. It passes through the cytoplasmic membrane via active transport and bind with RNA on the ribosome by chelating divalent cations like magnesium (Mg^{++}), which are attached to the phosphates on RNA. However, free magnesium ions in the cytosol may chelate with the drug prior to its binding, disabling their interaction with the ribosome (Takahashi *et al*., 1986). The

doxycycline hyclate has yellow powder. The chemical structure is shown in Figure 12. Doxycycline is used in periodontal treatment because it is bacteriostatic antibiotics and also inhibits tissue collagenase activity (Yu, 1993; Levy, 1984; Seymour and Heasman, 1995). It is used 20 mg orally twice daily for up to 9 months and is used subgingival individualized dosing depended on the shape, size and number of pocket for periodontitis treatment (Polson *et al.*, 1997). In the pharmaceutical science research, doxycycline was loaded in *in situ* forming gel. The system was used for periodontitis treatment (Gad *et al.*, 2008). In addition, the *in situ* forming gel system containing doxycycline hyclate is used as commercial product such as Atrdox®.

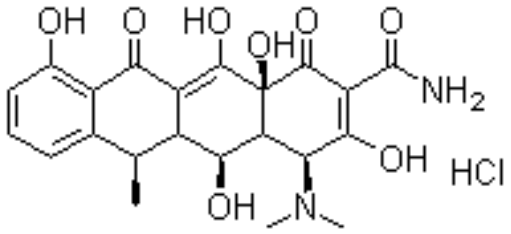


Figure 12 Chemical structure of doxycycline hyclate, $C_{22}H_{24}N_2O_8$, HCl.

2.4.6 Additive

2.4.6.1 Benzyl benzoate

Benzyl benzoate is the ester of benzyl alcohol and benzoic acid. The chemical structure of benzyl benzoate is shown in Figure 13. Benzyl benzoate has been used as a topical solution for antiparasitic insecticide (Landegren *et al*., 1979). Benzyl benzoate is a colorless liquid with faint aromatic odor. It has melting point at 18^{o}C and boiling point at 323^{o}C. Benzyl benzoate is insoluble in water but miscible in alcohol, chloroform, ether, oil and soluble in acetone and benzene. Benzyl benzoate has LD_{50} of 100 mg/kg in rat. The pharmaceutical research has been used benzyl benzoate for decreasing burst release in controlled release system such as injectable implant system. Effect of solvent on granisetron hydrochloride release was investigated for *in situ* forming implants containing 32% poly(DL-lactide-co-glycolide) 50:50, 64% solvent (benzyl benzoate) and 4% drug by weight. The drug

release of formula containing benzyl benzoate was very slow in early week and sustained for 21 days (Evren and Tamer 2008).

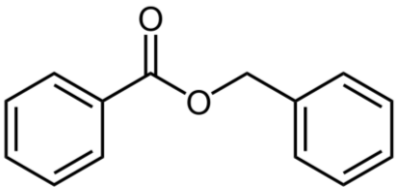


Figure 13 Chemical structure of benzyl benzoate, $C_{14}H_{12}O_2$

2.5 Rheological studies

Rheology is the study of the flow behavior of liquid and deformation of solid. The rheology can measure with rheometer or viscometer. The sample is recieved the shearing force after that it is deformed from breakage of the bond in the structure. The device can measure the viscosity of a material and the rheology behavior of material can be determined. The rheology can be divided into two groups as Newtonian behavior and non Newtonian behavior.

Newtonian behavior

Newtonian fluid is a power-law fluid with a flow index close to 1. The shear stress is directly proportional to shear rate. This behavior is typical for simple liquids such as water, mineral oil, alcohol and glycerin (Maheshwari *et al*., 2006).

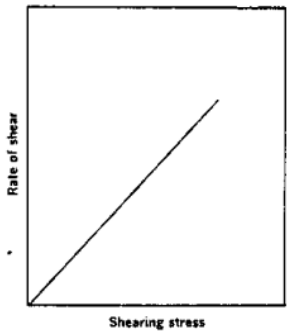


Figure 14 Newtonian behaviors. The shear stress means the applied force; the shear rate means the rate of deformation.

Non-Newtonian behavior

Non-Newtonian behavior is the flow properties which differ from Newtonian behavior. The viscosity of the most common of non-Newtonian fluid is dependent on shear rate but some non-Newtonian fluids with shear-independent viscosity, however, still exhibit normal stress-differences or other non-Newtonian behavior. The non-Newtonian behavior can divide into two groups as time independent and non time dependent (Malkin, 2013).

- **Time independent**

1. Pseudoplastic

For pseudoplastic flow a fluid's resistance flow decreases when rate of shear stress is increased because the change of particle size, the orient in flow direction, or an agglomerate dissolving. The samples of fluid display a decreasing viscosity with an increasing shear rate. Examples include suspending agent from nature such as acacia, methyl cellulose and sodium carboxymethylcellulose. This type of behavior is called shear-thinning.

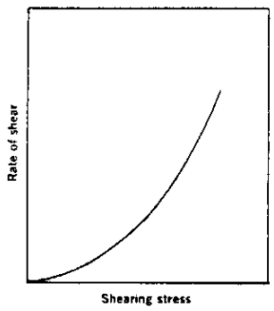


Figure 15 Non-Newtonian behaviors (Pseudoplastic flow). The shear stress means the applied force; the shear rate means the rate of deformation.

2. Dilatant

Dilatant fluid is one in which viscosity increases with the rate of shear strain. For example, the more effort you put into stirring a dilatant material, the more resistant it becomes to stirring. This is usually an indication that the applied

force is causing the material to adopt a more ordered structure, some examples including deflocculation suspension. This type of behavior is called as shear-thickening liquids.

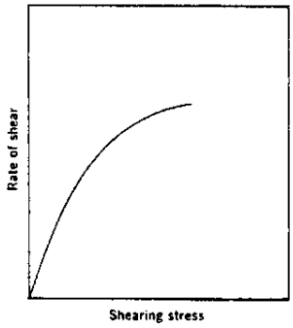


Figure 16 Non-Newtonian behaviors (Dilatant flow). The shear stress means the applied force; the shear rate means the rate of deformation.

3. Plastic (Bingham)

Plastic flow is a linear shear stress/shear strain relationship requiring a finite yield stress before they begin to flow (the plot of shear stress against shear strain does not pass through the origin). Example includes suspension that high concentration of powder.

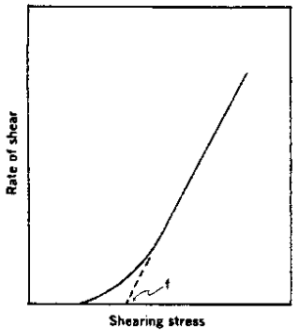


Figure 17 Non-Newtonian behaviors (Plastic flow). The shear stress means the applied force; the shear rate means the rate of deformation.

- **Time dependent**

The materials resist the shear flow and strain linearly with time when a stress is applied. The stress of elastic material is removed when it is stretched and

quickly returns to their original state. The thixotropic materials become more fluid with increasing time of the applied force, which called that “the work softening”. This system is reversible. On the other hands, the rheopectic materials become more viscous with increasing time of the applied force, which called that “the work hardending”. The Kelvin–Voigt material is a viscoelastic material having the properties both of elasticity and viscosity.

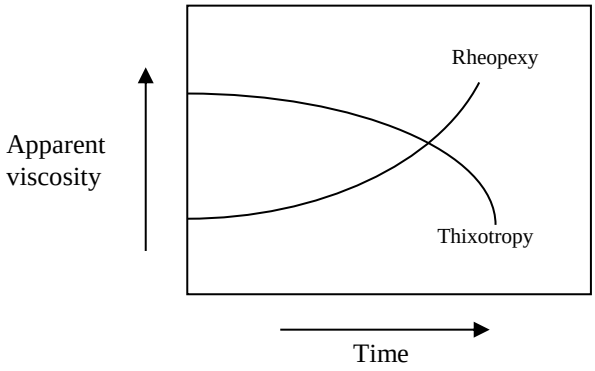


Figure 18 Viscosity and time curves of thixotropy and rheopexy (Malkin, 2013).

Rheological behavior was used as the criteria for evaluation of *in situ* forming gel and *in situ* forming implant because the injectable system should be a Newtonian or pseudoplastic flow because of its ease to injection. The *in situ* forming gel containing ethylene oxide-propylene oxide block copolymer as gelling agent and *N*-methyl-2-pyrrolidone as solvent was measured by rheometer. The flow curve of system was the pseudoplastic behavior because the up curve did not coincide with the down curve indicating the presence of thixotropy, with a hysteresis loop (Phaechamud *et al*., 2012).

2.6 Release kinetics

The kinetic models predict drug release from dosage form. The use of mathematical modeling can predict the best release kinetics of the systems for the

suitable controlled release systems. The release profiles can be divided into three types including zero order, square root time and first order release models (Baker *et al.*, 1987).

In the research, the release kinetic has been used for evaluation of system. The *in situ* forming gel containing poloxamer as polymer and articain as drug for periodontitis treatment sustained release for 7 h. The release profile was zero order kinetic (Kulkarni *et al*., 2012). The release of drug from *in situ* forming gel system containing gellan gum and sodium alginate as gelling agent was zero order kinetic (Kunche *et al*., 2012).

2.6.1 Zero order release model

Zero order release is the process that takes place at a constant rate independent of drug concentration such as rate of process does not increase when the drug concentration increases. The zero order release model is given as:

$$C = C_0 - k_0 t \qquad [1]$$

Where, C_0 is the initial drug concentration, C is drug concentration at time t, k_0 is the rate constant of zero order release, t is time

2.6.2 Square root time release model (Higuchi's model)

Higuchi described the release of a drug from an insoluble matrix by diffusion as the square root of a time-dependent process (Mukesh, *et al*., 2000). Higuchi's equation is usually desired and used as (Higuchi, 1963):

$$C = k_H \sqrt{t} \qquad [2]$$

Where, C is drug concentration at time t, k_H is the rate constant of Higuchi's, t is time

Convert into logarithmic

$$\log C = \log k_H + \tfrac{1}{2} \log t \qquad [3]$$

Where, C is drug concentration at time t, k_H is the rate constant of Higuchi's, t is time

$$C = [D\varepsilon/ \tau (2A- \varepsilon C_s) C_s t]^{1/2} \quad [4]$$

Where, C is weight of drug release (g), D is diffusion coefficient, ε is porosity of the matrix, τ is tortuosity of matrix, C_s is solubility of drug in the release medium and A is concentration of drug in the tablet.

2.6.3 First order release model

First order release is the process that is directly proportional to the drug concentration involving in process such as rate of process increases linearly when increase the drug concentration. The first order release model is given as:

$$C = C_0 e^{-k_1 t} \quad [5]$$

Where, C_0 is the initial drug concentration, C is drug concentration at time t, k_1 is the rate constant of first order release, t is time

Convert into logarithmic

$$\log C = \log C_0 - \frac{k_1 t}{2.303} \quad [6]$$

2.7 Kosmeyer-Peppas model

The release mechanism of the polymer can predict using the semi-empirical equation of Kosmeyer-Peppas model (Power law equation). The fractional release of drug is exponentially related to release time, given below (Dash *et al*, 2010).

$$\frac{M_t}{M_\infty} = Kt^n \quad [7]$$

Where, M_t and M_∞ are the cumulative drug release at time, k is kinetic constant and n is release exponent (Peppas, 1985).

The n value can characterize the release mechanism as shown in Table 1.

Table 1 Interpretation of diffusional release mechanism from drug release data from thin polymer film (Peppas, 1985 and Para, 2012).

Release exponent (n)	Drug transport mechanism	Rate as a function
0.5	Fickian diffusion	$t^{1/2}$
0.5<n<1.0	Anomalous (non-Fickian) Transport	t^{n-1}
1.0	Case-II transport	Zero-order (time-independent)
n>1.0	Super case-II transport	t^{n-1}

The empirical could be modified for application to analyze drug release from sheets, cylinders, spheres, tablets and polydisperse microspheres under perfect sink conditions. In non-swellable matrices, the diffusional exponent and mechanism of drug from various swellable controlled release systems are shown in Table 2 (Siepmann and Peppas, 2001).

Table 2 Diffusional exponent and mechanisms of diffusional release from various non-swellable controlled release systems (Rittger and Peppas, 1987).

Diffusional exponent (n)			Drug release mechanism
Thin film	Cylindrical sample	Spherical sample	
0.5	0.45	0.43	Fickian diffusion
0.5<n<1.0	0.45<n<1.0	0.43<n<1.0	Anomalous (non-Fickian) transport
1.0	1.0	1.0	Case II transport

In swellable matrices, the system does not swell more than 25% of its original volume. The diffusional exponent and mechanism of drug from various

swellable controlled release systems are shown in Table 3 (Siepmann and Peppas, 2001).

Table 3 Diffusional exponent and mechanisms of drug from various swellable controlled release systems (Siepmann and Peppas, 2001).

Diffusion exponent (n)			Drug release mechanism
Thin film	Cylindrical sample	Spherical sample	
0.5	0.45	0.43	Fickian diffusion
0.5<n<1.0	0.45<n<0.89	0.43<n<0.85	Anomalous (non-Fickian) transport
1.0	0.89	0.85	Case II transport

In the research, the korsmeyer-Peppas equation was used for characterization the drug transport mechanism. The drug release from *in situ* forming gel system from 0.4% w/v gellan gum and 0.17% w/v sodium alginate as gelling agent loading moxicloxacin was fickian release mechanism and that from the system containing 2.5% w/v sodium alginate and 0.17% w/v sodium citrate loading moxicloxacin was non fickian release mechanism (Kunche *et al*., 2012).

2.8 MicroMath Scientist® for Windows

MicroMath Scientist® for Windows is a program for solving equation and fitting mathematical model including release kinetic model. The cumulative drug release (10-80%) was carried out using a nonlinear computer program. The parameters of each model in the software were T, F, K, Tl and N. The T was as time in min of drug release, F was fractional drug release, K was the constant of each model, Tl was lag time of drug release and N was the n exponent value of power law model. These parameters are shown in Table 5. The coefficient of determination (r^2) was used to indicate the degree of curve fitting and goodness-of-fit was also evaluated using the Model Selection Criterion (MicroMath Scientist Handbook, 1995 and Mahadlek, 2012), given below.

$$msc = \ln\left\{\frac{\sum_{i=1}^{n} w_i (Y_{obs_i} - \overline{Y}_{obs})^2}{\sum_{i=1}^{n} w_i (Y_{obs_i} - Y_{cal_i})^2}\right\} - \frac{2p}{n} \quad [8]$$

Where Y_{obsi} and Y_{cali} are observed and calculated values of the i-th point, respectively, and w_i is weight that applies to the i-th point, n is number of points and p is number of parameters. MicroMath Scientist® for Windows was used for solving equation and fitting mathematical model including release kinetic model. It has been reported that the research of thermal decomposition used MicroMath Scientist® for least-squares analysis (Westhuizen *et al*., 2010).

Table 4 Model files used with Scientist®

// MicroMath Scientist Model File (ZERO ORDER) IndVars: T DepVars: F Params: K,Tl F=K*(T-Tl)
// MicroMath Scientist Model File (FIRST ORDER) IndVars: T DepVars: F Params: K,Tl F=1-EXP(-K*(T-Tl))
// MicroMath Scientist Model File (HIGUCHI's) IndVars: T DepVars: F Params: K,Tl F=K*((T-Tl)^(1/2))
// MicroMath Scientist Model File (POWER LAW EXPRESSION) IndVars: T DepVars: F Params: K,TI,N F=K*((T-TI)^N)

CHAPTER 3

MATERIALS AND METHODS

3.1. Materials

2-Pyrrolidone (lot no. BCBF5715V, Fluka, Germany)

Benzyl benzoate (BB) (Bheisajpanish Co., Ltd., Thailand)

Bleached shellac (BS) (Ake shellac Co., Ltd., Lumpang, Thailand)

acid value 70-95 mg KOH/g

loss on drying less than or equal 3.5%

colour index 2

Brain Heart Infusion (BHI) (lot no. 0270845, Bacto™, USA)

Brain Heart Infusion Agar (BHA) (lot no. 0298038, Bacto™, USA)

Camphor (P.C. Drug Center Co., Ltd., Bangkok, Thailand)

Cholesterol (lot no. 1324049, Fluka, Japan)

cholesterol from lanolin

assay more than or equal 95.0%

Dialysis tube (Spectra / Por® membrane MWCO: 6,000 - 8,000, lot no. 32644, Spectrum Laboratories, Inc., CAL, USA)

Dimethyl sulfoxide (lot no. 453035, Fluka, Switzerland)

Doxycycline hyclate (DH) (Batch No. 20071121, Huashu pharmaceutical corporation, Shijiazhuang, China)

Menthol (P.C. Drug Center Co., Ltd., Bangkok, Thailand)

Metronidazole (MT) (T.MAN Pharma Ltd., Part, Bangkok, Thailand)

Mitis Salivarius Agar (MSA) (lot no. 0118681, Difco™, USA)

N-methyl-2-pyrrolidone (NMP) (lot no. A0251390, Fluka, New Jersey, USA)

Pig's gum (Nakhon Pathom's market, Amphur Meuang, Nakhon Pathom, Thailand)

Potassium dihydrogen orthophosphate (lot no. E23W60, Ajax Finechem, Australia)

Sodium hydroxide (lot no. AF 310204, Ajax Finechem, Australia)

Tryptic Soy Agar (TSA) (lot no. 7341698, Difco™ , USA)

Tryptic Soy Broth (TSB) (lot no. 8091999, Difco™ , USA)

3.2 Microbials

3.2.1 Standard microbes (Aerobic microbes)

Staphylococcus aureus ATCC 6853P

3.2.2 Anaerobic microbes

Streptococcus mutans ATCC 27175

Porphyrommonas gingivalis ATCC 33277

3.3 Equipments

Analytical balance (Sartorius model BP2100S and Sartorius model CP224S, Germany)

Anaerobic incubator (Forma Anaerobic System, Thermo Scientific, Ohio, USA)

Autoclave (Rexall model LS-2D, Rexall industries co., Ltd, Taiwan)

Brookfield viscometer DV-III ULTRA (Brookfield Engineering Laboratories.Inc., USA)

Cryo-scanning electron microscopy (Cryo-SEM) (JOEL, JSM-6010lv, Japan)

Digital camera (Samsung ST50, Korea)

Freeze dryer (Triad™ Labconco, Missouri, USA)

Hot air oven (Binder, Scientific promotion co.Ltd, Thailand)

Inverted microscope (Nikon DXM 1200, Japan)

Image frame work software (Nikon DXM 1200, Japan)

Scanning electron microscope (Maxim 200 Camscan, Cambridge, England)

Shaking incubator Model SI4 (Shel Lab, Cornelius, USA)

Syringe connector (Qosina, USA)

Texture analyzer (TA.XT plus, Charpa Techcenter, Godalming, Stable micro Systems Ltd., UK)

UV-vis spectrophotometer (Perkin-Elmer, Germany)

Water bath (Buchi Heating bath B-490, New Hampshire, USA)

3.4 Methods

The meaning of abbreviation in formula is shown in Table 5.

Table 5 Meaning of abbreviation in formula.

Abbreviation	Meaning
Cho	Cholesterol
She	Bleached shellac
M	Microparticle
B	Benzyl benzoate

3.4.1 Preparation and evaluation of the injectable *in situ* forming gel systems prepared from cholesterol containing drugs

3.4.1.1 Preparation of the *in situ* forming gel from cholesterol

In situ forming gels using cholesterol (10% w/w) as gelling agent were prepared. *N*-methyl pyrrolidone (NMP) was used as the solvent whereas menthol was used as co solvent (20% w/w). The polarity of menthol is near to cholesterol (cholesterol about 2 (Javed *et al*., 2006) and menthol about 3.95 (Workman, 2001)). The 10%w/w metronodazole or doxycycline hyclate was employed as active compounds. All substances were mixed for 20 min until a clear solution was formed. The formula containing different drugs are shown in Table 6.

Table 6 Composition formula of gels containing different drugs in NMP.

Formula	Amount (%w/w)				
	Gelling agent (Cholesterol)	Drug		Solvent (NMP)	Cosolvent (Menthol)
		Metronidazole	Doxycycline hyclate		
Cho-1	10	10	-	60	20
Cho-2	10	-	10	60	20

3.4.1.2 Evaluation of the *in situ* forming gel from cholesterol

3.4.1.2.1 Viscosity and rheological behavior studies

The viscosity and rheology of the *in situ* forming gels was determined using Brookfield DV-III Ultra programmable rheometer (Brookfield Engineering Laboratories Inc, Middleboro, MA, USA) with spindles (CP-40 and CP-52) (n=3). Viscosity parameters were collected at different shear rates with 15 seconds equilibration time at every shear rate at room temperature. It has been reported that the flow property correlated with the viscosity and the viscosity about 1×10^4 mPas was found to be adequate for a proper flow property (Maheshwari *et al*., 2006).

The flow parameters were characterized using the exponential formula (Martin, 1993):

$$F^N = \eta G \quad [9]$$

$$\text{Log } G = N \text{ Log } F - \text{Log } \eta \quad [10]$$

Where shear stress (F) is the apply force to material, shear rate (G) is the rate which a progressive shearing deformation is applied to some material, exponential constant (N) and viscosity coefficient (ŋ) (Martin, 1993).

3.4.1.2.2 Antimicrobial activity studies

3.4.1.2.2.1 Preparation of microbial inoculum

Antimicrobial activities of the samples were evaluated. *Porphyromonas gingivalis* ATCC 33277 was used in this study. Antimicrobial activities of prepared systems were determined using agar-cup diffusion method (Ji *et al.*, 2010).

Microbes were inoculated to media (Brain heart infusion broth for *P. gingivalis*) and incubated in an anaerobic incubator at 37 °C, for

48 h. The culture was adjusted its turbidity by an UV-visible spectrophotometry at 540 nm. The suspension was further diluted to provide a final inoculum density of 10^8 cfu/mL. Relationship between the tubidity and absorbance of the inoculum is presented as equation 11 (Mahadlek, 2012).

P. gingivalis : y = 0.0056 x - 0.1335 [11]

3.4.1.2.2.2 Cup diffusion method

Antimicrobial activities of prepared systems were determined using agar-cup diffusion method (Ji *et al.,* 2010). The inoculum obtained from 3.4.1.2.2.1 was swabbed and spread onto the agar plate which was further dried. The sterilized cylinder cups were carefully placed on surface of the swabbed agar. The 150 µl prepared systems were filled into the cylinder cup (the inside diameter 6 mm, outside diameter 8 mm and height 10 mm) and incubated at 37°C for 48 h. The inhibition zone demonstrated the antimicrobial activity of the formula. The tests were carried in triplicate and the mean of inhibition zone ± S.D. were calculated. The positive control was 10 µg of ampicillin disc.

3.4.1.2.3 *In vitro* drug release studies

Drug release studies were evaluated by dialysis membrane method. A dialysis tube (Spectrapor, MW cutoff: 6,000- 8,000) containing 1 g formulation was immersed in 100 mL phosphate buffer pH 6.8 (to simulate the gingival crevicular fluid) (Esposito *et al.,* 1996) at 37 °C and maintained the rotational speed at 50 rpm. 10 mL of release medium were withdrawn the release medium at time intervals of 5, 15, 30, 45, 60, 90, 120, 180, 240, 360, 480, 720, 960, 1200, 1440, 1680, 1920, 2160 and 2880 min and replaced with 10 mL of fresh medium. Amount of drug release was determined by UV-vis spectrophotometer at the wavelength 320 nm for metronidazole and 349 nm for doxycycline hyclate. All of the experiments were triplicately done, and the mean cumulative drug release ± S.D. was calculated.

The relationship between the drug concentration (µg/mL) and absorbance of these two active compounds are presented as equation 12 and 13.

$$\text{Metronidazole} : y = 0.5206x - 0.0152 \quad [12]$$

$$\text{Doxycycline hyclate} : y = 0.0244\,x + 0.0022 \quad [13]$$

3.4.1.3 Selection of the most suitable *in situ* forming gel prepared from cholesterol

The most suitable *in situ* forming gel prepared from cholesterol was selected from its good appearance, proper rheology and high antimicrobial activity. The suitable system should be a clear solution that behaved as Newtonian or pseudoplastic flow indicating its ease to be injected. In addition it should inhibit *P. gingivalis* effectively.

3.4.2 Development of the prepared injectable *in situ* forming gel systems from cholesterol containing the additive

3.4.2.1 Preparation of the *in situ* forming gel form cholesterol containing the additive

Typically the drug liberation from *in situ* forming gel mostly exhibited a burst release therefore benzyl benzoate was incorporated to attempt for minimizing this undesired release behavior. First, cholesterol was dissolved in *N*-methyl pyrrolidone (NMP) and menthol and benzyl benzoate was dissolved as additive and then doxycycline hyclate was loaded in the system. Benzyl benzoate was aimed for decreasing burst release in the research work. For the experiment, all compounds were mixed for 15-20 min and then a clear solution was obtained. The formula containing different drugs are shown in Table 7.

Table 7 Composition formula of gels containing different amounts of benzyl benzoate in *N*-methyl-2-pyrrolidone.

Formula	Amount (%w/w)				
	Gelling agent (Cholesterol)	Drug (Doxycycline hyclate)	Additive (Benzyl benzoate)	Cosolvent (Menthol)	Solvent (NMP)
ChoB-1	10	-	0	20	qs to 100
ChoB-2	10	-	10	20	qs to 100
ChoB-3	10	-	20	20	qs to 100
ChoB-4	10	-	30	20	qs to 100
ChoBD-1	10	10	0	20	qs to 100
ChoBD-2	10	10	10	20	qs to 100
ChoBD-3	10	10	20	20	qs to 100
ChoBD-4	10	10	30	20	qs to 100

3.4.2.2 Evaluation of the *in situ* forming gel form cholesterol

3.4.2.2.1 Appearances

The appearances of formulations, color and turbidity were observed by visual observation.

3.4.2.2.2 Viscosity and Rheological behavior studies

The prepared systems were evaluated for the viscosity and rheological behavior with the methods as described in 3.4.1.2.1.

3.4.2.2.3 Syringeability studies

The difficulty of injection of the injectable system was identified by syringeability (Rungseevijitprapa and Bodmeier, 2009). Syringeability was evaluated using texture analyzer (TA.XT plus, Stable Micro Systems, UK) in compression mode. The sample was filled into 1 mL syringe with 18-guage needle that was clamped with stainless stand. The 18-gauge needle was widely used in the dental field (Sato *et al*., 2012). The upper probe of the texture analyzer was moved downwards gradually at constant speed (1.0 mm s^{-1}) until it contacted with the syringe barrel base. A constant force of 0.1 N was applied to the base and the distance required to expel the content for a barrel length of 20 mm. The tripicate measurement was carried out at room temperature. Force displacement profiles were recored, which the force at distance of 10 mm were selected for analysis. The area under the resulting curve was used to determine the work of expulsion (n=3) (Kelly *et al.*, 2004; Simoes *et al*., 2012).

3.4.2.2.4 *In vitro* gel formation

Gel formation was studied by injection. The samples (0.5 mL) filled in plastic syringe were injected from 18-gauge needle into 5 m phosphate buffer solution pH 6.8, 7.0 and 7.4 in test tube (5 mL). Then the gel formation was observed visually and taken its photo on black paper by digital camera (Samsung ST50, Korea) at various times (0, 1, 5 and 30 min) (Jong *et al*., 2011).

3.4.2.2.5 *In vitro* degradability studies

Degradability studies of the prepared gels were studied by incubating in phosphate buffer solution pH 6.8. The known amount of sample (about 0.5 g) was injected into the glass bottle containing 10 mL phosphate buffer pH 6.8 and incubated in shaking bath at 37 °C with rotational speed of 50 rpm. Fresh phosphate buffer solution was replaced every week for 4 weeks to mimic the oral

cavity condition. Then the sample was dried in hot air oven at 65 °C for 24 h and kept in desiccator for 72 h before weighing (final weight) (n=3). The percentage of weight loss was calculated as following:

$$\%\ \text{Weight loss} = \frac{\text{Initial weight} - \text{Final weight}}{\text{Initial weight}}\ x\ 100 \qquad [14]$$

3.4.2.2.6 Antimicrobial activity studies

3.4.2.2.6.1 Preparation of microbial

Antimicrobial activity study was prepared by method as described previously in 3.4.1.2.2.1. Three microbes such as *S.aureus, P. gingivalis and S. mutans* were selected in this study. Relationship between the turbidity and absorbance of these two inoculums are presented as equation 15, 16 and 17 (Mahadlek, 2012).

P. gingivalis : $y = 0.0056\ x - 0.1335$ [15]

S. mutans : $y = 0.0024x - 0.1067$ [16]

S. aureus : $y = 2 \times 10^{-5}\ x + 0.0282$ [17]

3.4.2.2.6.2 Cup diffusion method

Antimicrobial activitie of prepared systems was determined using agar-cup diffusion method (Ji *et al.*, 2010). The prepared inoculated media in 3.4.2.2.6.1 was spreaded onto the agar plate (Tryptic soy agar for for *S. aureus* and Brain heart infusion agar for *P. gingivalis and S. mutans*) and dried, then the sterilized cylinder cups were carefully placed on the surface of the swabbed agar. The 150 µL prepared systems were filled into the cylinder cup (inside

diameter 6 mm outside diameter 8 mm and height 10 mm) and incubated at 37°C for 24-48 h. The antimicrobial activities were measured as the diameter (mm) of inhibition zone. The tests were carried in triplicate and the mean of inhibition zone ± S.D. was calculated. The positives control were 7.5 µg of doxycycline hyclate solution for *in situ* microparticle and 15 µg of doxycycline hyclate solution for *in situ* forming gel.

3.4.2.2.7 *In vitro* drug release studies

In vitro drug release studies were evaluated using dialysis membrane method and membrane-less diffusion method as following.

3.4.2.2.7.1 Dialysis membrane method

A dialysis tube (Spectrapor, MW cutoff: 6,000-8,000) containing 1 g gel formulation was immersed in 100 mL phosphate buffer pH 6.8 (to simulate the gingival crevicular fluid) (Esposito *et al.*, 1996) at 37 °C and maintained the rotational speed at 50 rpm. Aliquots, each of 10 mL, were withdrawn from the release medium at time intervals of 5, 15, 30, 45, 60, 90, 120, 180, 240, 360, 480, 720, 960, 1200, 1440, 1680, 1920, 2160 and 2880 min and each aliquot was replaced with 10 mL of fresh medium. The amount of samples was determined by UV-vis spectrophotometer (wavelength 349 nm). All of the experiments were triplicately done, and the mean cumulative drug release ± S.D. was calculated.

3.4.2.2.7.2 Membrane-less diffusion method

A membrane-less diffusion system was used for studying drug release from prepared *in situ* gel systems. The 0.4 g sample was added into the ceramic cup (10 mm x 12 mm) and then placed in glass bottle containing100 mL of phosphate buffer solution pH 6.8 at 37°C and maintained the rotational speed at 50 rpm. Aliquots, each of 10 mL, were withdrawn from the release

medium at time intervals of 5, 15, 30, 45, 60, 90, 120, 180, 240, 360, 480, 720, 960, 1200, 1440, 1680, 1920, 2160 and 2880 min and each aliquot was replaced by 10 mL fresh medium. The amount of samples was determined by UV-vis spectrophotometer (wavelength 349 nm). All of the experiments were triplicately done, and the mean cumulative drug release ± S.D. was calculated.

3.4.2.2.8 Analysis drug release data.

The data obtained from the *in vitro* release experiments were analyzed by a nonlinear computer programe, Scientist® for Windows, version 2.1 (MicroMath Scientific Software, SaltLake City, UT, USA). The cumulative percentage of drug release profiles were fitted with different mathematical release equations. Least square fitting the experimental dissolution data (cumulative drug release > 10% and up to 80%) to the mathematical equations (power law, zero order, first order and Higuchi's) was carried out. The coefficient of determination (r^2) and model selection criteria (msc) were used to indicate the degree of curve fitting (MicroMath Scientist Handbook, 1995 and Mahadlek, 2012).

3.4.2.2.9 Determination of surface morphology (Cryo-SEM)

A dialysis tube (Spectrapor, MW cutoff: 6,000- 8,000) containing 1 g gel formulation was immersed in 100 mL phosphate buffer pH 6.8 (to simulate the gingival crevicular fluid) (Esposito *et al.*, 1996) at 37 °C and maintained the rotational speed at 50 rpm for 7 days. The samples were then stored in liquid nitrogen and transferred into the cryo stage (Gatan, Alto 1000, UK). Samples were investigated with Cryo-scanning electron microscopy (Cryo-SEM) (JOEL, JSM-6010lv, Japan) at -140^{o}C to -185^{o}C. Then micrographs were taken. The morphology of samples was observed for the structures.

3.4.2.2.10 *In vitro* gel formation (pig's gum)

The selected samples (about 0.1 mL) filled in plastic syringe were injected from 18-gauge needle into pig's gum. Pig's gum was operated after 30 min. The gel formation was observed visually and taken photo by digital camera (Samsung ST50, Korea).

3.4.3 Preparation and evaluation of the *in situ* forming gel systems from bleached shellac

3.4.3.1 Preparation of the *in situ* forming gel from bleached shellac and different solvents

In situ forming gels were prepared using (30% w/w) bleached shellac as polymer in systems whereas *N*-methyl pyrrolidone (NMP), dimethylsulfoside (DMSO), 2-pyrrolidone and eutectic (1:1 menthol : camphor) were used as the solvent. The 10% w/w doxycycline hyclate was loaded as active compound. Each of system was mixed and stirred for 24 h then a clear solution was formed. The formula containing different types of solvent are shown in Table 8.

Table 8 Composition formula of the systems fabricated from bleached shellac containing different solvents.

Formula	Amount (%w/w)					
	Gelling agent (Bleached shellac)	Drug (Doxyxycline hyclate)	Solvent			
			NMP	DMSO	2-Pyrrolidone	Eutectic
She-1	30	-	qs to 100	-	-	-
She-2	30	-	-	qs to 100	-	-
She-3	30	-	-	-	qs to 100	-
She-4	30	-	-	-	-	qs to 100
SheD-1	30	10	qs to 100	-	-	-
SheD-2	30	10	-	qs to 100	-	-
SheD-3	30	10	-	-	qs to 100	-
SheD-4	30	10	-	-	-	qs to 100

3.4.3.2 Evaluation of the *in situ* forming gel prepared from bleached shellac

The prepared systems were evaluated with the methods as described in 3.4.2.2.1 - 3.4.2.2.6 and 3.4.2.2.9.

3.4.3.2.7 *In vitro* drug release study

In vitro drug release study was evaluated by dialysis membrane method as described previously in 3.4.2.2.7.1.

3.4.3.2.8 Determination of surface morphology

3.4.3.2.8.1 Scanning electron microscope (SEM)

Samples were determined after the release studies under conditions identical to those described above in 3.4.2.2.8 and then they were dried using the freeze dryer for 48 h in order to avoid the collapse of porous structures. Samples were coated with gold prior to examine by scanning electron microscope (SEM). The surface and cross-sectional morphology of the dried samples were determined. Micrographs were taken with SEM at an accelerating voltage of 15 kV. The morphology of samples was observed for the porous structures, surface structure and drug crystalline.

3.4.3.2.8.2 Cryo-scanning electron microscope (Cryo-SEM)

Surface morphology was evaluated by Cryo-SEM as described previously in 3.4.2.2.10.

3.4.4 Preparation and evaluation of the ISM containing bleached shellac

3.4.4.1 Preparation of the ISM form bleached shellac

The internal phase of ISM was prepared with the same method of *in situ* forming gel in 3.4.3.1 and external phase was prepared by mixing between olive oil and glyceryl monosterate (GMS). GMS amount and internal/external phase ratio were varied. The prepared two phases were mixed by back-and-forth movement of the syringe plungers of 50 cycles in a two-syringe/connector system. The formula containing different solvents are shown in Table 9.

Table 9 Composition formula of the ISM prepared from bleached shellac using different solvents.

Formula	Amount (%w/w)							
	Internal phase						External phase	
	Gelling agent (Bleached shellac)	Drug (Doxycycline hyclate)	Solvent				Olive oil	GMS
			NMP	DMSO	2-pyrrolidone	Eutectic		
MShe-1	15	-	qs to 100	-	-	-	47.5	2.5
MShe-2	15	-	-	qs to 100	-	-	47.5	2.5
MShe-3	15	-	-	-	qs to 100	-	47.5	2.5
MShe-4	15	-	-	-	-	qs to 100	47.5	2.5
MSheD-1	15	5	qs to 100	-	-	-	47.5	2.5
MSheD-2	15	5	-	qs to 100	-	-	47.5	2.5
MSheD-3	15	5	-	-	qs to 100	-	47.5	2.5
MSheD-4	15	5	-	-	-	qs to 100	47.5	2.5

3.4.4.2 Evaluation of the ISM prepared form bleached shellac

3.4.4.2.1 Stability studies of emulsion

The system was increased amount of GMS from 0-5% and increased the ratio of external phase and internal phase. All system was stability

studied of emulsion by syring method and the suitable system was stability studied by microscopic method.

3.4.4.2.1.1 Syringe method

The phase separation of systems after mixing two phases together was determined by storing them in 1 mL syringes vertically. The separation of oil phase and creaming phase was measured. The distance of emulsion that excluded from oil phase was recorded in 0, 1, 5 and 30 min (n=3).

3.4.4.2.1.2 Microscope method

Stability studies were evaluated by inverted microscope and image frame work software from Japan. The obtained mixture was dropped onto glass slide and taken photo at different time intervals. The picture was analyzed by image frame work software for droplet size and droplet size distribution (n=3) (Voigt, *et al*. 2012).

3.4.4.2.2 Transformation of system from emulsion into microparticle

Transformation of system from emulsion into microparticle was evaluated under inverted microscope. The mixed component which was oil in oil (o/o) emulsion of 0.5 mL was dropped in glass slide and taken the photos every 10 sec after the phosphate buffer pH 6.8 of 0.5 mL was dropped onto the emulsion.

3.4.4.2.3 The other evaluations

The prepared gels were also evaluated with the other methods as described in 3.4.4.2.

3.4.5 Statistical analysis

All experimental measurements were collected in triplicate. Values were expressed as mean ± standard deviation (S.D.). Statistical significance of the drug release studies was examined using one-way analysis of variance (ANOVA) followed by the least significant difference (LSD) post hoc test or Duncan. The significance level was set at $p < 0.05$.

CHAPTER 4
RESULTS AND DISCUSSION

4.1 Evaluation of the injectable *in situ* forming gel system prepared from cholesterol

The gels comprising 10% metronidazole were clear and colorless whereas the gels comprising 10% doxycycline hyclate were clear and yellowish. The gel systems were easy to prepare because the substance could dissolve in NMP within 15 min.

4.1.1 Viscosity and rheological behavior studies

The viscosity of *in situ* forming gels prepared from cholesterol comprising doxycycline hyclate and metronidazole at 25^{o}C is shown in Figure 19. The consistency index of doxycycline hyclate and metronidazole *in situ* forming gels was not significantly different ($p<0.05$). Interestingly, the viscosity of all formula was quitely low. The low viscosity of system was good for filling it into periodontal pocket by injection. The consistency index (K is the constant valve from Herschel-Bulkley model which it indicates the viscosity of shear rate at ln γ =0) is shown in Table 10. The prepared gels exhibited Newtonian flow behavior because the consistency index was constant when the shear rate was increased.

The flow index (N value) indicates the flow type. The flow type of all formula was Newtonian because the flow index was close to 1 (Bjorn *et al*., 2012). These indicated that all formulations exhibited the Newtonian flow behavior. The flow index of gel systems is shown in Table 10. For the injectable gel, it should be the Newtonian or pseudoplastic flows to promote its ease for injection (Allmendinger *et al.*, 2014).

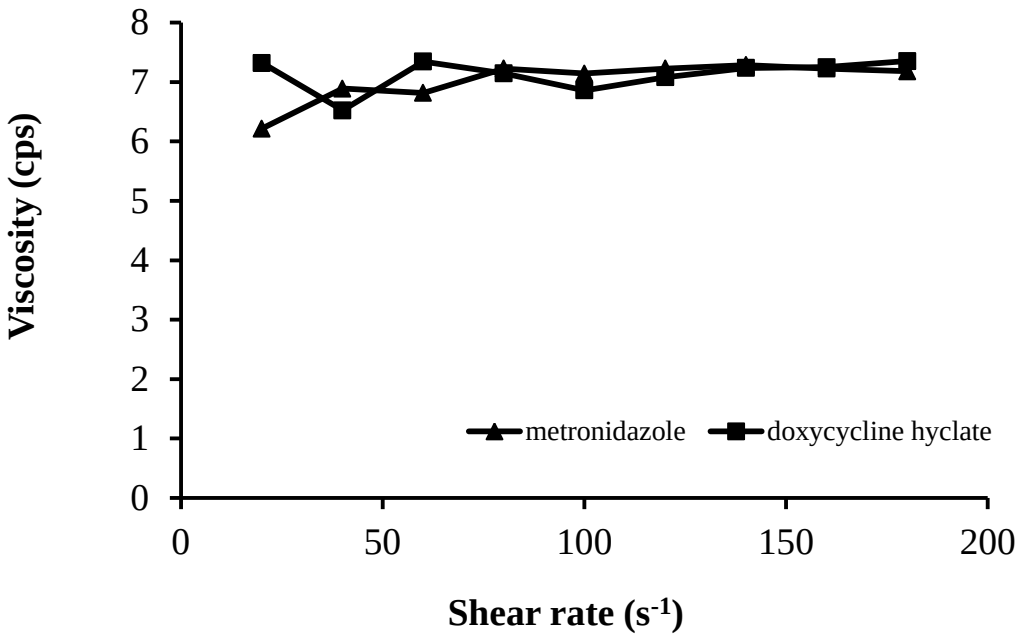


Figure 19 Shear rate-viscosity curves of *in situ* forming gel systems containing cholesterol with an addition of metronidazole (cho-1) and doxycycline hyclate (cho-2) at 25°C (n=3)

Table 10 Effect of type of drug on flow index and consistency index (n=3).

Sample	Flow index (N)	Consistency index (ŋ)
	mean ± S.D.	mean ± S.D.
Cho-1 (metronidazole)	1.07 ± 0.03	6.60 ± 0.11
Cho-2 (doxycycline hyclate)	1.05 ± 0.06	5.64 ± 1.39

Figure 20 showed the shear stress versus shear rate flow curves of *in situ* forming gels prepared from cholesterol comprising doxycycline hyclate and metronidazole at 25°C. All formula showed Newtonian flow behavior since the up curve and down curve was overlapped. For the injectable gel the rheological behavior should be a Newtonian or pseudoplastic flow for expediency of injection (Elnaggar et al., 2014).

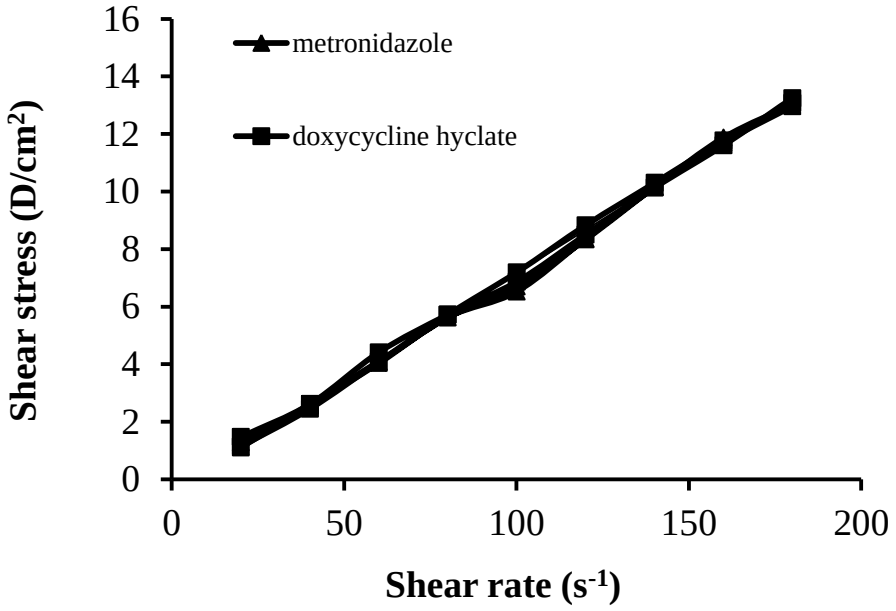


Figure 20 Flow curves of *in situ* forming gel systems prepared from cholesterol with an addition of metronidazole (cho-1) and doxycycline hyclate (cho-2) at 25°C (n=3)

4.1.2 Antimicrobial activity studies

The inhibition zone diameter of *in situ* forming gels containing doxycycline hyclate or metronidazole against *P. gingivalis* using agar diffusion method are shown in Figure 21. The inhibition zones of the system containing metronidazole, doxycycline hyclate or ampicillin disc (positive control) were significantly higher than that of the cholesterol gel base ($p<0.05$). The inhibition zone of the system containing doxycycline hyclate was significantly higher than that of the system containing metronidazole ($p<0.05$) because the aqueous solubility of doxycycline hyclate (50 mg/mL in water) (Wagil *et al*., 2014) was higher than metronidazole (10 mg/mL in water) (Chien, 1984).

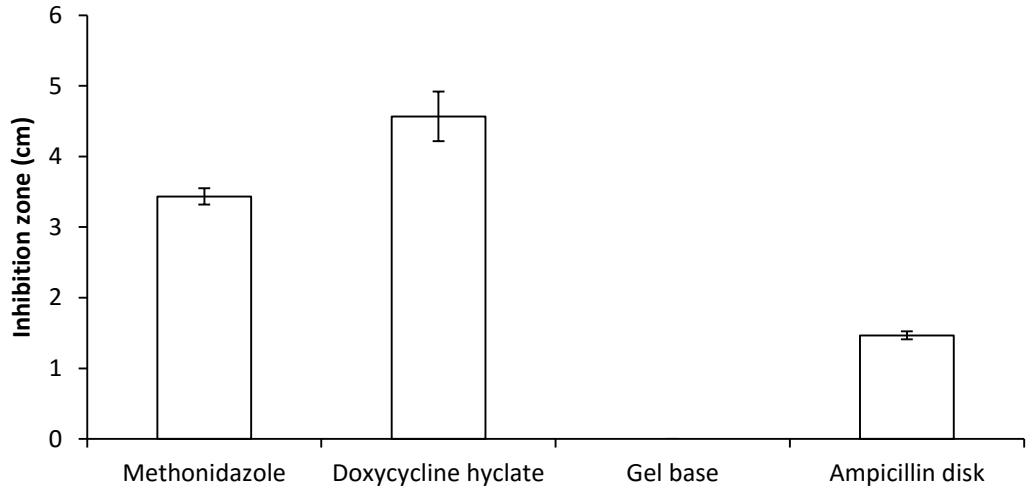


Figure 21 Inhibition zone diameter of the *in situ* forming gel prepared from cholesterol containing doxycycline hyclate and metronidazole against *P. gingivalis* (n=3).

4.1.3 *In vitro* drug release studies

The drug release from the systems was tested in phosphate buffer pH 6.8 to simulate the environment of periodontitis using the dialysis membrane method (Kulkarni *et al*., 2012). Drug release from systems loaded with doxycycline hyclate and metronidazole were fast and nearly not different as presented in Figure 22. The drug released from systems containing metronidazole and doxycycline hyclate were 95.54% and 94.69% at 7 h., respectively. The system exhibited the burst release because the hydrophilic solvent of system which was NMP promoted the drug release. The preparation containing poly(DL-lactide and NMP as gelling agent and solvent, respectively, was previously reported about its burst drug release (Evren and Tamer 2008). In addition, the quite low viscosity of these developed systems promoted the drug release. The preparation containing HPMC and carbopol as gelling agent loading articaine hydrochloride was previously reported about the low viscosity caused burst drug release (Kulkarni 2012).

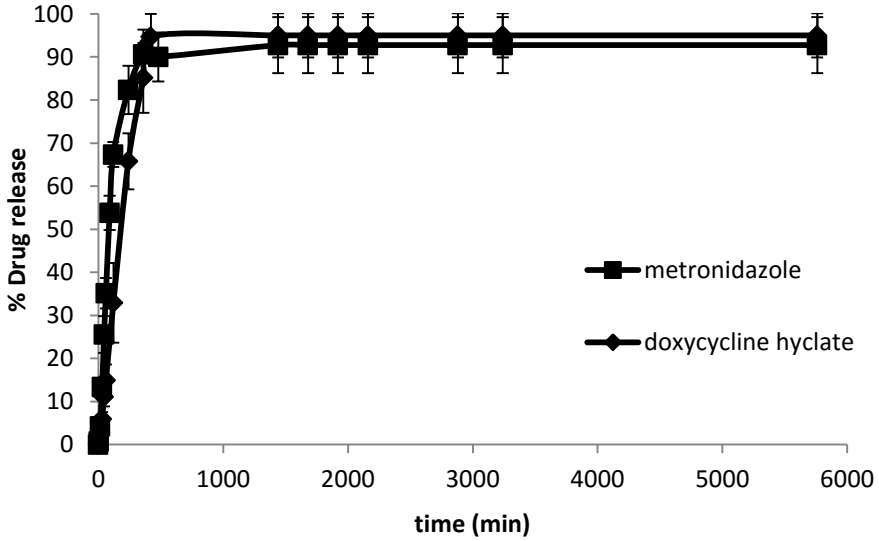


Figure 22 Release of metronidazole (Cho-1) and doxycycline hyclate (Cho-2) from *in situ* forming gel systems containing cholesterol using dialysis method (n=3).

The physical properties such as viscosity, rheology, antimicrobial activity and drug release of *in situ* forming gel loading doxycycline hyclate and metronidazole were not different. Both of release profiles of the gels exhibited burst release because hydrophilic solvent used in system and low viscosity of system promoted the drug release. Thereafter the *in situ* forming gel loading doxyxycline hyclate was selected for further development to minimize the burst release and to prolong the drug release because doxycycline hyclate is more favor than metronidazole in periodontitis treatment.

4.2 Evaluation of *in situ* forming gel system prepared from cholesterol containing the additive

The *in situ* forming gel loading doxyxycline hyclate was selected to minimize the burst release and to prolong the drug release by addition of benzyl benzoate. Benzyl benzoate is insoluble in water so the decreased rate of water diffuse into the system and the decreased drug release. The pharmaceutical research used

benzyl benzoate for decreasing burst release in controlled release system such as injectable implant system of granisetron hydrochloride. This formulation contained 32% poly(DL-lactide-co-glycolide) and benzyl benzoate (BB) was used as solvents. The system showed very slow drug release in the first week and the drug release was sustainable for 21 days (Evren and Tamer 2008).

4.2.1 Appearance

The gel bases contained with or without benzyl benzoate were clear and colorless. The drug-loaded formula with and without benzyl benzoate were light yellow as shown in Table 11. The physical appearance was not different as the amount of benzyl benzoate was increased.

Table 11 Appearance of *in situ* forming gel system prepared from cholesterol containing benzyl benzoate.

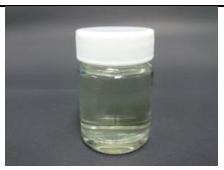
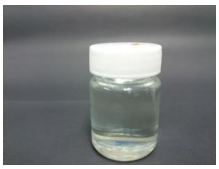
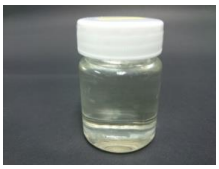
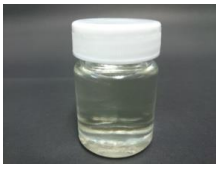
Formula	Appearance	Photo
ChoB-1	Clear, colorless, low viscosity	
ChoB-2	Clear, colorless, low viscosity	
ChoB-3	Clear, colorless, low viscosity	
ChoB-4	Clear, colorless, low viscosity	

Table 11 Appearance of *in situ* forming gel system prepared from cholesterol containing benzyl benzoate (continued)

ChoBD-1	Clear dark yellow, low viscosity	
ChoBD-2	Clear dark yellow, low viscosity	
ChoBD-3	Clear dark yellow, low viscosity	
ChoBD-4	Clear dark yellow, low viscosity	

4.2.2 Viscosity and rheological behavior

The relationship between shear rate and consistency index are shown in Figure 23 and the consistency index is shown in Table 12. The consistency index of formulation increased as the amount of benzyl benzoate was increased. Since the viscosity of benzyl benzoate (8.292 cp at 25^{o}C) (Blanco et al., 1993) was higher than that of the gel base (3 cp at 25^{o}C). The consistency index of gel without benzyl benzoate was significantly different from the others ($p<0.05$). The consistency index of gel with 10-30% benzyl benzoate was not significantly different ($p>0.05$). The prepared gels showed Newtonian behavior because the consistency index was constant when the shear rate was increased. This result is similar to previeous result. *In situ* forming gel containing poly (lactide) (PLA) and poly (lactide-co-glycolide)

(PLGA) as polymer loading with doxycycline hydrochloride or secnidazole for periodontitis treatment exhibited nearly Newtonian flow (Gad *et al*., 2008).

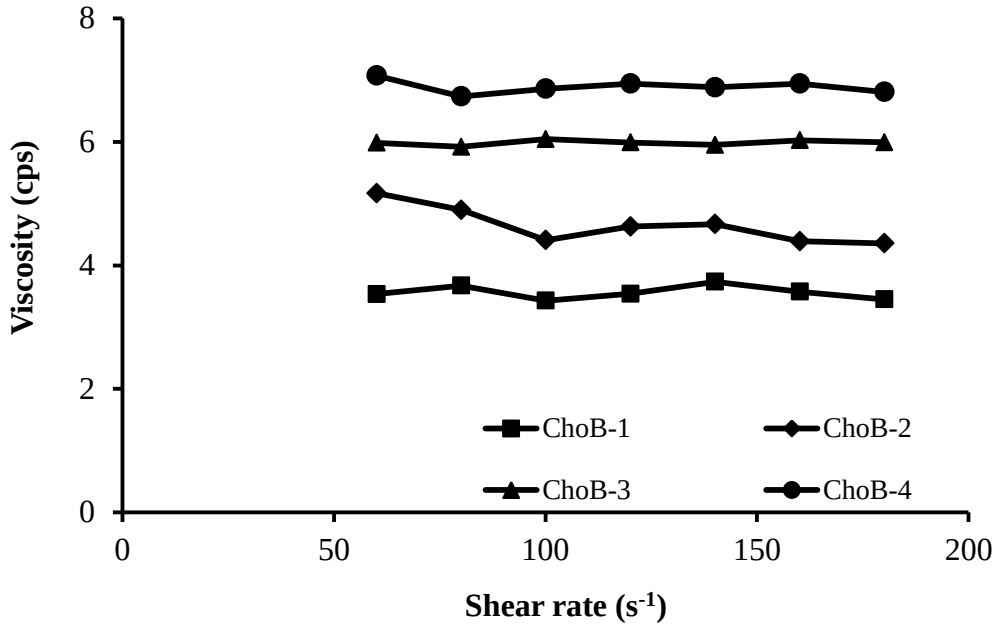


Figure 23 Shear rate-viscosity curves of *in situ* forming gel systems containing cholesterol and different amounts of benzyl benzoate without doxycycline hyclate at 25°C (n=3)

All formula showed Newtonian flow because the flow index was close to 1 (Bjorn et al., 2012). The flow index of gels is shown in Table 12. For the injectable gel the rheological behavior should be a Newtonian or pseudoplastic flow for expediency of injection.

Table 12 Effect of benzyl benzoate concentration on flow index and consistency index of *in situ* forming gels prepared from cholesterol without doxycycline hyclate (n=3).

Sample	Flow index (N) (mean ± S.D.)	Consistency index (ŋ) (mean ± S.D.)
ChoB-1	1.02 ± 0.02	3.38 ± 0.31
ChoB-2	0.91 ± 0.02	9.79 ± 1.31
ChoB-3	0.93 ± 0.01	8.29 ± 0.08
ChoB-4	0.97 ± 0.01	8.07 ± 0.47

The rheological behavior of *in situ* forming gels prepared from cholesterol without doxycycline hyclate was investigated at 25°C. The shear stress versus shear rate flow curve is shown in Figure 24. The shear stress increased when the amount of benzyl benzoate was increased because of the rather high viscosity of benzyl benzoate (8.292 cp at 25°C) (Blanco *et al*., 1993). All formula showed Newtonian behavior since the up curve and down curve was overlapped.

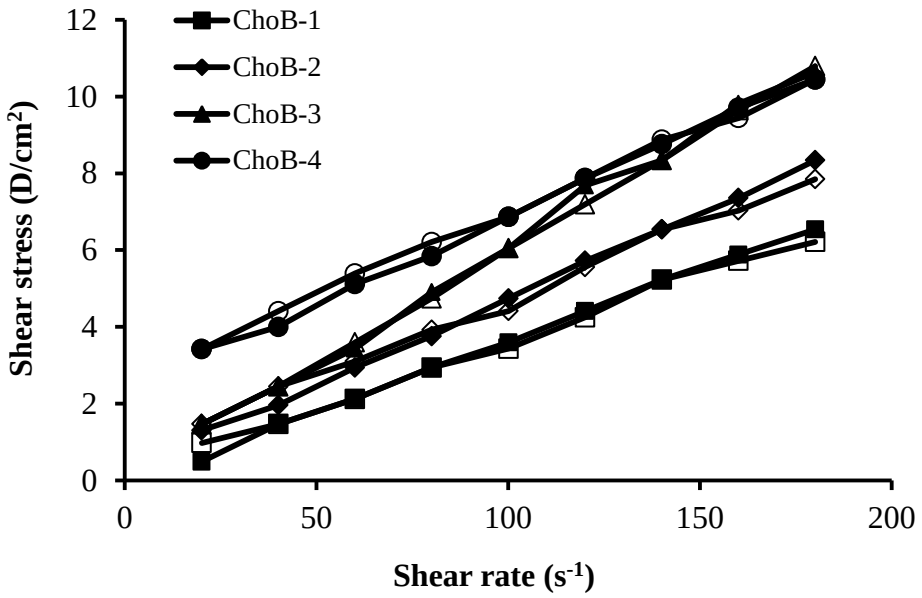


Figure 24 Flow curve of *in situ* forming gel systems prepared from cholesterol and different amounts of benzyl benzoate without doxycycline hyclate at 25°C. Open symbols represent the up-curve, and closed symbols represent the down-curve (n=3).

The consistency index of formulation containing doxycycline hyclate increased as the amount of benzyl benzoate was increased significantly ($p<0.05$). Except the viscosity of formula containing 20% benzyl benzoate was not significantly different ($p>0.05$) from that containing 30% benzyl benzoate. The relationship between shear rate and consistency index is shown in Figure 25 and the consistency

index is shown in Table 13. The prepared gel showed Newtonian behavior because the consistency index was constant when the shear rate was increased.

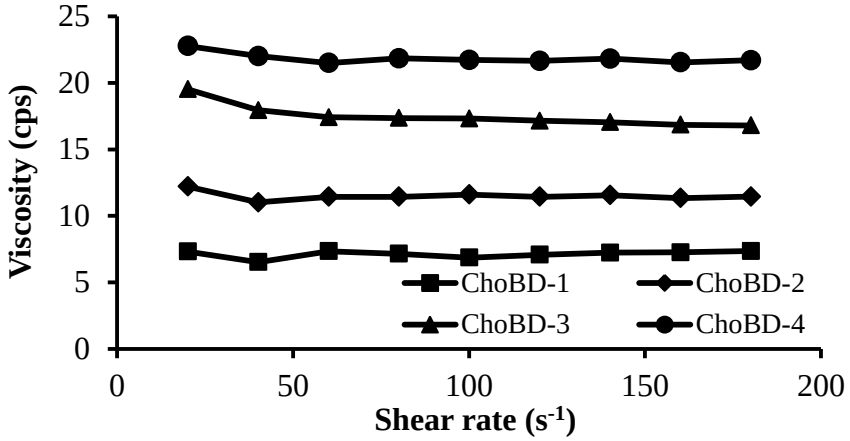


Figure 25 Shear rate-viscosity curves of *in situ* forming gel systems containing cholesterol and different amounts of benzyl benzoate and addition of 10% doxycycline hyclate at 25°C (n=3).

The flow type of all formula was Newtonian because the flow index was close to 1. The flow index of gels is shown in Table 13. The suitable gel for injection was selected from flow property that should be newtonian or pseudoplastic flows. The consistency index of *in situ* forming gel comprising doxycycline hyclate was significantly higher than that of the system without doxycycline hyclate. The viscosity of system comprising doxycycline hyclate increased when amount of benzyl benzoate was increased except the system containing 20% benzyl benzoate was not significantly different ($p>0.05$) from that of 30% benzyl benzoate. These results were owing to the lower amount of solvent in the system comprising doxycycline hyclate therefore the viscosity of the system comprising doxycycline hyclate was higher than the system without doxycycline. The shear stress versus shear rate flow curve of *in situ* forming gels prepared from cholesterol is shown in Figure 26. The shear stress increased when the additive was increased. Some research also reported the increment of shear stress when the additive amount was increased (Parent *et al.*, 2013). All formula showed Newtonian behavior since the up curve and down curve was overlaped.

Table 13 Effect of benzyl benzoate concentration on flow index and consistency index of *in situ* forming gels prepared from cholesterol with doxycycline hyclate (n=3).

Sample	Flow index (N) (mean ± S.D.)	Consistency index (ŋ) (mean ± S.D.)
ChoBD-1	1.05 ± 0.06	5.64 ± 1.39
ChoBD-2	0.98 ± 0.01	12.37 ± 0.31
ChoBD-3	0.95 ± 0.02	21.83 ± 2.10
ChoBD-4	0.95 ± 0.03	25.47 ± 5.48

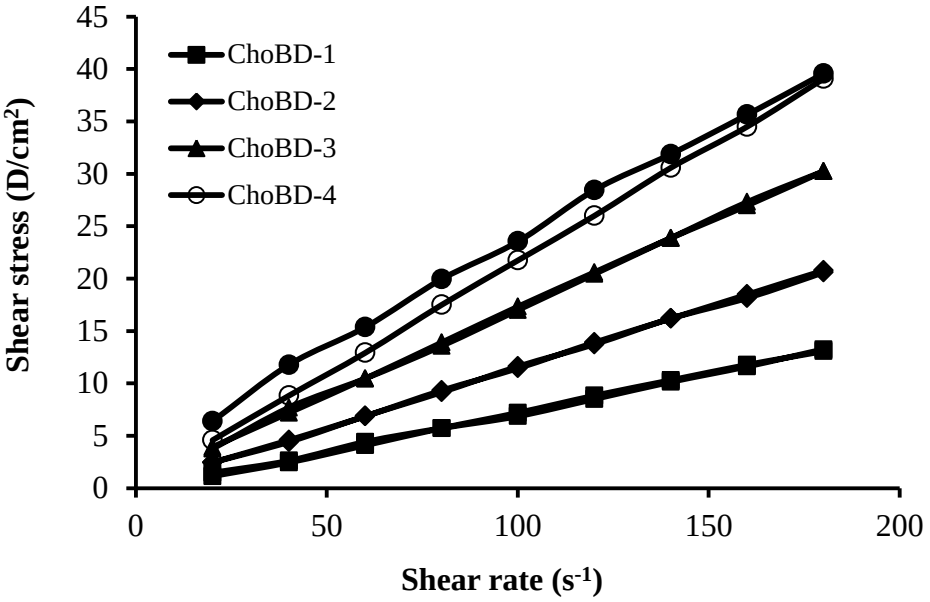


Figure 26 Flow curve of *in situ* forming gel systems prepared from cholesterol and different amounts of benzyl benzoate with an addition of 10% doxycycline hyclate at 25°C. Open symbols represent the up-curve, and closed symbols represent the down-curve (n=3).

4.2.3 Syringeability studies

The syringeability relates to ejection force of a formulation from the syringe via a needle to the injection site. The force was not significantly different ($p>0.05$) for *in situ* forming gels prepared from cholesterol without drug and without benzyl benzoate (Table 14). For the formula with doxycycline hyclate and without benzyl benzoate was significantly different from the others ($p<0.05$) except the

formula with 10-30% benzyl benzoate was not significantly different ($p>0.05$). The force used for the formula without drug was not significantly different from the drug-loaded formula for the system without benzyl benzoate ($p>0.05$). However this force of the system without drug was significantly different ($p<0.05$) from the drug-loaded formula that contained same amount of benzyl benzoate except the formula with 30% benzyl benzoate. The force applied for all formula was apparently low about 1-2 N therefore they should be very easy for injection (Rungseevijitprapa and Bodmeier, 2009).

Table 14 Effect of the amount of benzoyl benzoate and doxycycline hyclate on the force of syringeability of *in situ* forming gels prepared from cholesterol (n=3).

Formula	**Force** (mean ± S.D.)
ChoB-1	1.081 ± 0.05
ChoB-2	1.295 ± 0.25
ChoB-3	1.262 ± 0.13
ChoB-4	1.452 ± 0.22
ChoBD-1	1.448 ± 0.13
ChoBD-2	1.915 ± 0.32
ChoBD-3	1.919 ± 0.11
ChoBD-4	2.025 ± 0.51

4.2.4 *In vitro* gel formation

In situ forming gel transforms from solution into gel by solvent exchange after injection into periodontal pocket (Parent *et al*., 2013). The *in vitro* gel formation of *in situ* forming gel prepared from cholesterol in phosphate buffer pH 6.8, 7.0 and 7.4 are shown in Tables 15, 16 and 17, respectively. The effect of amount of additive on the *in vitro* gel formation was demonstrated. The formulation with more amount of benzyl benzoate showed the longer time to transform into gel because hydrophobic manner of benzyl benzoate retarded the medium penetration or solvent exchange. The hydrophobic substance retards water diffusion because the substance is immiscible with water (Parent *et al*., 2013). However the pH of phosphate buffers (pH 6.8, 7.0 and 7.4) did not affect the gel formation because the pH did not affect apparently the cholesterol solubility or structure.

Table 15 *In vitro* gel formation of *in situ* forming gels prepared from cholesterol with benzyl benzoate (0-30%w/w) at pH 6.8.

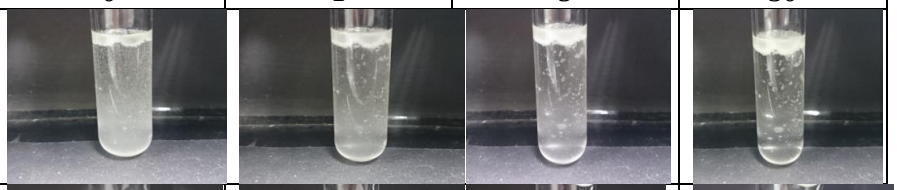
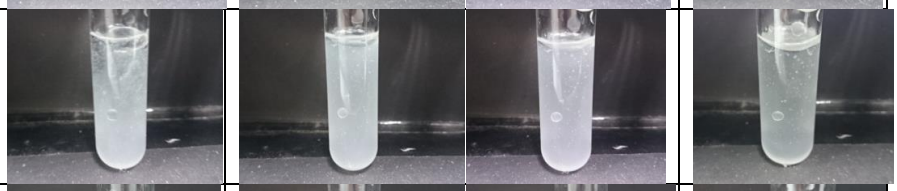
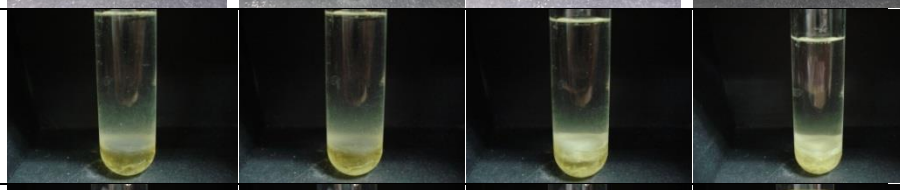
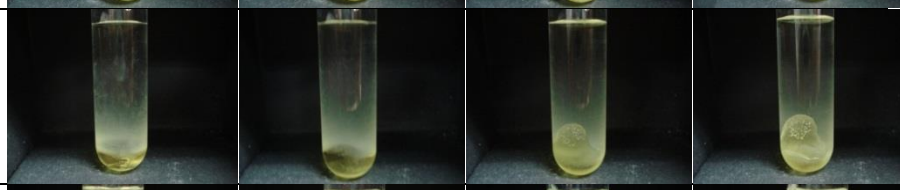
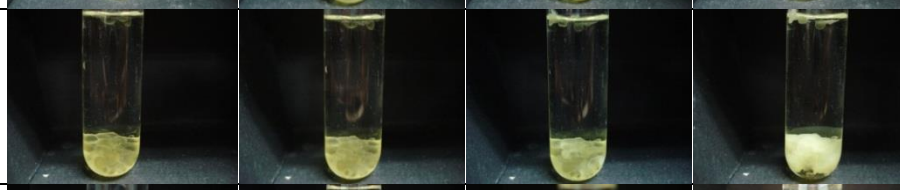
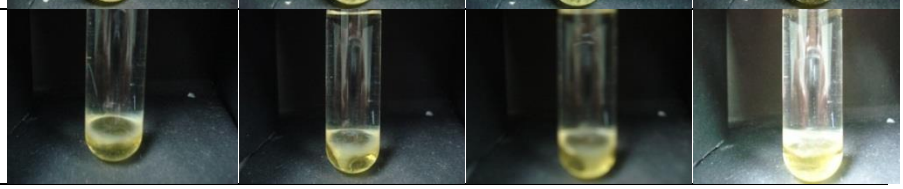
Formulation	Time (min)			
	0	1	5	30
ChoB-1				
ChoB-2				
ChoB-3				
ChoB-4				
ChoBD-1				
ChoBD-2				
ChoBD-3				
ChoBD-4				

Table 16 *In vitro* gel formation of *in situ* forming gels prepared from cholesterol with benzyl benzoate (0-30%w/w) at pH 7.0.

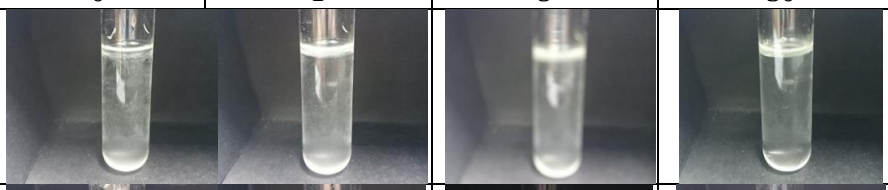
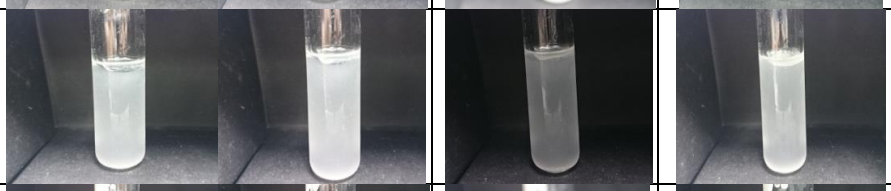
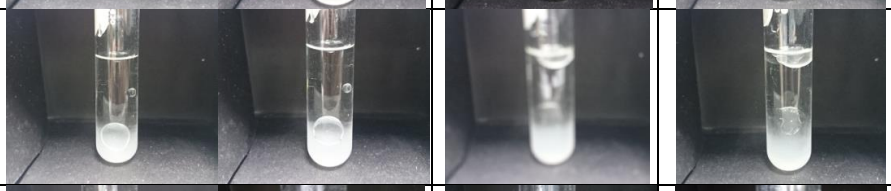
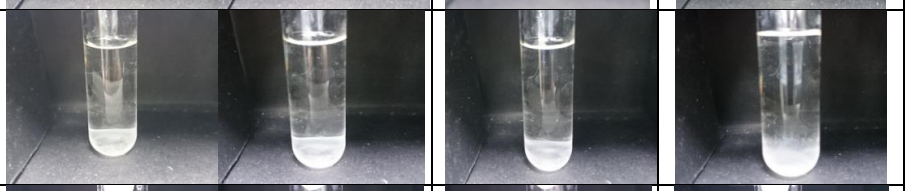
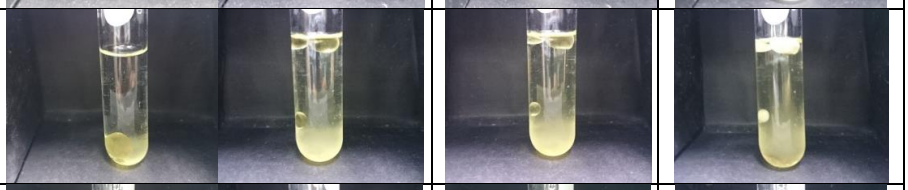
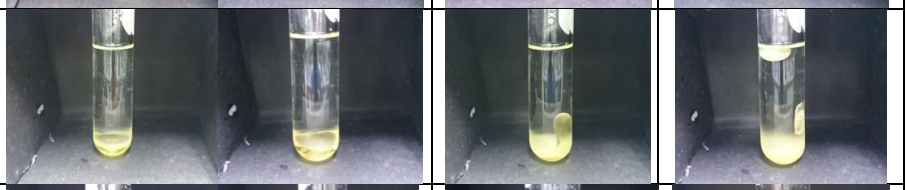
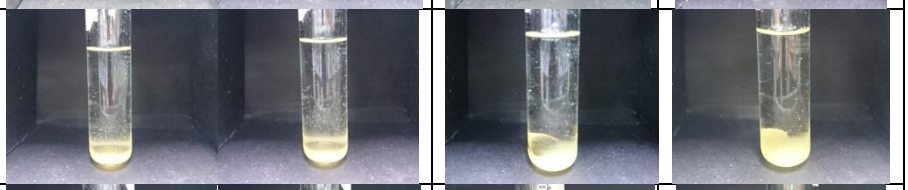
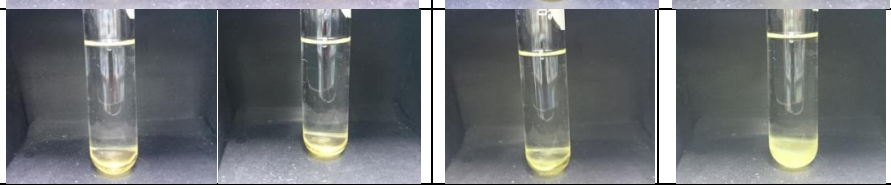
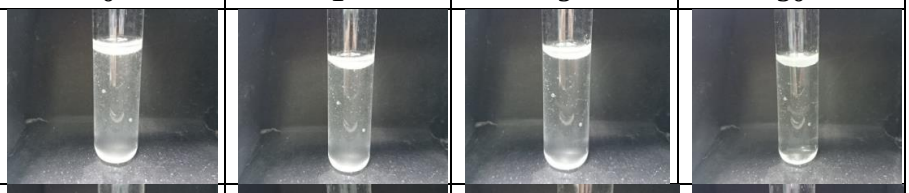
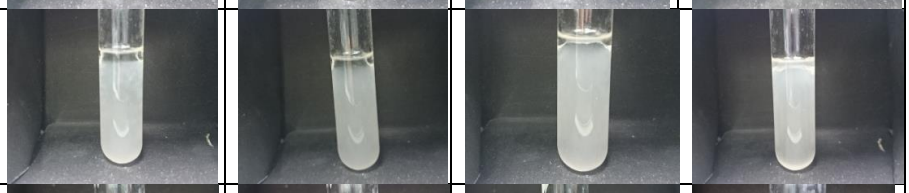
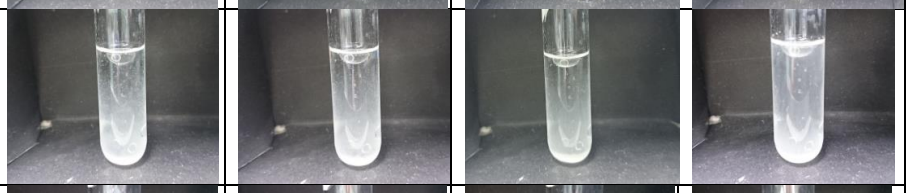
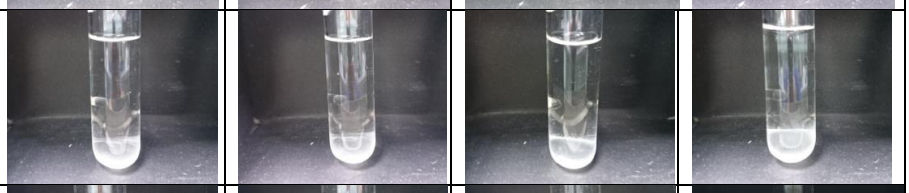
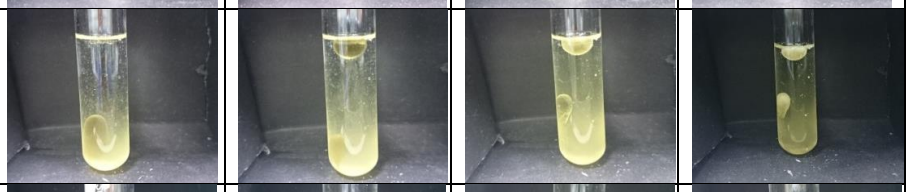
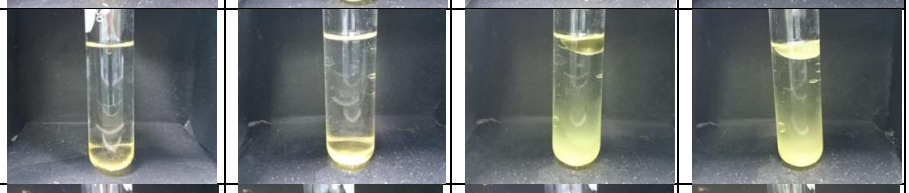
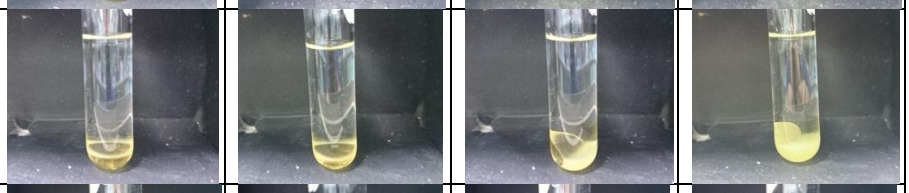
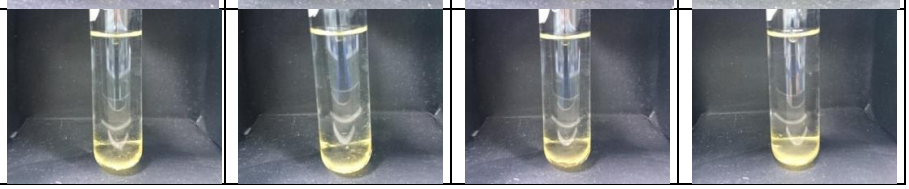
Formulation	Time (min)			
	0	1	5	30
ChoB-1				
ChoB-2				
ChoB-3				
ChoB-4				
ChoBD-1				
ChoBD-2				
ChoBD-3				
ChoBD-4				

Table 17 *In vitro* gel formation of *in situ* forming gels prepared from cholesterol with benzyl benzoate (0-30%w/w) at pH 7.4

Formulation	Time (min)			
	0	1	5	30
ChoB-1				
ChoB-2				
ChoB-3				
ChoB-4				
ChoBD-1				
ChoBD-2				
ChoBD-3				
ChoBD-4				

In situ forming gel was injected into PBS (Figure 27-A). Initially, the system was sol form (Figure 27-B) thereafter the water diffused into the gel and solvent diffused out into the environment (Figure 27-C). Then the gelling agent was precipitated into insoluble matter (Figure 27-D) (Thakur *et al*., 2014). This characteristic was found in system of *in situ* forming gel containing PLA and PLGA dissolved in NMP (Gad *et al.*, 2008). The step of gel formation was presented in Figure 27.

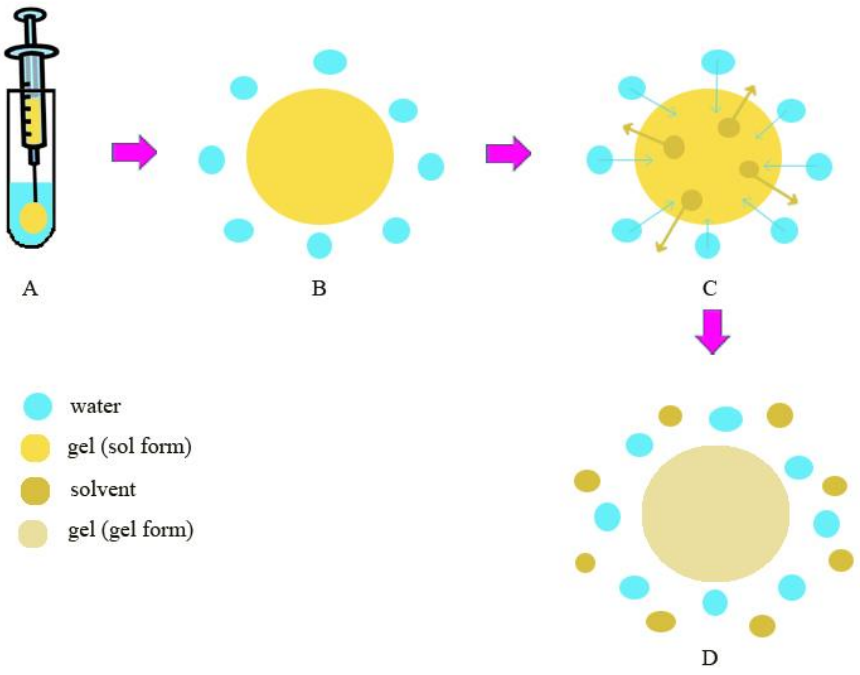


Figure 27 Diagram of gel formation in PBS

4.2.5 *In vitro* degradability studies

The weight loss of the prepared gels was evaluated in phosphate buffer pH 6.8 after 1 month of incubation at 37^{o}C and the percentage of weight loss was calculated by equation 14. For percentage of weight loss, the prepared gel without doxycycline hyclate was not significantly different when benzyl benzoate amount was increased ($p>0.05$) whereas the doxycycline hyclate prepared gel was significantly different when benzyl benzoate amount was increased ($p<0.05$). The percentage of weight loss of the prepared gels comprising the same amount of benzyl benzoate was not significantly different except the prepared gel with 30% benzyl benzoate. Because the prepared gels comprising the drug was harder than the gel base. The percentage of weight loss of prepared gels without and with doxycycline hyclate are shown in Tables 18 and 19, respectively.

Table 18 Percentage of weight loss of *in situ* forming gels prepared from cholesterol without doxycycline hyclate (n=3).

Formula	Percentage of weight loss (mean ± S.D.)
ChoB-1	89.85 ± 0.86
ChoB-2	90.18 ± 0.74
ChoB-3	90.60 ± 2.03
ChoB-4	90.50 ± 1.08

Table 19 The percentage of weight loss of *in situ* forming gels prepared from cholesterol containing doxycycline hyclate (n=3).

Formula	Percentage of weight loss (mean ± S.D.)
ChoBD-1	99.50 ± 0.83
ChoBD-2	90.39 ± 0.46
ChoBD-3	81.79 ± 15.93
ChoBD-4	70.78 ± 7.03

4.2.6 Antimicrobial activity studies

The inhibition zone diameter of *in situ* forming gels containing doxycycline hyclate using agar diffusion method is shown in Figure 28. The inhibition zone diameter of the system containing 10% doxycycline hyclate against *S. mutans* using agar diffusion method was significantly higher than that of gel base ($p<0.05$) but was not significantly different from positive control (cup loading with 150 microlitter of 10% doxycycline hyclate solution) ($p>0.05$). The inhibition zones against *P. gingivalis* and *S. aureus* of the system with 10% doxycycline hyclate was significantly higher than that of gel base ($p<0.05$) and significantly lower than that of the positive control ($p<0.05$) because the drug in prepared gel might diffuse slower than that from positive control. The increased in benzyl benzoate amount up to 30% did not influence the antimicrobial activity against all microbes ($p>0.05$). The gel base also exhibited inhibition zone because NMP exhibited an antimicrobial activity (Phaechamud *et al*., 2012).

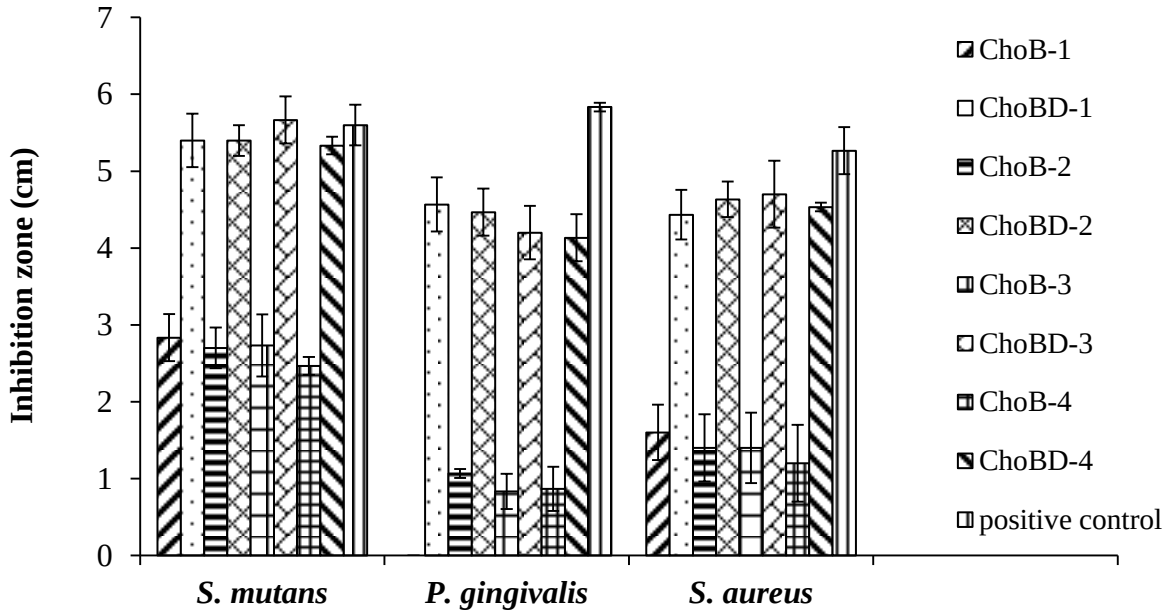


Figure 28 Inhibition zone diameter of the *in situ* forming gels prepared from cholesterol containing benzyl benzoate against *S. aureus, S. mutans* and *P.gingivalis* (n=3).

4.2.7 *In vitro* drug release studies

Dialysis membrane method

The doxycycline hyclate release was tested in phosphate buffer pH 6.8 to simulate the environment of periodontitis using the dialysis membrane method (Kulkarni *et al*., 2012). Drug release from system loaded with 0-20% benzyl benzoate was not different except that of the system with 30% benzyl benzoate was the slowest as presented in Figure 29. The drug release of system containing 10% doxycycline hyclate with 0, 10 and 20% benzyl benzoate were 78.80%, 79.25% and 82.14% at 720 min, respectively whereas the system with 30% benzyl benzoate was about 66.19% drug release at 12 h.

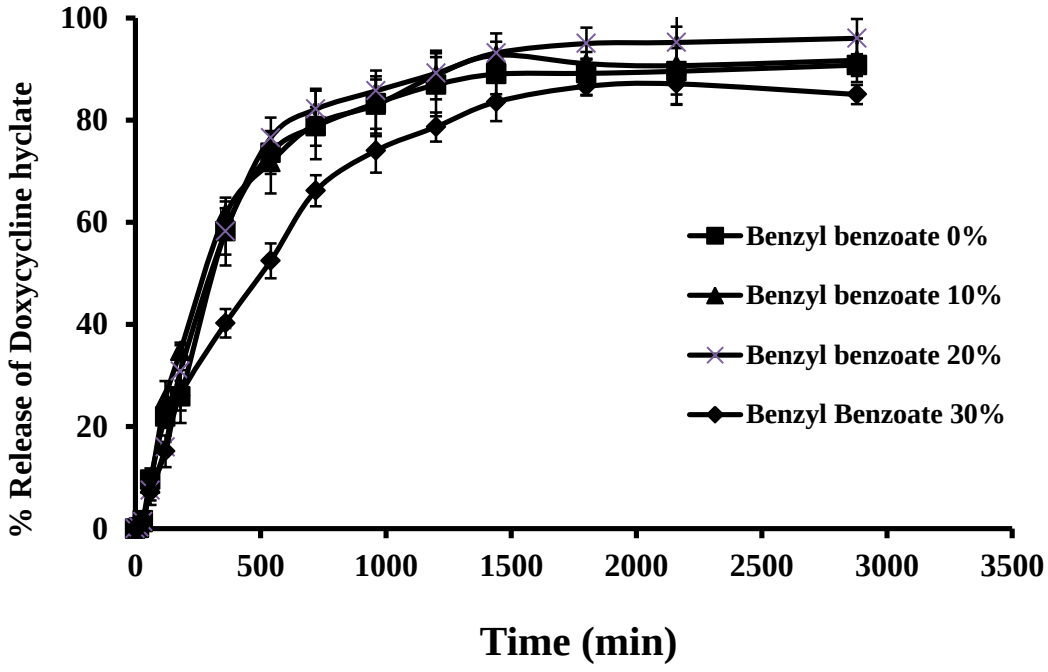


Figure 29 Release of doxycycline hyclate from *in situ* forming gel system containing cholesterol and benzyl benzoate using dialysis method (n=3).

Membrane-less method

The membrane-less diffusion method was studied to allow the medium solution to directly contact with the gel surface and thus dissolved the gel. The doxycycline hyclate release profile was tested in phosphate buffer pH 6.8 to simulate the environment of periodontitis. The drug release from the formula decreased when amount of benzyl benzoate was increased as presented in Figure 30. The drug release profile of system containing 10% doxycycline hyclate with 0, 10, 20 and 30% benzyl benzoate were 99.41%, 58.47%, 26.23% and 17.81% drug release for 5760 min, respectively. Hydrophobic substance could reduce the penetration of NMP in the system to outside and also retard the water penetration into the system therefore the drug release was decreased (Ganesh *et al.,* 2008). In addition the viscosity was increased when the concentration of benzyl benzoate was increased. Typically, the viscosity affects the drug release from the gel. When viscosity is increased the drug release decreases from the gel (Kulkarni *et al*., 2012 and Yaper and Baykara, 2008).

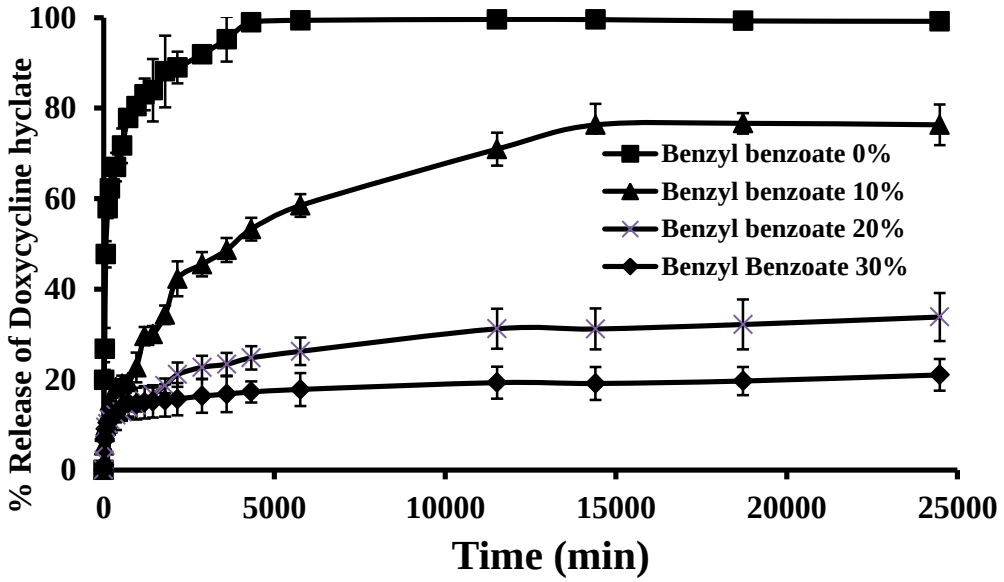


Figure 30 Release of doxycycline hyclate from *in situ* forming gel systems containing cholesterol and benzyl benzoate using membrane less method (n=3).

4.2.8 Analysis of drug release data

Release data of all formula were fitted to mathematical models such as first order, Higuchi's, zero order and power law model to characterize the mechanism of drug release. The high value of coefficient of determination (r^2) and model selection criteria (msc) indicated a superiority of the release profile fitting to mathematical equations as shown in Table 20. The doxycycline hyclate release from *in situ* forming gel containing cholesterol without benzyl benzoate using dialysis membrane method were fitted well with zero order. Whereas, the doxycycline hyclate release from *in situ* forming gel containing cholesterol with 10-30% benzyl benzoate using dialysis membrane method were fitted well with first order. It has been reported that the drug release from hydrophobic polymer matrix in water provided the first order release profile (Khairuzzaman *et al*., 2006).

The release exponent values (n) for power law of all formulations from the release studies using dialysis membrane method are shown in Table 21. The n values of all systems were less than or close to 0.45. The results indicated that the formula showed drug release by Fickian diffusion mechanism which a rate of drug release decreased as a function of time, due to a decrease in the concentration gradient. The kinetic constant (Kunche *et al*., 2012) from the equation 8 indicated the drug release rate from the system. The drug release rate (k) parameter after releasing studies using dialysis membrane method was investigated. The drug release rate of the system without benzyl benzoate was significantly different ($p<0.05$) with the others formula.

Table 20 Comparison of degree of goodness-of-fit from curve fitting of the release profiles of doxycycline hyclate from the system with 0-30% benzyl benzoate in phosphate buffer pH 6.8 using dialysis membrane method to different release models.

Formula (%w/w)	First order		Higuchi's		Zero order		Power law	
	r^2	msc	r^2	msc	r^2	msc	r^2	msc
ChoBD-1	0.9934	3.61	0.9719	2.05	0.9948	3.91	0.9810	2.42
ChoBD-2	0.9951	3.84	0.9592	2.00	0.9770	2.59	0.9883	3.08
ChoBD-3	0.9967	4.55	0.9621	1.98	0.9942	3.96	0.9954	3.69
ChoBD-4	0.9936	3.81	0.9841	2.97	0.9887	3.35	0.9905	3.22

Table 21 Estimate parameter from curve fitting of doxycycline hyclate from the system with 0-30% benzyl benzoate release in phosphate buffer pH 6.8 using dialysis membrane method to power law expression.

Formula (%w/w)	k ± S.D.	n ± S.D.	Release mechanism
ChoBD-1	0.2095 ± 0.1031	0.3945 ± 0.03	Fickian diffusion
ChoBD-2	0.1497 ± 0.0113	0.2077 ± 0.06	Fickian diffusion
ChoBD-3	0.0628 ± 0.0169	0.2508 ± 0.01	Fickian diffusion
ChoBD-4	0.0550 ± 0.0060	0.3594 ± 0.01	Fickian diffusion

k = constant, and n = diffusional exponent

The high value of coefficient of determination (r^2) and model selection criteria (msc) indicated a superiority of the release profile fitting to mathematical equations as shown in Table 22. The doxycycline hyclate release from *in situ* forming gel containing cholesterol without benzyl benzoate using dialysis membrane method were fitted well with zero order (Table 22). Whereas, the doxycycline hyclate release from *in situ* forming gel containing cholesterol with 10-30% benzyl benzoate using dialysis membrane method were fitted well with Higuchi's model. It has been reported that the drug release from *in situ* forming gel provided the Higuchi's model (Phelps *et al*., 2011).

The release exponent values (n) from power law of all formulations from the release studies using dialysis membrane method are shown in Table 23. The n value of the system without and with 20-30% benzyl benzoate was less than 0.45. The results indicated that the formula showed drug release by Fickian diffusion mechanism. And the n value of the system with 10% benzyl benzoate was $0.45<n<1.0$. The results indicated that the formula showed drug release by anomalous (non-Fickian) transport. The drug release rate (k) parameter after releasing studies using dialysis membrane method was investigated. The drug release rate of the system without benzyl benzoate did significantly different ($p<0.05$) with the others formula.

Table 22 Comparison of degree of goodness-of-fit from curve fitting of the release profiles of doxycycline hyclate from the system with 0-30% benzyl benzoate in phosphate buffer pH 6.8 using membrane-less method to different release models.

Formula (%w/w)	First order		Higuchi's		Zero order		Power law	
	r^2	msc	r^2	msc	r^2	msc	r^2	msc
ChoBD-1	0.9450	1.95	0.8784	1.95	0.9491	1.31	0.9960	4.32
ChoBD-2	0.9887	1.30	0.9899	3.62	0.9696	2.46	0.9883	3.30
ChoBD-3	0.9080	1.06	0.9863	1.61	0.9021	1.37	0.9887	3.49
ChoBD-4	0.8552	1.91	0.9902	1.85	0.9221	1.04	0.9939	4.11

Table 23 Estimate parameter from curve fitting of doxycycline hyclate from the system with 0-30% benzyl benzoate release in phosphate buffer pH 6.8 using membrane less method to power law expression.

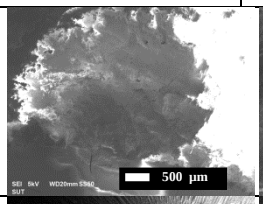
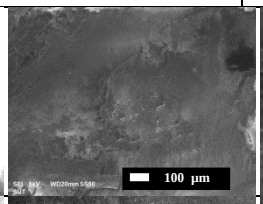
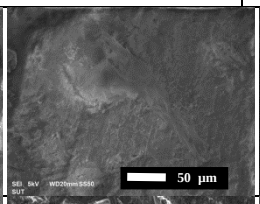
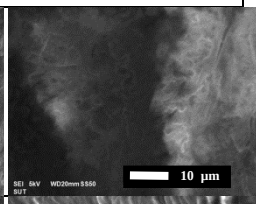
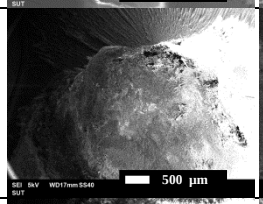
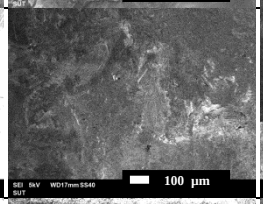
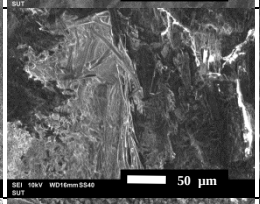
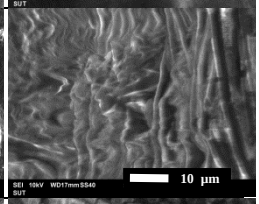
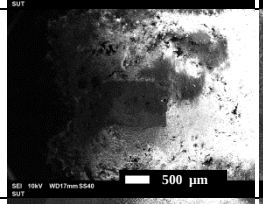
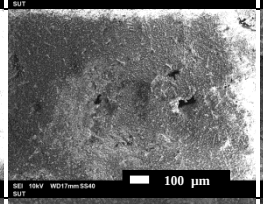
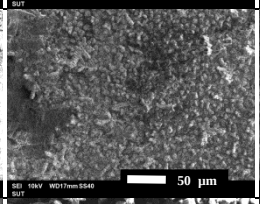
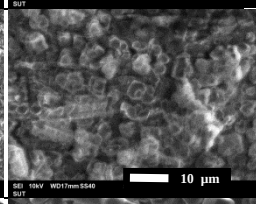
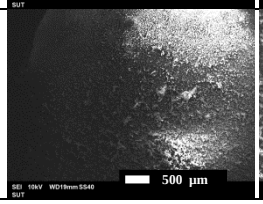
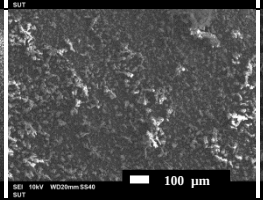
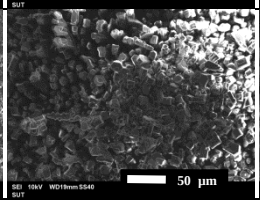
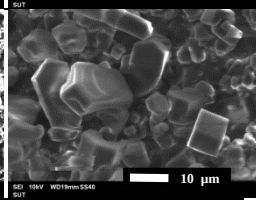
Formula (%w/w)	k ± S.D.	n ± S.D.	Release mechanism
ChoBD-1	0.1956 ± 0.0797	0.22 ± 0.06	Fickian diffusion
ChoBD-2	0.0746 ± 0.0119	0.55 ± 0.31	Anomalous transport
ChoBD-3	0.0431 ± 0.0168	0.21 ± 0.06	Fickian diffusion
ChoBD-4	0.0159 ± 0.0157	0.10 ± 0.04	Fickian diffusion

k = constant, and n = diffusional exponent

4.2.9 Determination of surface morphology (Cryo-SEM)

For the cryo-SEM, the samples were cut and imaged at temperatures below -140^{o}C. This method was maintained the morphology between the formula components. The morphology of the gels prepared from cholesterol with different amount of benzyl benzoate are shown in Table 24. The crystalline in prepared gel increased when benzyl benzoate amount was increased. The cholesterol prepared with 10%w/w benzyl benzoate was the most suitable because the burst release was decrease.

Table 24 Cryo-SEM micrograph of the gels prepared from cholesterol containing different amount of benzyl benzoate with different magnifications (25X, 100X, 400X and 2000X).

Formula	25X	100X	400X	2000X
ChoB-1				
ChoB-2				
ChoB-3				
ChoB-4				

4.2.10 *In vitro* gel formation (pig's gum)

The system containing cholesterol as gelling agent and comprising 10% benzyl benzoate and doxycycline hyclate was injected from 18-gauge needle into pig's gum. Pig's gum was operated with surgical blades after injection for 30 min of injection. The system could transform into gel inside pig's gum. It has been reported that the gel formation containing multiblock poly(ester amino urethane)s was injected in the rat. The system could form gel within 15 min (Dayananda *et al.*, 2008).

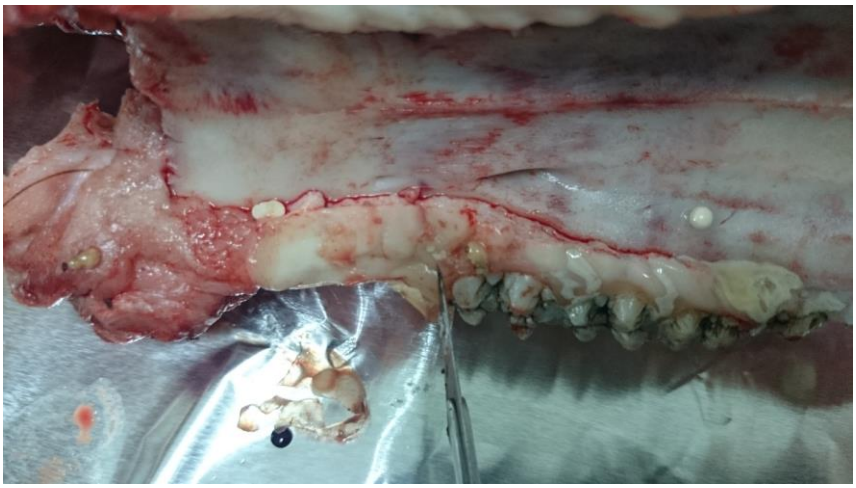


Figure 31 Gel formation inside pig's gum of the *in situ* forming gel system prepared from cholesterol

The appearance and rheology of the *in situ* forming gels with 0-30% benzyl benzoate were not different. The viscosity and time of gel formation of the gel increased when the amount of benzyl benaozte was increased. On the other hand, the syringeability and degradation was decreased when the amount of benzyl benzoate was increased. The amount of benzyl benzoate did not affect to antimicrobial activity. The drug release profile showed the *in situ* forming gel system with 10% benzyl benzoate was the most suitable. Therefore the system containing doxycycline hyclate and 10% benzyl benzoate seemed to be the proper formulation.

4.3 Evaluation of the *in situ* forming gel system from bleached shellac.

Bleached shellac is non-toxic and physiologically harmless. Bleached shellac was used in pharmaceutical product for controlled drug release. Bleached shellac is water insoluble but soluble in NMP, DMSO, 2-pyrrolidone and eutectic. Therefore bleached shellac was used as gelling agent in *in situ* forming gel system.

4.3.1 Appearance

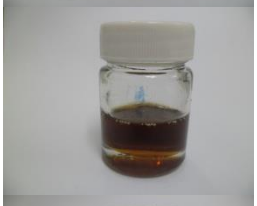
The appearance of *in situ* forming gel prepared from bleached shellac using NMP, DMSO, 2-pyrroridone and eutectic (menthol and camphor) as solvent were clear and light brown and the system with 10% of doxycycline hyclate in NMP, DMSO and 2-pyrroridone were clear and dark brown (Table 25). But the formula using eutectic as solvent was turbid because doxycycline hyclate did not dissolve.

4.3.2 Viscosity and rheological behavior studies

The viscosity of *in situ* forming gel prepared from bleached shellac without doxycycline hyclate using different solvents were investigated at 25^{o}C. The consistency index of formula with 2-pyrrolidone was significantly different and higher than that of DMSO and NMP ($p<0.05$). But the consistency index of the system using eutectic was not determined owing to its too viscous to measure. The relationships between shear rate and consistency index is shown in Figure 32 and the consistency index is shown in Table 26. The prepared systems exhibited Newtonian flow behavior because the apparent viscosity was constant when the shear rate was increase.

The rheological behaviors of *in situ* forming gel systems prepared from bleached shellac without doxycycline hyclate were investigated at 25^{o}C. The flow curve of shear stress versus shear rate is shown in Figure 33. The shear stress value was as followed: 2-pyrrolidone > DMSO > NMP. All formula showed Newtonian behavior because the up curve and down curve was overlapped.

Table 25 Appearance of *in situ* forming gel system from bleached shellac in different solvents.

Formula	Appearance	Picture
She-1	Light brown, clear, low viscosity	
She-2	Light brown, clear, low viscosity	
She-3	Light brown, clear, low viscosity	
She-4	Light brown, clear, low viscosity	
SheD-1	brown, clear, high viscosity	
SheD-2	brown, clear,high viscosity	
SheD-3	brown, clear,high viscosity	
SheD-4	drug could not dissolve	

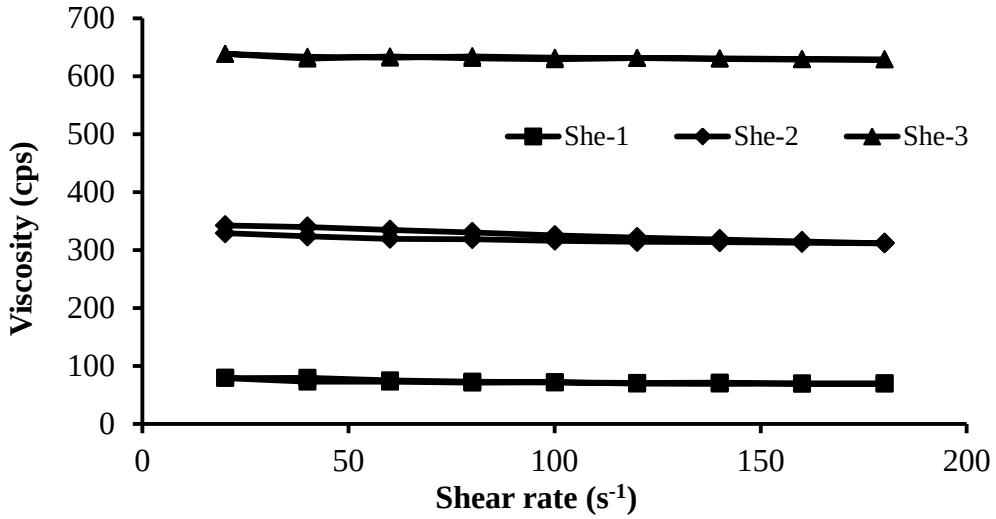


Figure 32 Shear rate-viscosity curves of *in situ* forming gel systems containing bleached shellac without doxycycline hyclate in different solvents at 25°C (n=3).

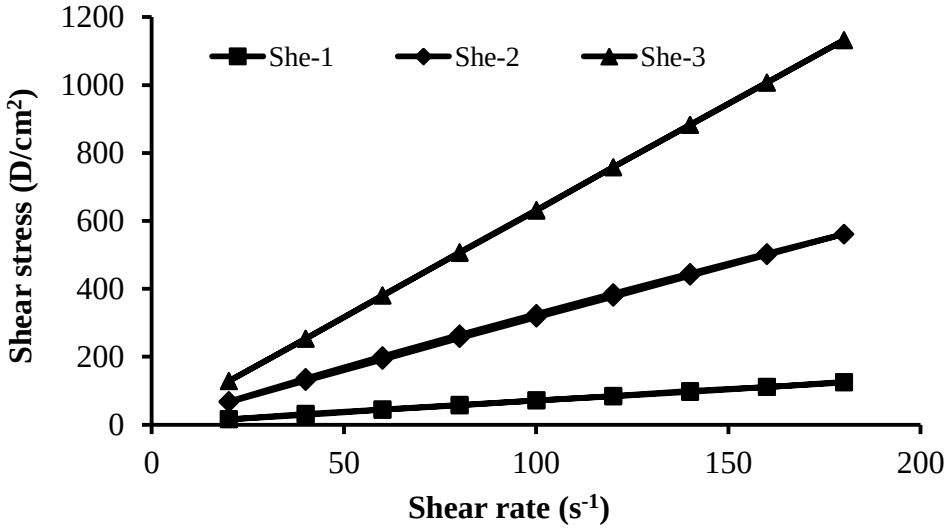


Figure 33 Flow curve of *in situ* forming gel system prepared from bleached shellac without doxycycline hyclate in different solvents at 25°C (n=3).

The flow type of all formula was Newtonian because the flow index was close to 1. These results indicated Newtonian behavior of all formula. The flow index of systems is shown in Table 26. For the injectable gel it should be a Newtonian or pseudoplastic flow for easiness of injection.

Table 26 Effect of type of solvent on flow index and consistency index (n=3). (without doxycycline hyclate)

Sample	Flow index (N) (mean ± S.D.)	Consistency index (ŋ) (mean ± S.D.)
She-1	0.94 ± 0.01	95.03 ± 2.63
She-2	0.97 ± 0.01	375.57 ± 57.65
She-3	0.99 ± 0.01	648.00 ± 15.53
She-4	N/A	N/A

The viscosity of *in situ* forming gel prepared from bleached shellac with doxycycline hyclate using different solvents was investigated at 25°C. The consistency index of formula using 2-pyrrolidone as solvent was significantly different and higher than that of system prepared with DMSO and NMP ($p<0.05$). But the consistency index of the system comprising eutectic was not determined as above mentioned reason. The graph between shear rate and consistency index is shown in Figure 34 and the consistency index is shown in Table 27. The prepared systems showed Newtonian behavior because the apparent viscosity was constant when the shear rate was increased.

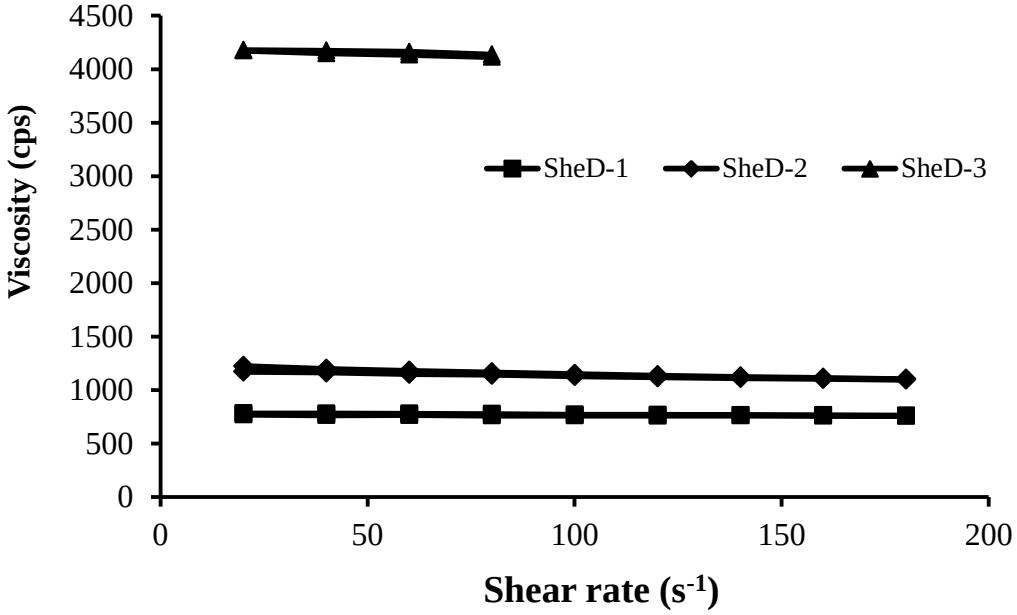


Figure 34 Shear rate-viscosity curves of *in situ* forming gel system containing bleached shellac and addition of doxycycline hyclate in different solvents at 25°C (n=3).

The rheological behaviors of *in situ* forming gel systems prepared from bleached shellac containing doxycycline hyclate were investigated at 25^{o}C. The flow curve between shear stress versus shear rate is shown in Figures 35. The curve show shear stress value was as followed: 2-pyrrolidone > DMSO > NMP. All formula showed Newtonian behavior because the up curve and down curve was overlapped.

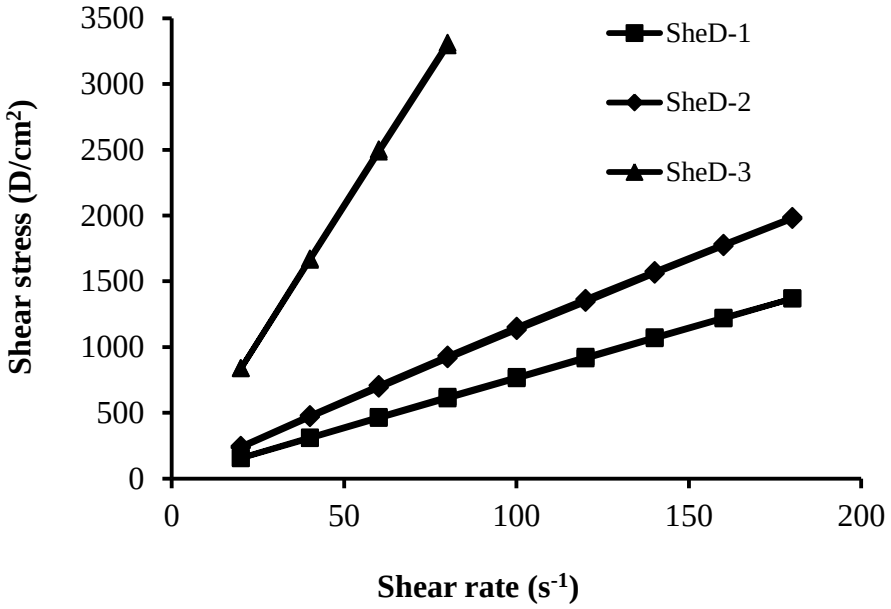


Figure 35 Flow curve of *in situ* forming gel system prepared from bleached shellac with an addition of doxycycline hyclate in different solvents at 25°C (n=3).

The flow type of all formula was Newtonian because the flow index was close to 1. These results indicated Newtonian behavior of all formula. The flow index of gel is shown in Table 27. For the injectable gel, it should be a Newtonian or pseudoplastic flow to promote its ease for injection. In addition the consistency index of *in situ* forming gel with doxycycline hyclate was significantly higher than that of the system without doxycycline hyclate ($p<0.05$) because the amount of solvent in the system comprising doxycycline hyclate was less than that of the system without doxycycline hyclate.

Table 27 Effect of type of solvent on flow index and consistency index (n=3) (with doxycycline hyclate).

Sample	Flow index (N) (mean ± S.D.)	Consistency index (ŋ) (mean ± S.D.)
SheD-1	0.99 ± 0.00	801.47 ± 15.42
SheD-2	0.96 ± 0.01	1362.33 ± 76.37
SheD-3	0.99 ± 0.00	4284.67 ± 73.66
SheD-4	N/A	N/A

4.3.3 Syringeability

The syringeability of *in situ* forming gel systems prepared form bleached shellac is shown in Table 28. Force of *in situ* forming gel systems prepared from bleached shellac was not significantly different ($p>0.05$) except the system using eutectic as solvent which its value was higher than that of the others significantly ($p<0.05$). For the system that used eutectic as solvent and containing doxycycline hyclate could not be injected because of its high viscosity. The force applied for all formula was apparently low about 1-2 N, therefore they should be very easy for injection (Rungseevijitprapa and Bodmeier, 2009).

Table 28 Effect of the type of solvent and doxycycline hyclate on the force of syringeability of formulations containing bleached shellac (n=3).

Formula	Force (N) (mean ± S.D.)
She-1	1.131 ± 0.09
She-2	1.275 ± 0.17
She-3	1.058 ± 0.24
She-4	2.628 ± 0.78
SheD-1	1.179 ± 0.21
SheD-2	1.251 ± 0.06
SheD-3	1.344 ± 0.16
SheD-4	N/A

4.3.4 *In vitro* gel formation

The *in vitro* gel formation of *in situ* forming gel fabricated from bleached shellac in phosphate buffers pH 6.8, 7.0 and 7.4 are shown in Tables 29, 30 and 31, respectively. The effect of type of solvent on the *in vitro* gel formation was demonstrated. The formulation containing DMSO as solvent exhibited the fastest transformation into gel. The velocity of gel formation was as followed: DMSO > NMP > 2-pyrrolidone > eutectic. Since the ranking of polarity of used solvent was ranked as following: DMSO > NMP > 2-pyrrolidone > eutectic, respectively (Sastry, 2004; Hollingsworth, 1952). Therefore the transformation of the system containing high polar solvent was faster than that of the system containing low polar solvent. The pH of phosphate buffers (pH 6.8, 7.0 and 7.4) did not affect the formation of gel. The drug loading did not influence the gel formation except the system using eutectic as solvent which the system containing drug exhibited the faster transformation into gel than the system without drug. Owing to high polarity of loaded drug the water could diffuse easier into the system and thereafter the dissolved drug molecules could diffuse outward into the test medium.

Table 29 *In vitro* gel formation of *in situ* forming gel system prepared from bleached shellac using different solvents at pH 6.8.

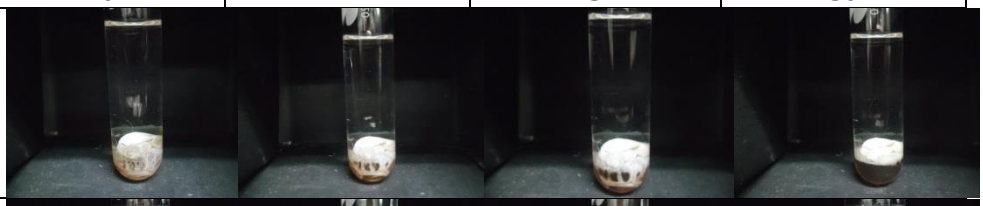
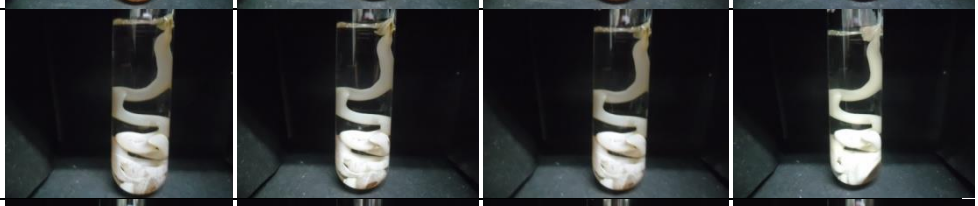
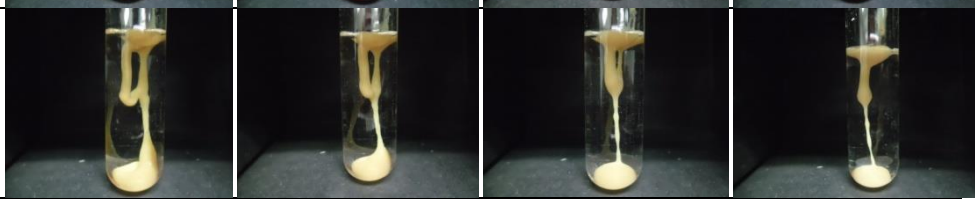
formula	Time (min)			
	0	1	5	30
She-1				
She-2				
She-3				
She-4				
SheD-1				
SheD-2				
SheD-3				
SheD-4				

Table 30 *In vitro* gel formation of *in situ* forming gel using different solvents at pH 7.0.

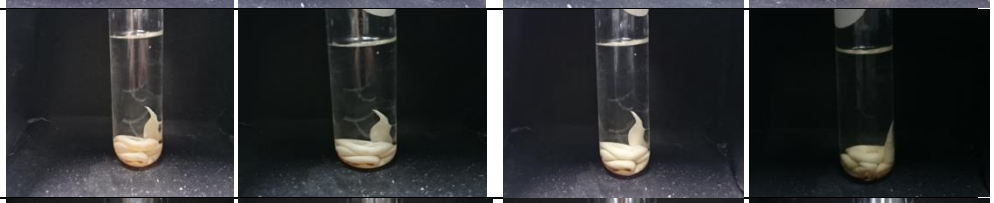
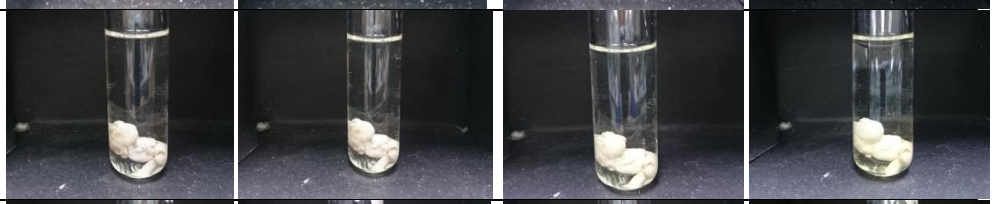
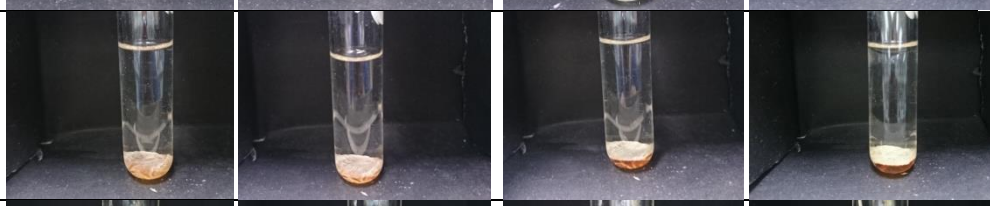
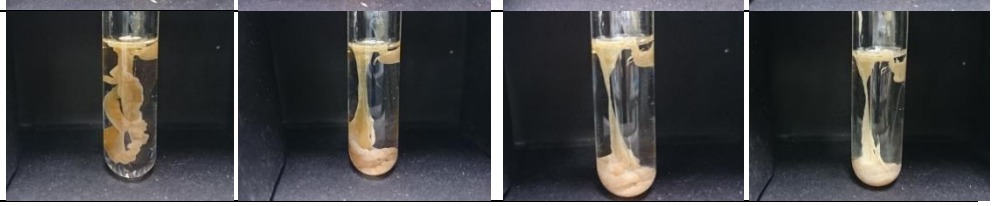
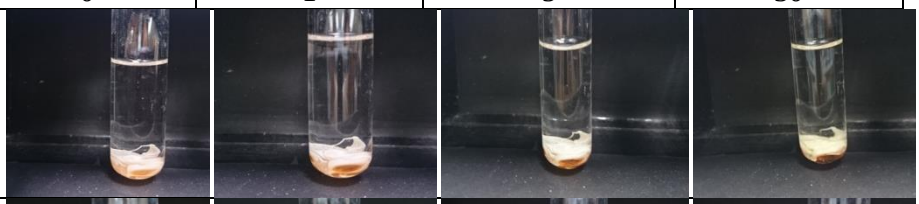
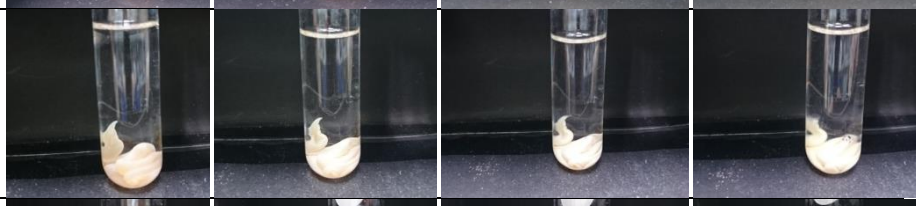
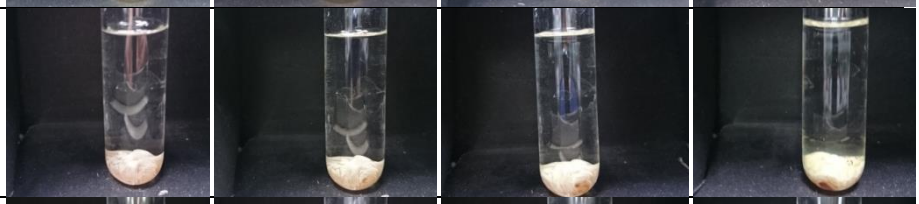
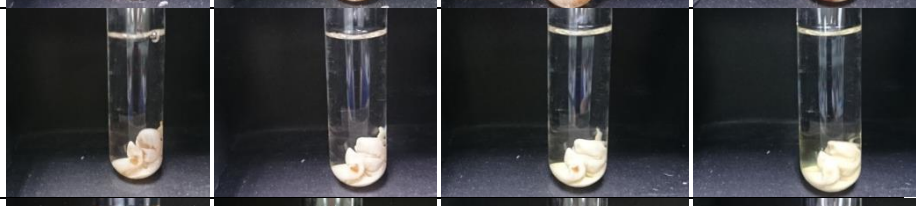
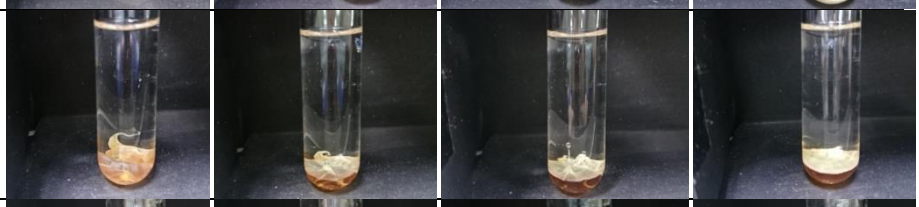
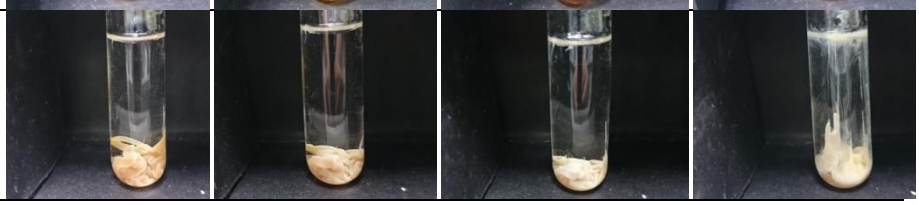
formula	Time (min)			
	0	1	5	30
She-1				
She-2				
She-3				
She-4				
SheD-1				
SheD-2				
SheD-3				
SheD-4				

Table 31 *In vitro* gel formation of *in situ* forming gel using different solvents at pH 7.4.

Formula	Time (min)			
	0	1	5	30
She-1				
She-2				
She-3				
She-4				
SheD-1				
SheD-2				
SheD-3				
SheD-4				

In situ forming gel prepared from bleached shellac was sol form before inject into PBS. Afterthat the water diffused into the gel and solvent diffused out into the environment. The system was precipitated into insoluble matter (Thakur *et al*., 2014). This characteristic was found in system of *in situ* forming gel containing PLA and PLGA dissolved in NMP (Gad *et al.*, 2008). The gel formation of *in situ* forming gel prepared from bleached shellac was similar to *in situ* forming gel prepared by cholesterol.

4.3.5 *In vitro* degradability studies

Percentage of weight loss of the prepared gels without doxycycline hyclate was not significantly different for the prepared gels using NMP and DMSO as solvent ($p>0.05$) but was significantly different from the others ($p<0.05$). Percentage of weight loss of the prepared gels containing doxycycline hyclate with different solvents was significantly different ($P<0.05$). Percentage of weight loss of the prepared gels with doxycycline hyclate was significantly different with the prepared gel without doxycycline hyclate ($p<0.05$). The percentage of weight loss of prepared gels without and with doxycycline hyclate are shown in Tables 32 and 33 respectively.

Table 32 The percentage of weight loss of *in situ* forming gel prepared from bleached shellac without doxycycline hyclate (n=3).

Formula	Percentage of weight loss (mean ± S.D.)
She-1	63.42 ± 2.46
She-2	62.49 ± 3.10
She-3	99.98 ± 0.00
She-4	92.90 ± 5.69

Table 33 The percentage of weight loss of *in situ* forming gel prepared from bleached shellac with doxycycline hyclate (n=3).

Formula	Percentage of weight loss (mean ± S.D.)
SheD-1	41.18 ± 7.48
SheD-2	32.40 ± 2.26
SheD-3	80.05 ± 4.38
SheD-4	64.81 ± 4.44

4.3.6 Antimicrobial activity studies

The inhibition zone diameters of *in situ* forming gels prepared from bleached shellac containing doxycycline hyclate using agar diffusion method are shown in Figure 36. The inhibition zone diameter of the system containing 10% of doxycycline hyclate using different solvents against *S. mutans, P. gingivalis* and *S. aureus* was significantly higher than that of the gel base ($p<0.05$) and was significantly lower than positive control, 10% of doxycycline hyclate solution ($p<0.05$). The system with bleached shellac could sustain the drug release therefore in the initial stage the inhibition zone of the developed system was lower than the doxycycline hyclate solution, positive control. The antimicrobial activity of prepared gel containing doxycycline hyclate and employing DMSO (SheD-2) as solvent against *S. aureus, P. gingivalis* and *S. mutans* was significantly higher than that of the others (Sastry, 2014). This might be owing to the higher polarity of DMSO rather than other solvents used in gel system. While the eutectic has low polarity, then it might reduce the diffusion rate of drug from gel system. This activity for prepared gel using eutectic as solvent against *P.gingivalis* and *S.mutans* was significantly lower than that of the others (Hollingsworth, 1952).

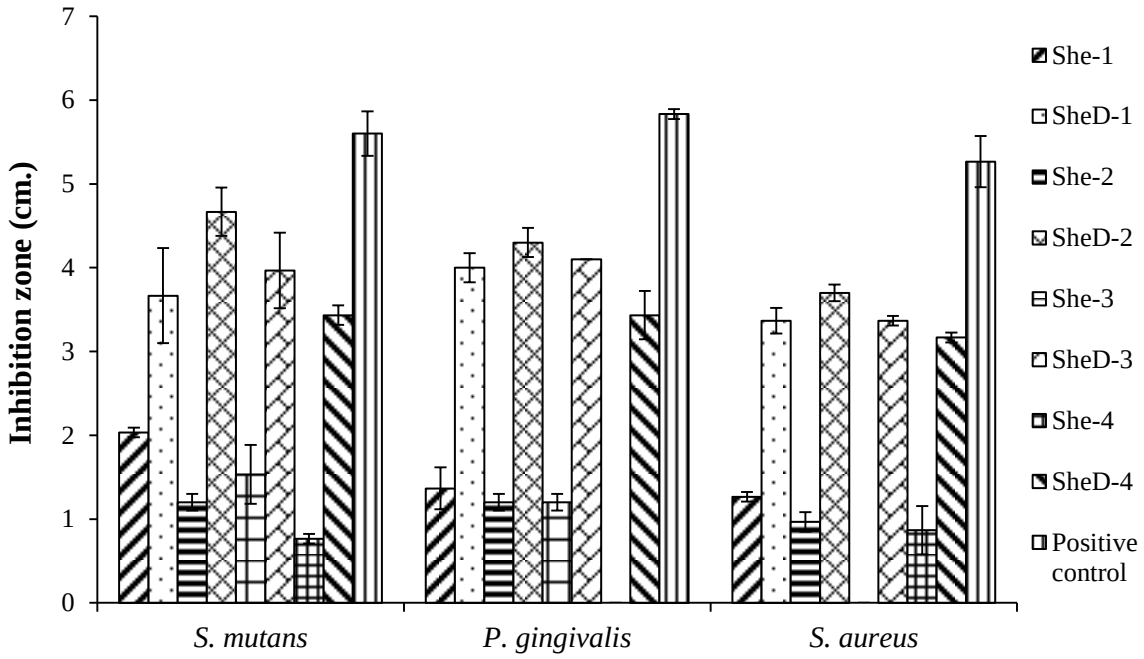


Figure 36 Inhibition zone diameter of the *in situ* forming gels prepared from bleached shellac containing doxycycline hyclate against *S. aureus, S. mutans* and *P. gingivalis*.

4.3.7 *In vitro* drug release studies

Dialysis membrane method

The system containing DMSO showed the fastest drug release because DMSO has the highest polarity. The system containing eutectic exhibited the slowest drug release because it contained 1:1 menthol and camphor which its polarity was low. The system containing 2-pyrrolidone showed the highest drug release due to its dominent erosion. The result is shown in Figure 37. The drug release of systems employing NMP, DMSO, 2-pyrrolidone and eutectic as solvents were 54.01%, 61.72%, 43.91% and 33.67% drug release, respectively, for 7 d.

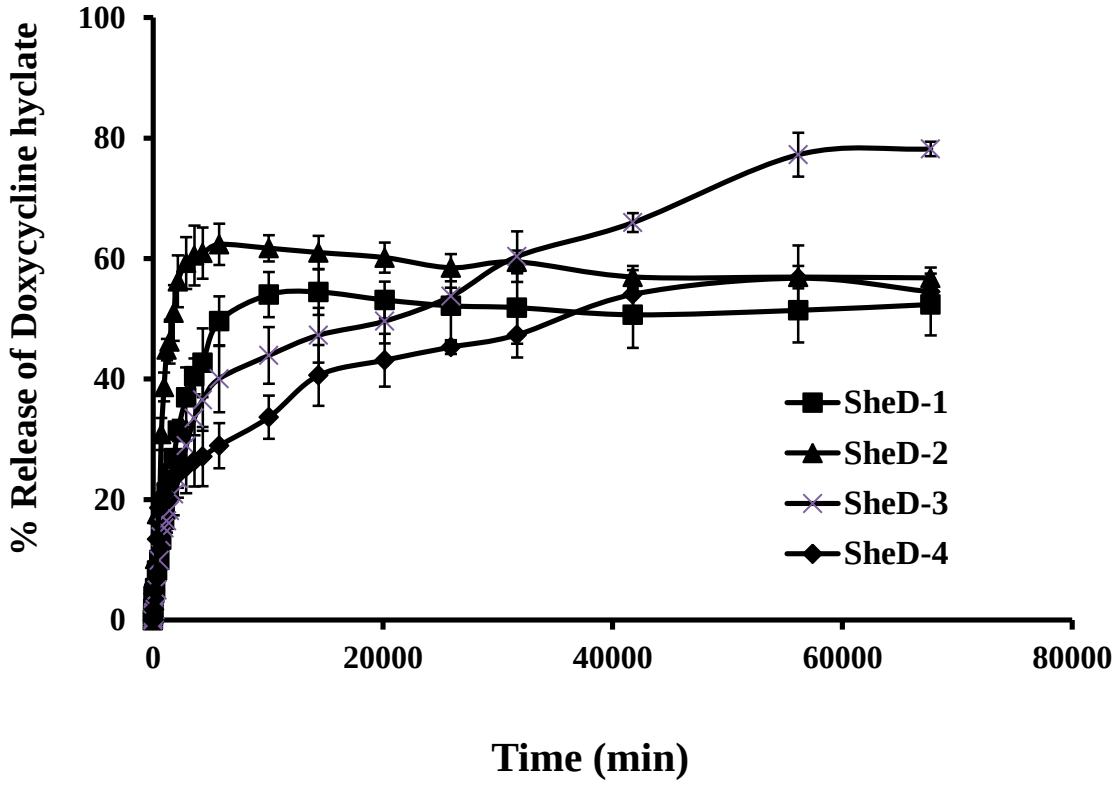


Figure 37 Release of doxycycline hyclate from *in situ* forming gel system containing bleached shellac using dialysis method (n=3).

4.3.8 Analysis of drug release data

The curve fitting of release profiles of doxycycline hyclate from the system using different solvents in phosphate buffer pH 6.8 using dialysis membrane method to different release models are shown in table 34. The doxycycline hyclate release profiles from *in situ* forming gel containing bleached shellac were fitted well with zero order. It has been reported that the drug release from *in situ* forming gel containing gellan gum and sodium alginate provided the zero order release profile (Kunche *et al*., 2012).

The n value of the bleached shellac system containing NMP and 2-pyrrolidone as solvent were $0.45<n<1.0$ (Table 35). The drug release by anomalous transport indicated that the drug release was controlled by both mechanisms of diffusion and polymeric chain relaxation (Pahwa *et al*., 2011; Perioli *et al*., 2004).

The n value of the system containing DMSO and eutectic as solvent were less than 0.45. The formula showed drug release by fickian diffusion. The kinetic constant from the equation 8 indicated the drug release rate from the system. The drug release rate (k) parameter after release study using dialysis membrane method was investigated. The drug release rate of the system containing eutectic as solvent was significantly different ($p<0.05$) with the others.

Table 34 Comparison of degree of goodness-of-fit from curve fitting of the release profiles of doxycycline hyclate from the system using different solvents in phosphate buffer pH 6.8 using dialysis membrane method to different release models.

Formula (%w/w)	First order		Higuchi's		Zero order		Power law	
	r^2	msc	r^2	msc	r^2	msc	r^2	msc
SheD-1	0.9948	2.77	0.9909	3.42	0.9951	3.83	0.9829	2.77
SheD-2	0.9720	0.58	0.9935	3.92	0.9974	4.78	0.9905	3.63
SheD-3	0.9933	1.68	0.9883	3.21	0.9980	3.20	0.9905	3.63
SheD-4	0.9729	0.59	0.9074	1.60	0.9967	3.60	0.9921	3.85

Table 35 Estimate parameters of curve fitting of doxycycline hyclate released from the system using different solvents in phosphate buffer pH 6.8 using dialysis membrane method to power law expression.

Formula (%w/w)	k ± S.D.	n ± S.D.	Release mechanism
SheD-1	0.0524 ± 0.0026	0.56 ± 0.14	Anomalous transport
SheD-2	0.0530 ± 0.0291	0.32 ± 0.08	Fickian diffusion
SheD-3	0.0166 ± 0.0262	0.57 ± 0.30	Anomalous transport
SheD-4	0.0070 ± 0.0081	0.20 ± 0.02	Fickian diffusion

k = constant, and n = diffusional exponent

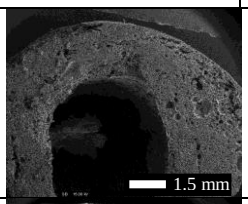
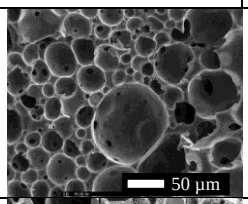
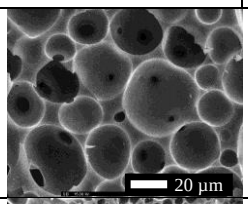
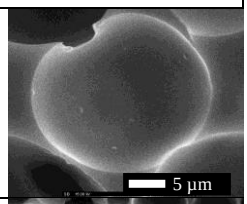
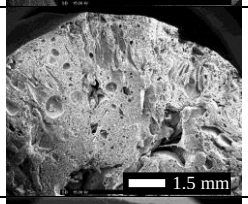
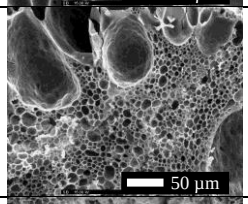
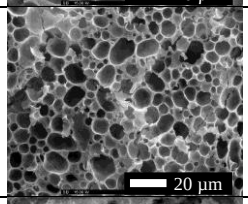
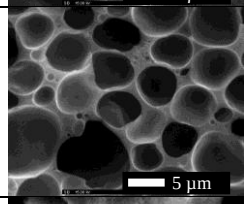
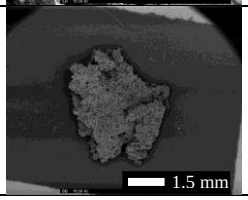
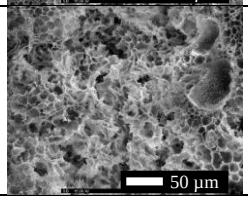
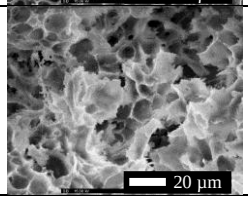
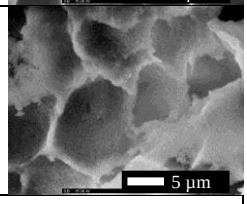
4.3.9 Determination of surface morphology

4.3.9.1 Scanning electron microscopy (SEM)

The samples were collected after release test of doxycycline hyclate in PBS pH 6.8 at 37 °C, frozen and dried by freeze-dryer. Then the sample was characterized by scanning electron microscopy (Table 36), except the system with eutectic as solvent could not be dried because the solvent did not diffuse into the

medium and was not evaporated. The structures were clarified at magnification of 6X, 200X, 500X and 2000X. There were many pores formed throughout the matrix indicated that the solvent of system diffused into the medium and water diffused into the system and then the drug diffused out from the system. The transformation rate was slow for system using 2-pyrrolidone as solvent (Parent *et al*., 2013) hence the structure of gel was not hard and easy to erode. The transformation rate of the system using NMP and DMSO as solvent was fast (Parent *et al*., 2013) therefore the structure of gel was hard.

Table 36 SEM micrograph of the dried gels prepared from bleached shellac containing different type of solvent with different magnifications (6X, 200X, 500X and 2000X).

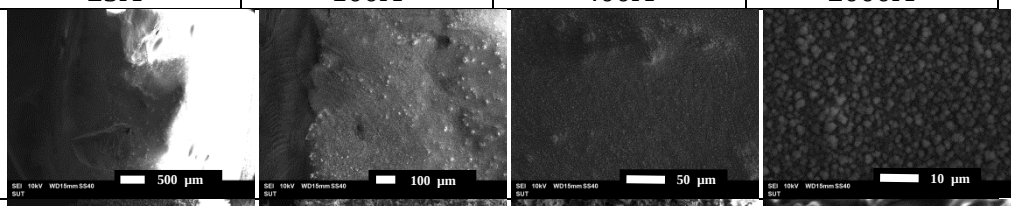
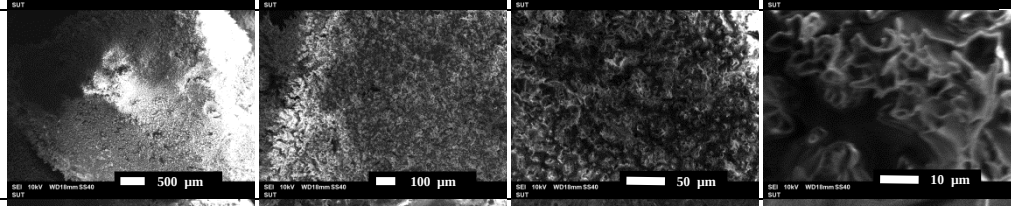
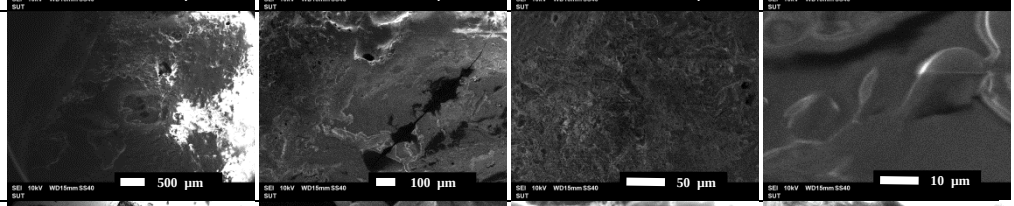
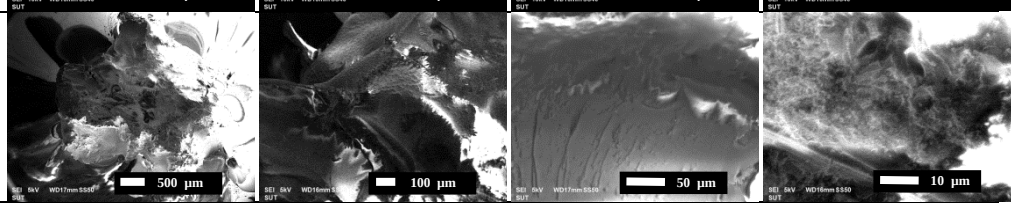
Formula	6X	200X	500X	2000X
SheD-1				
SheD-2				
SheD-3				
SheD-4	N/A			

4.3.9.2 Cryo-scanning electron microscopy (Cryo-SEM)

The cryo-SEM micrograph of the gels prepared from bleached shellac with doxycycline hylate and different types of solvent are shown in Table 37. The bleached shellac-doxycycline hyclate prepared with 2-pyrrolidone was

homogenous structure while the bleached shellac-doxycycline hyclate prepared with NMP, DMSO and eutectic were agglomerative particles and non-homogenous structure.

Table 37 Cryo-SEM micrograph of the gels prepared from bleached shellac dissolved in different type of solvent with different magnifications (25X, 100X, 400X and 2000X).

Formula	25X	100X	400X	2000X
SheD-1				
SheD-2				
SheD-3				
SheD-4				

4.3.10 *In vitro* gel formation (pig's gum)

The *in situ* forming gel system containing bleached shellac as gelling agent comprising 2-pyrrolidone and doxycycline hyclate was injected from 18-gauge needle into pig's gum. Pig's gum was operated with surgical blades after 30 min of injection. The injected system could transform into intact gel in pig's gum.

Figure 38 Gel formation in pig's gum of the *in situ* forming gel system prepared from bleached shellac

The appearance and rheology of the *in situ* forming gels containing bleached shellac in various solvent were not different except the system using eutectic as solvent. The viscosity of the system was followed: eutectic > 2-pyrrolidone > DMSO > NMP. The velocity of gel formation was followed: DMSO > NMP > 2-pyrrolidone > eutectic. The syringeability of *in situ* forming gel was not different except the system using eutectic as solvent that could not be injected because of its too viscous. The system using 2-pyrrolidone as solvent showed the highest degradation. The system using DMSO as solvent exhibited the highest antimicrobial activity for *P. gingivalis, S. mutans* and *S. aureus*. Moreover the drug release profile and degreadability studies signified that the *in situ* forming gel system using 2-pyrrolidone as solvent was the most suitable. Therefore the system containing doxycycline hyclate employing 2-pyrrolidone as solvent was suitable for periodontitis treatment.

4.4 Evaluation of the ISM system prepared from bleached shellac.

The ISM were prepared from *in situ* forming gel using bleached shellac as polymeric matter and 2.5% GMS dispersed in olive oil mixing with 2 syringes connector. Olive oil was used as external phase due to the incompatible with internal phase. The emulsions were oil in oil (o/o) emulsion (Luan *et al*., 2006). The ISM was brown and turbid because bleached shellac was brown and the external phase was

turbid. The ISM containing polymer dissolved in NMP, DMSO and 2-pyrrolidone as internal phase were easy to prepare. But the ISM containing eutectic as dispersing medium for polymer of internal phase could not be prepared because the viscosity of *in situ* forming gel containing eutectic was apparently high.

4.4.1 Stability studies of emulsion

4.4.1.1 Stability test by syringe method

Increasing the amount of GMS from 0% to 5% further improved the emulsion stability since this substance could against the droplet coalescence (Voigt *et al*., 2012). All systems prepared using NMP as solvent without doxycycline hyclate and containing GMS at different amount were instable at 5 min. On the other hand the system prepared using DMSO and 2-pyrrolidone as solvent containing 2.5-5% GMS without doxycycline hyclate did stable at 3 d.

Table 38 Effect of amount of GMS on the stability of emulsion (without doxyxycline hyclate) (n=3).

Formula	% of GMS	% Separation at different time intervals (min) (mean ± S.D.)				
		0	1	5	10	30
MShe-1	0	0	0	3.33 ± 1.15	18.00 ± 3.46	52.67 ± 1.15
	1.25	0	0	11.33 ± 3.06	36.67 ± 3.06	42.67 ± 1.15
	2.5	0	0	27.33 ± 9.24	39.33 ± 3.21	44.33 ± 3.51
	3.75	0	0	21.33 ± 0.58	35.00± 0.00	36.33 ± 0.58
	5	0	0	20.00 ± 4.58	32.67 ± 1.15	36.67 ± 0.58
MShe-2	0	0	0	3.33 ± 1.15	1.67 ± 4.16	35.33 ± 3.06
	1.25	0	0	3.67 ± 1.52	10.67 ± 2.31	29.33 ± 2.31
	2.5	0	0	0	0	0
	3.75	0	0	0	0	0
	5	0	0	0	0	0
MShe-3	0	0	0	6.67 ± 3.06	25.33 ± 7.02	41.33 ± 3.06
	1.25	0	0	1.67 ± 1.53	18.00 ± 5.29	25.00 ± 5.20
	2.5	0	0	0	0	0
	3.75	0	0	0	0	0
	5	0	0	0	0	0

For the system with doxycycline hyclate, increasing the amount of GMS from 0% to 5% further improved the emulsion stability since this substance could against the droplet coalescence. The system prepared using NMP as solvent was instable at 5 min in 0-2.5% GMS but the system with 3.75 and 5% GMS did not show their instability observed at 30 min. The system prepared using DMSO and 2-pyrrolidone as solvent containing 2.5-5% GMS did stable at 30 min. However, the system prepared using NMP as solvent containing 3.75 and 5% GMS were stored and did stable at 12 h and 2 days, respectively. The system prepared using DMSO and 2-pyrrolidone as solvent containing 2.5-5% GMS without doxycycline hyclate were stable after storing for 3 days and did not show the phase separation. GMS has been used for decreasing droplet coalescence in emulsion (Hodge and Rousseau, 2005) because the viscosity of continuous phase increased and the there was the formation of liquid crystalline GMS layer at the interface (Voigt *et al*., 2012).

Table 39 Effect of amount of GMS on the stability of emulsion (with doxyxycline hyclate) (n=3).

formula	% of GMS	% Separation at time (min) (mean ± S.D.)				
		0	1	5	10	30
MSheD-1	0	0	0	12.00 ± 0.00	50.00 ± 0.00	57.33 ± 1.15
	1.25	0	0	35.00 ± 5.00	41.00 ± 3.61	54.00 ± 1.73
	2.50	0	0	28.00 ± 5.29	38.00 ± 2.00	47.67 ± 2.51
	3.75	0	0	0	0	0
	5.00	0	0	0	0	0
MSheD-2	0	0	0	3.33 ± 0.58	10.67 ± 2.52	47.67 ± 1.55
	1.25	0	0	8.67 ± 1.15	13.67 ± 2.08	40.67 ± 1.15
	2.50	0	0	0	0	0
	3.75	0	0	0	0	0
	5.00	0	0	0	0	0
MSheD-3	0	0	0	20.00 ± 0.00	50.00 ± 0.00	55.33 ± 0.58
	1.25	0	0	3.00 ± 1.73	25.33 ± 0.58	48.33 ± 2.89
	2.50	0	0	0	0	0
	3.75	0	0	0	0	0
	5.00	0	0	0	0	0

The effect of GMS amount on the stability of emulsion was studied. The systems prepared using DMSO and 2-pyrrolidone containing 2.5% GMS were selected for further development. For decreasing the percent of internal phase and increasing the percent of external phase, stability was observed within 30 min. Therefore the system prepared using DMSO and 2-pyrrolidone containing 2.5% GMS with ratio of 1:1 internal phase: external phase were selected because the system could be loaded the highest drug amount.

4.4.1.2 Stability test by microscope method

The particle size of the system prepared using 2-pyrrolidone and DMSO as solvent without GMS was increased with time. The particle size of the system prepared using 2-pyrrolidone and DMSO as solvent comprising GMS was not increased with time. Therefore, GMS could improve the emulsion stability. The particle size of the system containing 2.5% GMS with and without doxycycline hyclate was not significantly different ($p>0.05$) at 30 min.

Table 40 Mean size of emulsion droplet (n=3).

Formula	Size (μm) (mean ± S.D.)				
	0 min	1 min	5 min	10 min	30 min
MShe-2 Without GMS	52.69 ± 5.07	58.27 ± 4.61	82.25 ± 5.51	372.77 ± 32.69	400.78 ± 7.94
MSheD-2 Without GMS	33.26 ± 2.04	41.31 ± 1.34	46.37 ± 2.50	93.04 ± 2.42	243.12 ± 7.00
MShe-2 With GMS	26.97 ± 1.80	28.03 ± 2.62	28.33 ± 2.33	28.53 ± 4.28	28.80 ± 3.70
MSheD-2 With GMS	26.65 ± 1.72	26.03 ± 0.88	26.35 ± 3.09	26.37 ± 1.71	23.59 ± 6.36
MShe-3 Without GMS	44.73 ± 1.93	50.51 ± 1.12	81.11 ± 1.52	395.15 ± 7.02	440.30 ± 16.60
MSheD-3 Without GMS	32.28 ± 1.93	40.38 ± 1.12	51.60 ± 1.52	91.52 ± 7.02	243.96 ± 16.6
MShe-3 With GMS	27.15 ± 1.82	26.01 ± 2.33	25.97 ± 1.93	26.67 ± 1.27	27.57 ± 1.83
MSheD-3 With GMS	25.98 ± 0.57	26.29 ± 2.19	26.34 ± 2.35	25.04 ± 1.97	25.32 ± 2.07

4.4.2 Transformation from emulsion into microparticle

The viscosity of system prepared using DMSO as solvent was low. DMSO has high affinity with water therefore the diffusion rate of water into gel and transformation rate of sol into gel was also rapid. The system containing 2-pyrrolidone was high viscous. 2-Pyrrolidone has low affinity with water since the diffusion rate of water into gel and transformation rate of sol into gel was also slow (Parent *et al*., 2013). The transformation rate of sol into gel of system with or without drug was not different.

Table 41 Transformation from o/o emulsion into microparticle of emulsion droplets containing 2.5% GMS.

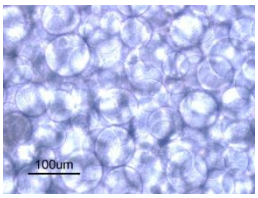
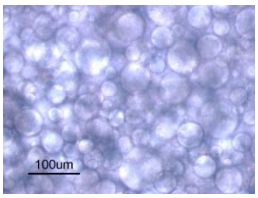
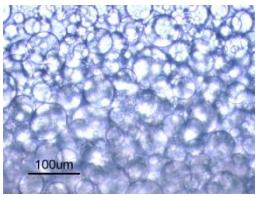
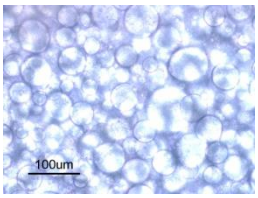
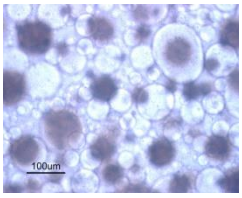
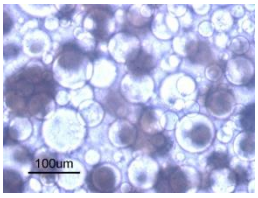
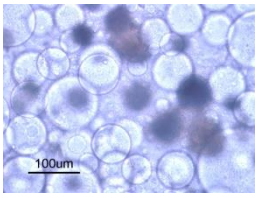
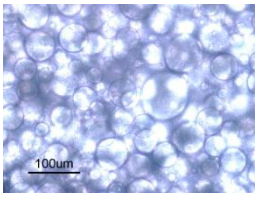
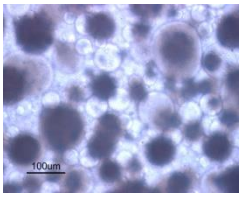
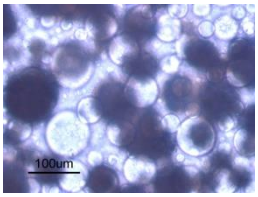
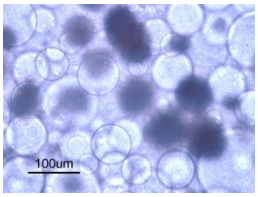
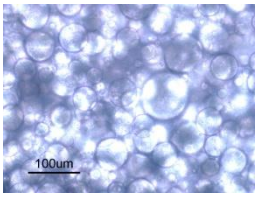
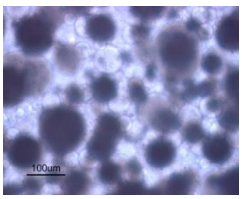
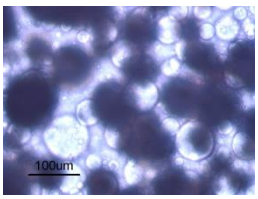
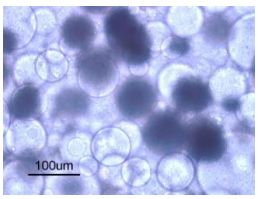
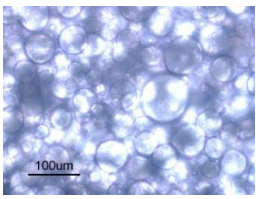
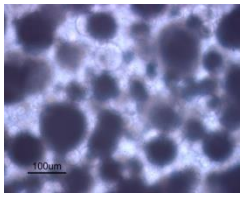
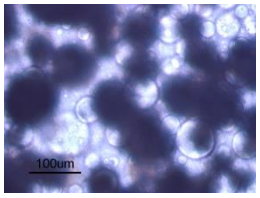
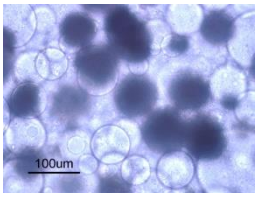
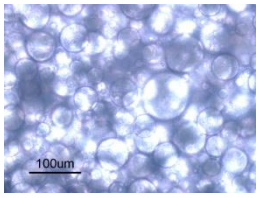
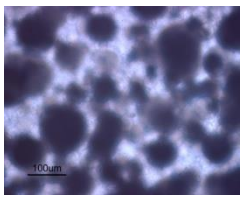
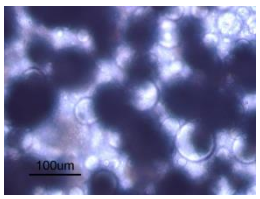
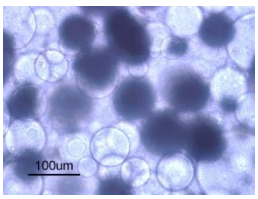
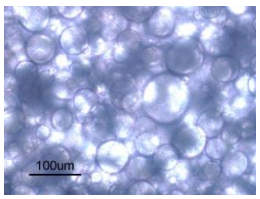
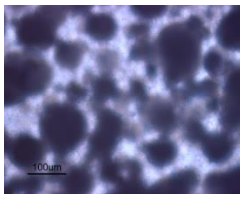
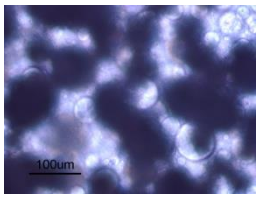
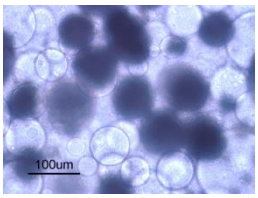
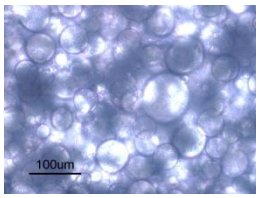
Time (sec)	Formula			
	MShe-2	MSheD-2	MShe-3	MSheD-3
0				
10				
20				
30				

Table 41 Transformation from o/o emulsion into microparticle of emulsion droplets containing 2.5% GMS. (continued)

Time (sec)	Formula			
	MShe-2	MSheD-2	MShe-3	MSheD-3
40				
50				
60				

4.4.3 Appearance

The appearance of external phase (olive oil and 2.5% GMS) was white and turbid. The appearance of ISM from bleached shellac with and without doxycycline hyclate in DMSO and 2-pyrroridone were turbidity and brown (Table 42).

Table 42 Appearance of ISM from bleached shellac in different solvents.

Formula	Appearance	Picture
MShe-1	brown, turbid	
MShe-2	brown, turbid	
MSheD-1	brown, turbid	
MSheD-2	brown, turbid	
Olive oil + 2.5% GMS	White, turbid	

4.4.4 Viscosity and rheological behavior studies

The viscosity of ISM prepared from bleached shellac with and without doxycycline hyclate containing different solvents at 25^{o}C is presented in Figure 39. The consistency index of formula prepared with 2-pyrrolidone was higher than that prepared with DMSO statistical significantly ($p<0.05$) and that of formula loaded with doxycycline hyclate was higher than that of the system without drug significantly different ($p>0.05$). The consistency index is shown in Table 43. The prepared gel exhibited pseudoplastic flow behavior because the consistency index was decreased with an increased rate of shear stress (Malkin, 2013).

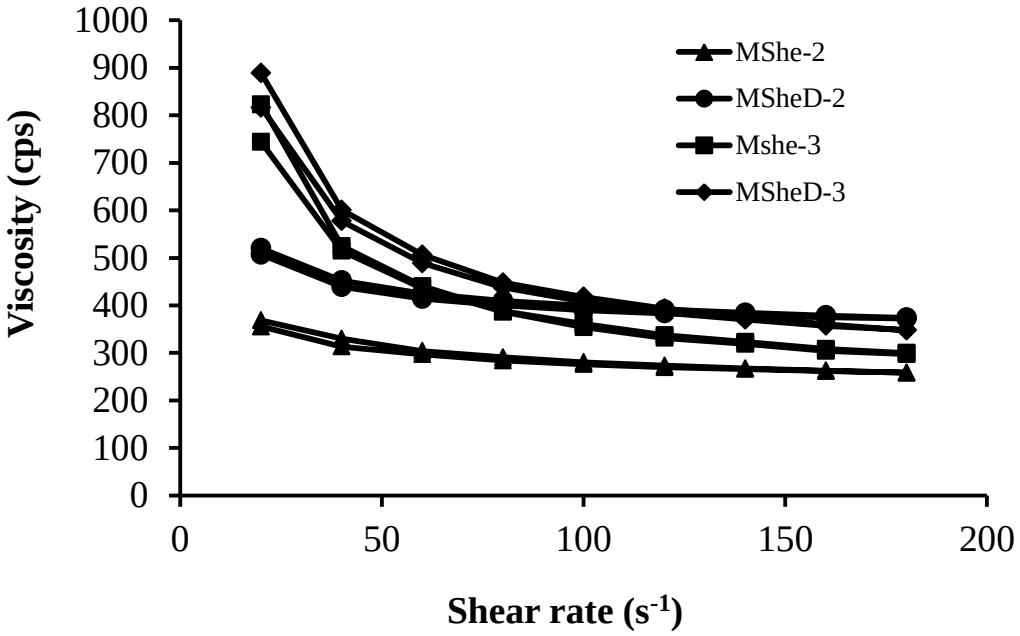


Figure 39 Shear rate-viscosity curves of ISM containing bleached shellac with and without doxycycline hyclate prepared with DMSO and 2-pyrrolidone) at 25°C (n=3).

The shear stress versus shear rate flow curves of ISM prepared from bleached shellac at 25°C are shown in Figure 40.

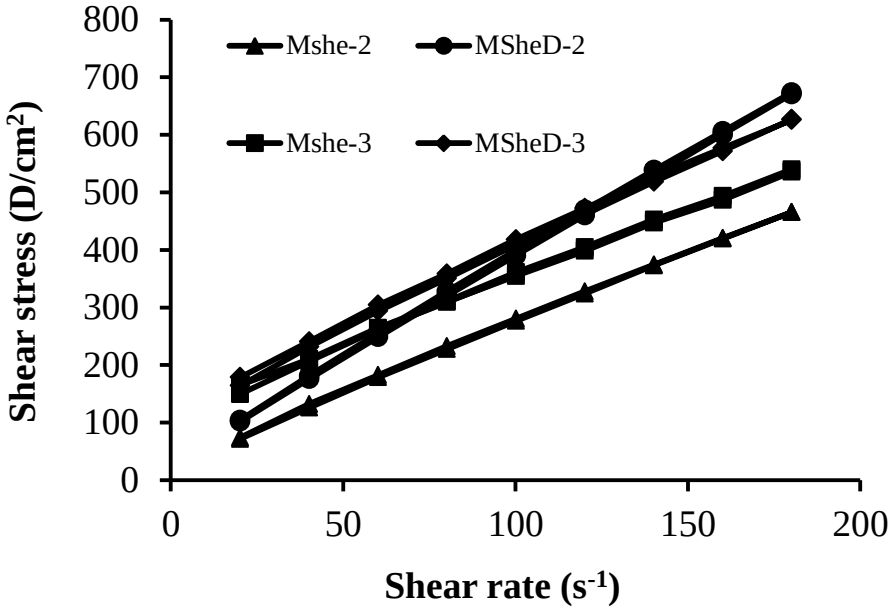


Figure 40 Flow curve of ISM containing bleached shellac with and without doxycycline hyclate in two solvents (DMSO and 2-pyrrolidone) at 25°C (n=3).

Typically, the flow index (N value) indicats the flow type. All formula showed pseudoplastic flow because the flow index was less than 1. The flow index of ISM is shown in Table 43. For the injectable gel the rheological behavior should be a newtonian or pseudoplastic flow due to easiness for injection.

Table 43 Effect of type of solvent and drug on flow index and consistency index (n=3).

Sample	Flow index (N) (mean ± S.D.)	Consistency index (ŋ) (mean ± S.D.)
Mshe-2	0.85 ± 0.01	566.87 ± 63.70
MsheD-2	0.86 ± 0.01	761.60 ± 28.18
Mshe-3	0.57 ± 0.03	2659.00 ± 157.09
MsheD-3	0.60 ± 0.05	2676.33 ± 588.27

4.4.5 Syringeability studies

The syringeability of ISM prepared form bleached shellac is presented in Table 44. Force of ISM systems prepared from bleached shellac was not significantly different ($p>0.05$). The force of all formula was rather low about less than 1 N therefore they should be very easy for injection (Rungseevijitprapa and Bodmeier, 2009).

Table 44 Effect of the type of solvent and drug on the force of syringeability (n=3).

Formula	Force (N) (mean ± S.D.)
Mshe-2	0.466 ± 0.07
MSheD-2	0.681 ± 0.13
Mshe-3	0.682 ± 0.31
MSheD-3	0.737 ± 0.18

4.4.6 *In vitro* gel formation

In situ forming gel transforms from solution into microparticle by solvent exchange after injection into periodontal pocket (Parent *et al*., 2013). The *in vitro* gel formation of ISM fabricated from bleached shellac in phosphate buffers pH 6.8, 7.0 and 7.4 are shown in Tables 45, 46 and 47, respectively. The effect of type of solvent on the *in vitro* microparticle formation was demonstrated. The formulation containing DMSO as solvent exhibited the fastest transformation into gel because the polarity of DMSO was higher than that of 2-pyrrolidone (Sastry, 2004; Hollingsworth, 1952). The pH of phosphate buffers (pH 6.8, 7.0 and 7.4) did not affect to the gel formation. The drug loading did not influence the gel formation.

Table 45 *In vitro* gel formation of ISM prepared from bleached shellac with different solvents at pH 6.8.

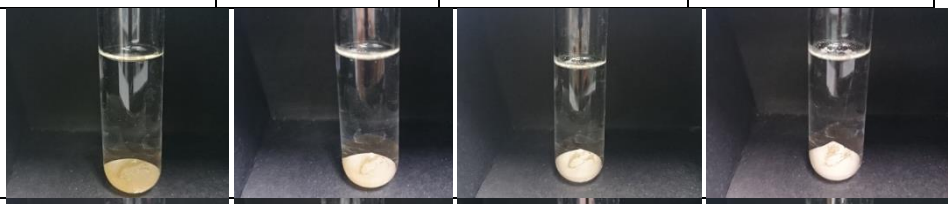
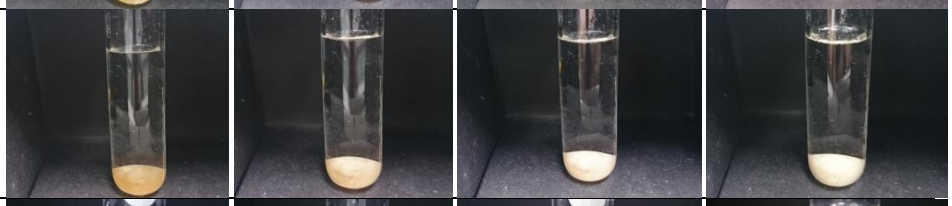
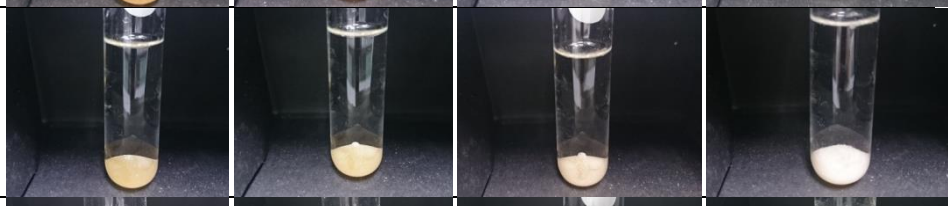
Formula	Time (min)			
	0	1	5	30
MShe-2				
MSheD-2				
MShe-3				
MSheD-3				

Table 46 *In vitro* gel formation of ISM prepared from bleached shellac with different solvents at pH 7.0.

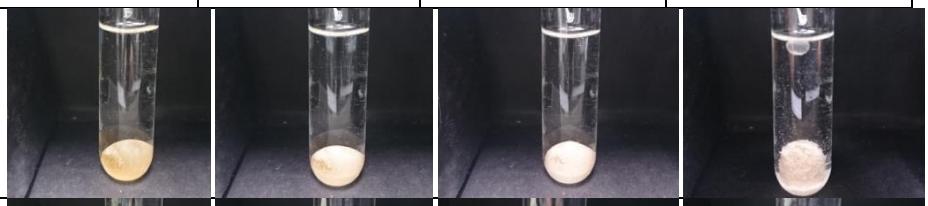
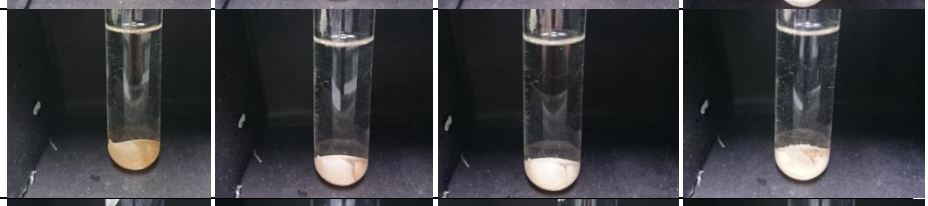
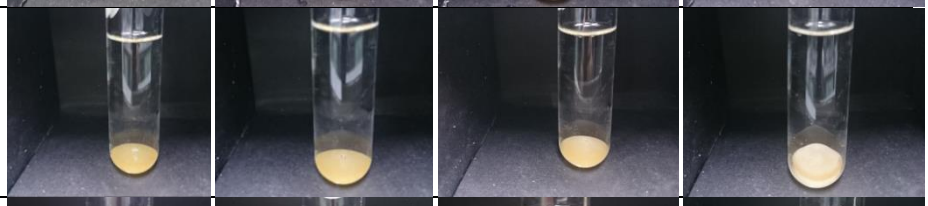
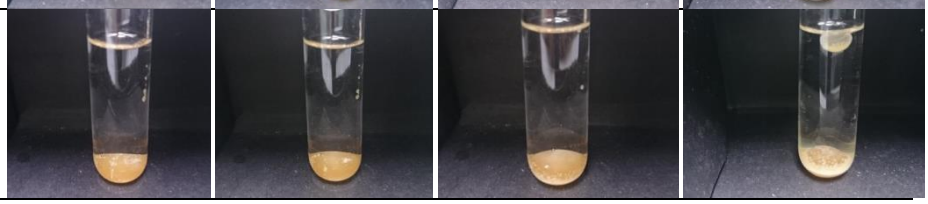
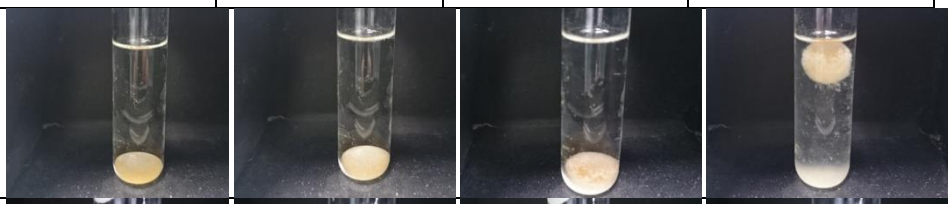
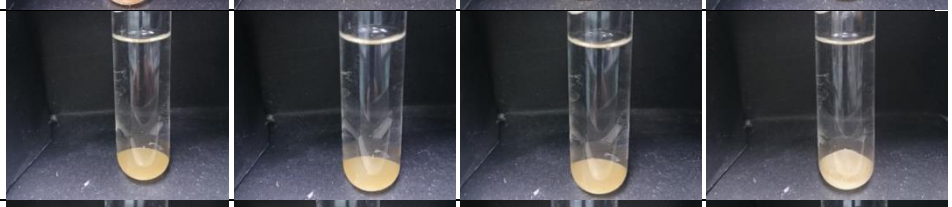
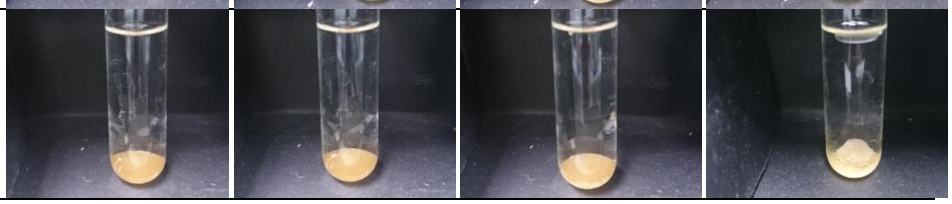
Formula	Time (min)			
	0	1	5	30
MShe-2				
MSheD-2				
MShe-3				
MSheD-3				

Table 47 *In vitro* gel formation of ISM prepared from bleached shellac with different solvents at pH 7.4.

Formula	Time (min)			
	0	1	5	30
MShe-2				
MSheD-2				
MShe-3				
MSheD-3				

Internal phase and external phase was mixed together by 2-syringe connector (Figure 41-A). ISM was injected into PBS (Figure 41-B). Initially the ISM was sol form (Figure 41-C) and then the water diffused through the external phase into the internal phase and solvent diffused out through the external phase into the environment (Figure 41-D). Then the gelling agent was precipitated into insoluble matter (Figures 41-E and F) (Giyoong *et al*., 2005). This characteristic was found in system of *in situ* forming microparticle containing PLGA dissolved in NMP as internal phase and peanut oil as external phase (Luan *et al.*, 2006). The step of gel formation is illustrated in Figure 41.

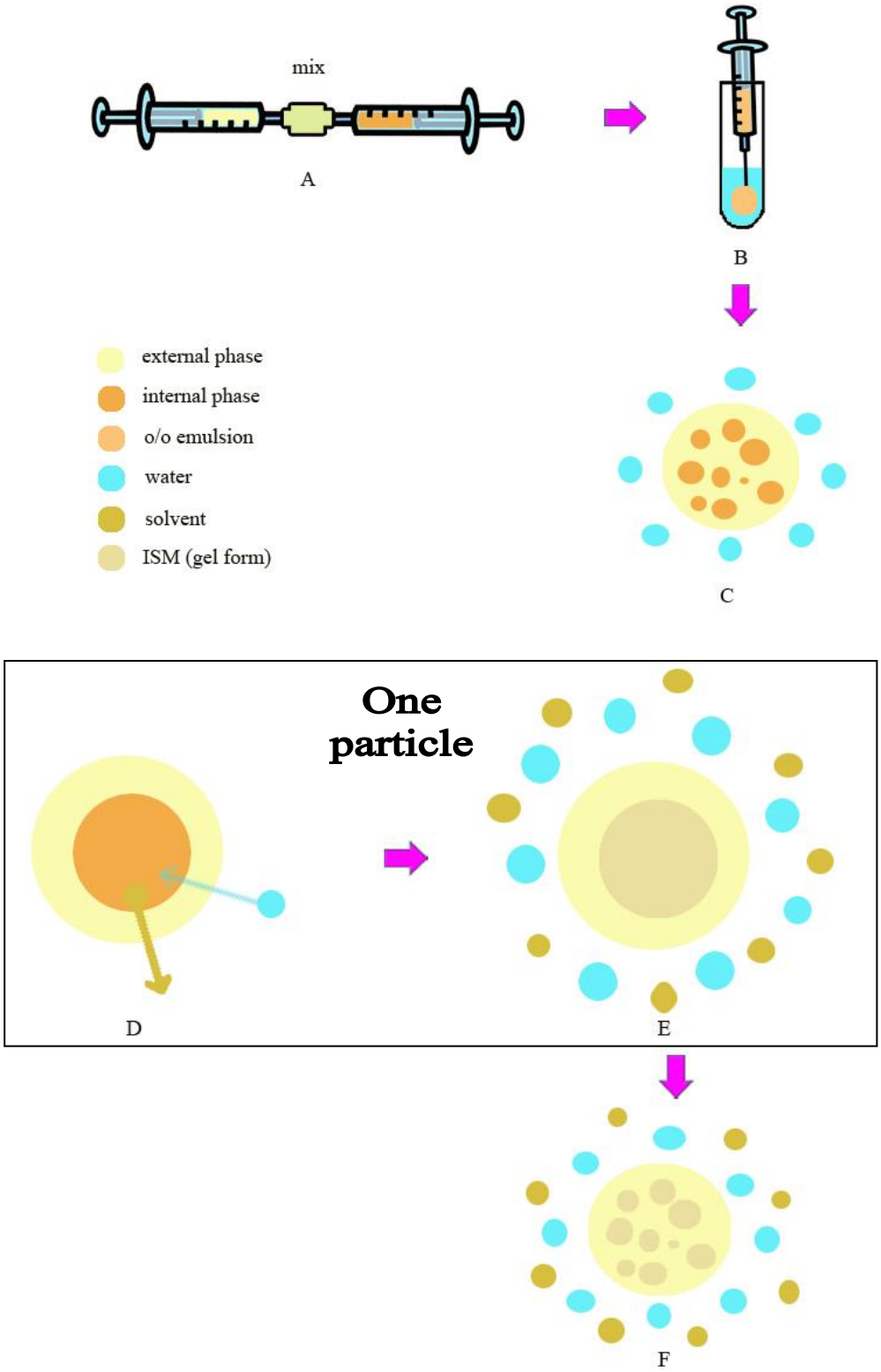


Figure 41 Diagram of gel formation of ISM

4.4.7 *In vitro* degradability studies

The weight loss of the ISM was evaluated in phosphate buffer pH 6.8 after 1 month of incubation at 37^{o}C and the percentage of weight loss was calculated by equation 14. For percentage of weight loss, the system prepared using DMSO as solvent without doxycycline hyclate was significantly different with the system with drug ($p<0.05$). The system prepared using 2-pyrrolidone as solvent with drug was not significantly different with the system without drug ($p>0.05$). The percentage of weight loss of ISM without and with doxycycline hyclate is shown in Table 48. Bleached shellac was used in pharmaceutical product such as enteric coated tablet and pellet (Roda *et al*., 2007). Bleached shellac can disslove in alkali solution.

Table 48 The percentage of weight loss of ISM from bleached shellac (n=3).

Formula	Percentage of weight loss (mean ± S.D.)
Mshe-2	83.59 ± 1.86
MsheD-2	78.90 ± 2.27
Mshe-3	99.61 ± 0.13
MsheD-3	96.64 ± 3.07

4.4.8 Antimicrobial activity studies

The inhibition zone diameter of *in situ* forming microparticles prepared from bleached shellac containing doxycycline hyclate using agar diffusion method are shown in Figure 42. The inhibition zone diameter against *S. aureus, S. mutans* and *P. gingivalis* of ISM fabricated from bleached shellac loading 5% doxycycline hyclate prepared with different solvents were significantly larger than that of the gel base ($p<0.05$). However the inhibition zone diameter ofhe system containing doxycycline hyclate was significantly lower than that of positive control, 75 mL of 5% w/v doxycycline hyclate solution ($p<0.05$). The system prepared with bleached shellac gradually transformed into gel that could control the drug release therefore the

inhibition zone of the developed systems was lower than that of the positive control (without bleached shellac).

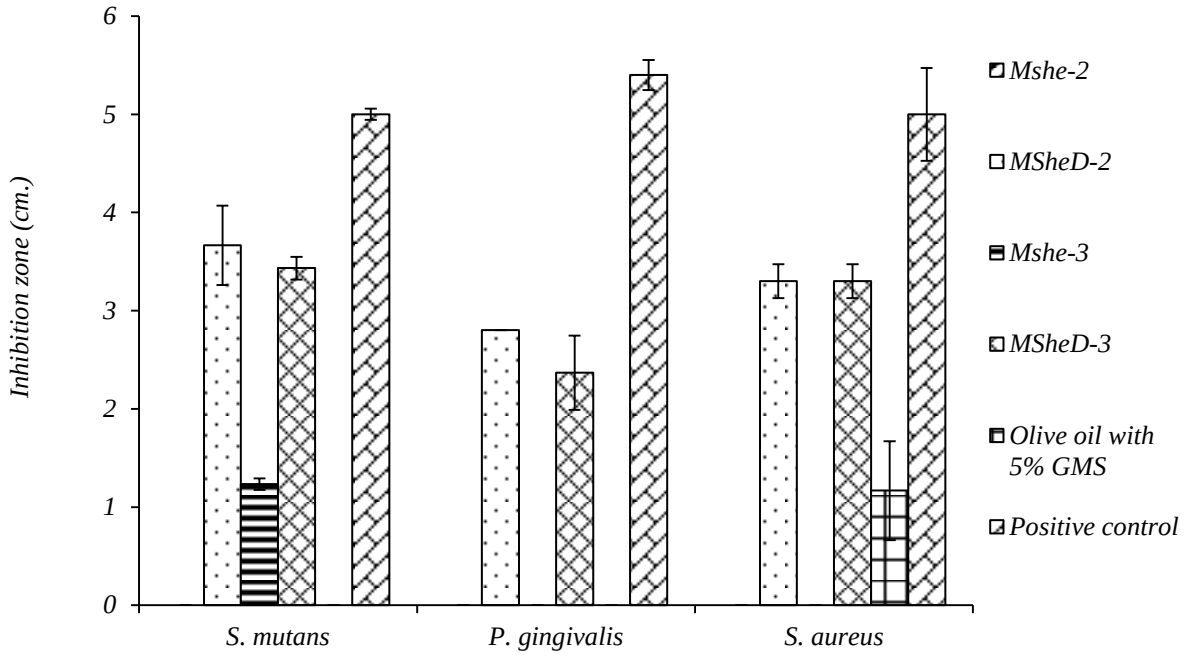


Figure 42 Inhibition zone diameter of the ISM prepared from bleached shellac containing doxycycline hyclate against *S. aureus*, *S. mutans* and *P. gingivalis* (n=3).

4.4.9 *In vitro* drug release studies

Dialysis membrane method

The doxycycline hyclate release was tested in phosphate buffer pH 6.8 to simulate the environment of periodontitis (Kulkarni *et al*., 2012) using the dialysis membrane method (Luan *et al*., 2006). Drug release from systems comprising DMSO and 2-pyrrolidone was not different at the initial phase. In the last the system prepared using DMSO as solvent exhibited the higher drug release than the system prepared using 2-pyrrolidone as solvent because of higher polarity of DMSO than 2-pyrrolidone (Sastry, 2004; Hollingsworth, 1952) (Figure 43). The drug release from system using DMSO and 2-pyrrolidone as solvent were 66.22% and 52.31% at 47 days, respectively.

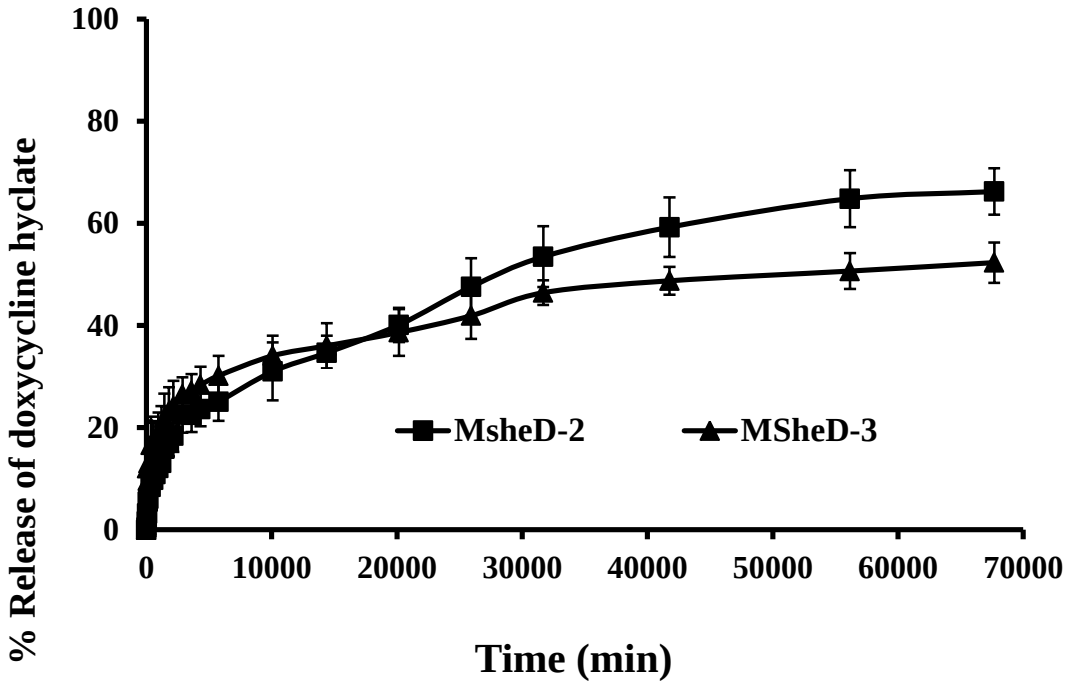


Figure 43 Release of doxycycline hyclate from ISM systems containing bleached shellac using dialysis method (n=3).

4.4.10 Analysis of drug release data

Release data of all formula were fitted to mathematical models such as first order, Higuchi's, zero order and power law model to characterize the mechanism of drug release. The high value of coefficient of determination (r^2) and model selection criteria (msc) indicated a superiority of the release profile fitting to mathematical equations as shown in Table 49. The doxycycline hyclate release from ISM prepared using DMSO as solvent were fitted well with Higushi's model whereas, the doxycycline hyclate release from ISM prepared using 2-pyrrolidone as solvent were fitted well with zero order. It has been reported the zero order drug release from poloxamer-base *in situ* gelling in phosphate buffer pH 6.8 (Kulkarni *et al*., 2012). Drug release from ISM containing PLA and NMP in phosphate buffer pH 7.0 exhibited the low initial release and linear continueous release (Luan *et al*., 2006).

The release exponent values (n) from power law are shown in Table 50. The n value of all systems was less than 0.45. The results indicated that the formula showed drug release by Fickian diffusion mechanism. The kinetic constant from the equation 8 indicated the drug release rate from the system (Kunche *et al*., 2012). The drug release rate (k) parameter after releasing studies using dialysis membrane method was investigated. The drug release rate of the system prepared using DMSO as solvent was not significantly different with the system prepared using 2-pyrrolidone as solvent ($p>0.05$).

Table 49 Comparison of degree of goodness-of-fit from curve fitting of the release profiles of doxycycline hyclate from the ISM in phosphate buffer pH 6.8 using dialysis membrane method to different release models.

Formula (%w/w)	First order		Higuchi's		Zero order		Power law	
	r^2	msc	r^2	msc	r^2	msc	r^2	msc
MSheD-2	0.9917	0.92	0.9947	3.05	0.9921	3.58	0.9945	4.13
MSheD-3	0.9569	0.76	0.9860	1.02	0.9982	3.07	0.9930	3.95

Table 50 Estimate parameter from curve fitting of doxycycline hyclate from the ISM release in phosphate buffer pH 6.8 using dialysis membrane method to power law expression.

Formula (%w/w)	k ± S.D.	n ± S.D.	Release mechanism
MSheD-2	0.0111 ± 0.0072	0.39 ± 0.09	Fickian diffusion
MSheD-3	0.0147 ± 0.0030	0.23 ± 0.05	Fickian diffusion

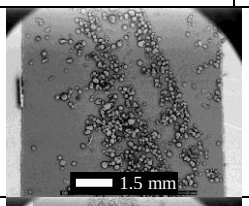
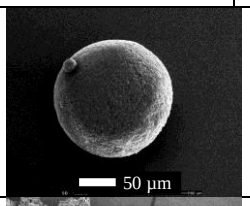
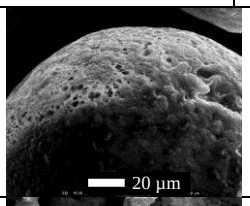
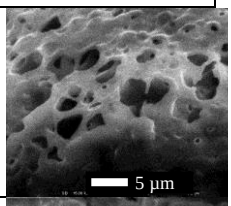
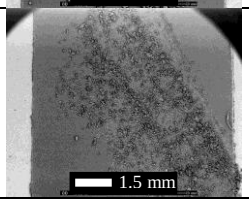
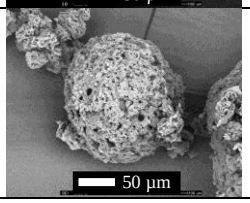
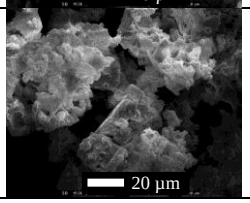
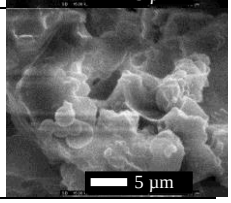
k = constant, and n = diffusional exponent

4.4.11 Determination of morphology

4.4.11.1 Scanning electron microscopy (SEM)

The sample of prepared ISM was collected after immersion in 100 mL phosphate buffer pH 6.8 for 3 h, washed with 50 mL of 1% sodium lauryl sulfate solution and distilled water, frozen and dried by freeze-dryer. The external phase of the system was removed by 50 mL of 1% sodium lauryl sulfate solution. Subsequently, the sample was characterized by SEM (Table 51). The structures were clarified at magnifications of 6X, 200X, 500X and 2000X. The particles of the system prepared with DMSO were spherical and there were many pores throughout the matrix like a microsponge. For the microparticles of the system prepared with 2-pyrrolidone was rather irregular because of their softness characteristic and efficient erosion which the degradability result confirmed this structure of the system. It has been reported that the morphology of ISM containing PLA and PLGA as gelling agent and peanut oil as external phase showed a porous topography (Luan *et al*., 2006).

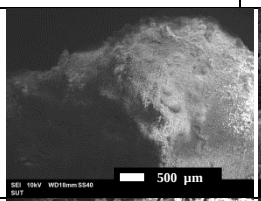
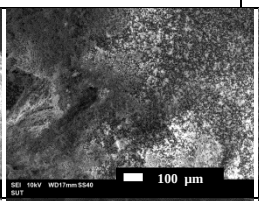
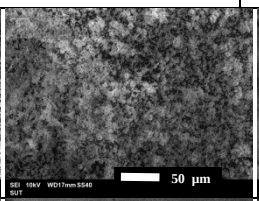
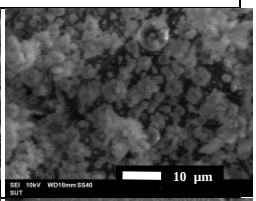
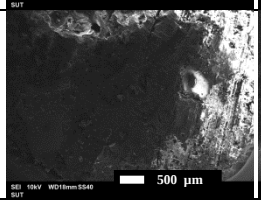
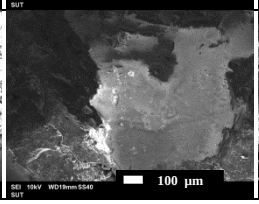
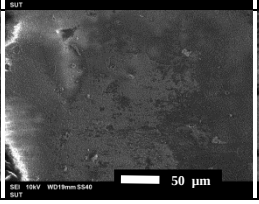
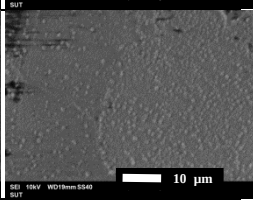
Table 51 SEM micrograph of the dried microparticle systems with different magnifications (6X, 200X, 500X and 2000X).

Formula	6X	200X	500X	2000X
MSheD-2				
MSheD-3				

4.4.11.2 Cryo-scanning electron microscopy (Cryo-SEM)

The cryo-SEM micrographs of the microparticle prepared from bleached shellac with doxycycline hylate and different types of solvent are shown in Table 52. The bleached shellac-doxycycline hyclate prepared with 2-pyrrolidone was homogenous structure while the bleached shellac-doxycycline hyclate prepared with DMSO were agglomerative particles.

Table 52 Cryo-SEM micrograph of the microparticle prepared from bleached shellac with different magnifications (25X, 100X, 400X and 2000X).

Formula	25X	100X	400X	2000X
MSheD-2				
MSheD-3				

4.4.12 *In vitro* gel formation (pig's gum)

The ISM containing bleached shellac as gelling agent comprising 2-pyrrolidone and doxycycline hyclate was injected from 18-gauge needle into pig's gum. Pig's gum was operated with surgical blades after 30 min of injection. The system could transform into gel inside pig's gum. It has been reported that the gel formation containing multiblock poly(ester amino urethane)s was injected in the rat. The system could form gel within 15 min (Dayananda *et al.*, 2008).

Figure 44 Gel formation in pig's gum of the *in situ* forming microparticle system prepared from bleached shella

From previous studies indicated the reason for choose the suitable system. The system containing eutectic could not mix with external phase because of its high viscosity and the system containing NMP was instable. Therefore, the system containing DMSO and 2-pyrrolidone was more suitable for further development. The appearance, rheology, drug release and syringeability of the ISMs containing bleached shellac were not different apparently. The viscosity the gel prepared with 2-pyrrolidone was higher than that prepared with DMSO. The gel formation of the system containing DMSO was faster than that containing 2-pyrrolidone. The system containing 2-pyrrolidone as solvent exhibited the higher degradation than that containing DMSO. Hence the system containing 2-pyrrolidone as solvent was the most suitable for periodontitis treatment.

CHAPTER 5
CONCLUSION

In situ forming gel systems containing cholesterol as gelling agent loaded with doxycycline hyclate and metronidazole showed the similar trend for the viscosity, rheology, antimicrobial activity and drug release behavior. The system containing doxycycline hyclate was selected for further development to minimize the burst release with an addition of benzyl benzoate. The burst release of the system could be decreased when the amount of benzyl benzoate was increased. The viscosity of the system was increased when the amount of benzyl benzoate was increased with newtonian flow. Surprisingly, the systems were easy to be injected into the desired site because of their minimal syringeability. They could transform from solution into gel but the formulation with more concentration of benzyl benzoate took longer time. However the degradability was decreased when the amount of benzel benzaote was increased. These systems inhibited *P. gingivalis, S. mutans* and *S. aureus* effectively. From the drug release profile the *in situ* forming gel system with 10% benzyl benzoate was the most suitable owing to sustainable release manner for 10 days. And the releases study suggested first order kinetic and fickian diffusion as possible mechanism of drug release. Therefore the system containing 10% doxycycline hyclate and 10% benzyl benzoate was the proper formulation.

In situ forming gel systems prepared with bleached shellac as gelling agent using NMP, DMSO, 2-pyrrolidone and eutectic mixture as solvent were also developed. The viscosity of the system containing eutectic mixture was apparently higher than that containing 2-pyrrolidone > DMSO > NMP, respectively. These systems exhibited newtonian flow with low syringeability of 1-3 N except the system containing eutectic mixture. System containing 2-pyrrolidone exhibited the highest degradability. These developed systems containing doxycycline hyclate could inhibit *P. gingivalis, S. mutans* and *S. aureus*. By comparison, the drug release profile of the

in situ forming gel system with 2-pyrrolidone as solvent was the most suitable since the sustainable drug release was attained for 40 days. Zero order kinetic and anomalous transport was as possible mechanism of drug release from this system. Therefore the system containing 10% doxycycline hyclate employing 2-pyrrolidone as solvent was suitable for periodontitis treatment.

The *in situ* forming gel containing bleached shellac was used as internal phase of ISM and olive oil comprising GMS was used as external phase. The ISM was prepared by 2-syringes connector. The 2.5% glycerol monosterate (GMS) dissloved in olive oil and the ratio 1:1 external phase : internal phase were the most suitable components. The system containing eutectic mixture as solvent could not mix with external phase because of its high viscosity whereas the system containing NMP was instable due to the system was low viscosity. The system containing DMSO and 2-pyrrolidone was more suitable for further development. The viscosity of the system containing 2-pyrrolidone was higher than that of system containing DMSO as solvent. The rheology of the system containing bleached shellac as gelling agent was pseudoplastic flow with notably low syringeability force of less than 1 N. The system containing 2-pyrrolidone showed higher degradability than that containing DMSO. Developed doxycycline hyclate ISM could inhibit *P. gingivalis*, *S. mutans* and *S. aureus*. Drug release from systems comprising DMSO and 2-pyrrolidone was not different at the initial. The *in vitro* release study indicated the sustained drug release for 40 d. Release kinetic of system containing DMSO and 2-pyrrolidone was Higuchi's model and zero order, respectively and mechanism of drug release was Fickian diffusion. Therefore the *in situ* forming gel containing bleached shellac dissolved in 2-pyrrolidone as internal phase of ISM and olive oil comprising GMS as external phase loading doxycycline hyclate prepared as ISM was suitable formulation for periodontitis treatment.

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APPENDICES

(A)

Standard curve for the *in vitro* release study

1. Determination of the amount of doxycycline hyclate released

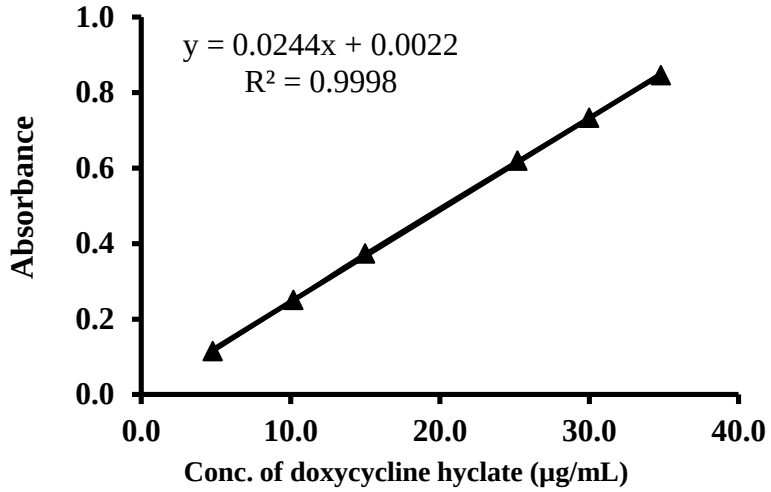

Figure 45 Standard curve of doxycycline hyclate in phosphate buffer pH 6.8 for the *in vitro* release study (UV-vis spectrophotometry at 379 nm).

2. Determination of the amount of metronidazole released

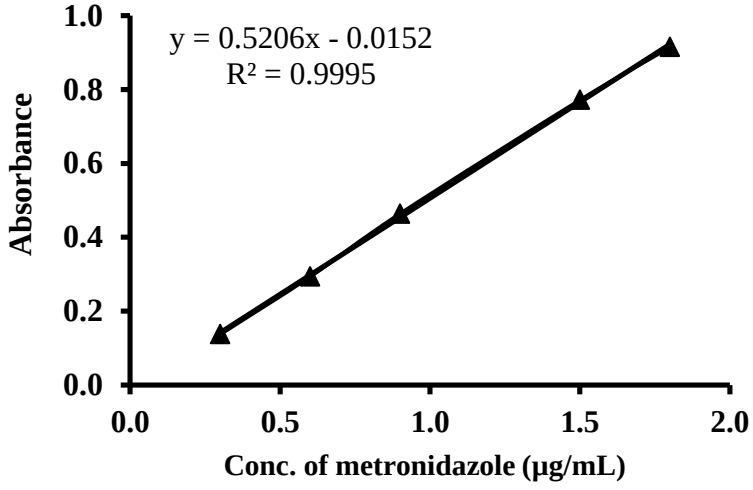

Figure 46 Standard curve of metronidazole in phosphate buffer pH 6.8 for the *in vitro* release study (UV-vis spectrophotometry at 320 nm).

(B)

Antimicrobial activity against *P. gingivalis, S. mutans and S. aureaus* measurement

Table 53 Inhibition zone diameter of the *in situ* forming gel prepared from cholesterol containing doxycycline hyclate and metronidazole against *P. gingivalis* (n=3).

Formula	Clear zone (mean ± SD)
10% cholestrol , 10% metronidazole in NMP	3.4 ± 0.1
10% cholestrol , 10% doxycycline hyclate in NMP	4.6 ± 0.4
10% cholestrol in NMP (no drug)	0.7 ± 0.0
NMP	1.3 ± 0.2
Ampicillin disk	1.5 ± 0.1

Table 54 Inhibition zone diameters of the *in situ* forming gels prepared from cholesterol containing benzyl benzoate against *S. aureus, S. mutans* and *P.gingivalis* (n=3).

Formula	Clear zone (mean ± SD)		
	Streptococcus mutans	*Porphyromonas gingivalis*	*Staphylococcus aureus*
10% cholestrol in NMP	2.8 ± 0.3	0.7 ± 0.0	1.6 ± 0.4
10% cholestrol, 10% benzyl benzoate in NMP	2.7 ± 0.3	1.1 ± 0.1	1.4 ± 0.4
10% cholestrol, 20% benzyl benzoate in NMP	2.7 ± 0.4	0.8 ± 0.2	1.4 ± 0.5
10% cholestrol, 30% benzyl benzoate in NMP	2.5 ± 0.1	0.9 ± 0.3	1.2 ± 0.5
10% cholestrol , 10% doxycycline hyclate in NMP	5.4 ± 0.3	4.6 ± 0.4	4.4 ± 0.3
10% cholestrol, 10% benzyl benzoate , 10% doxycycline hyclate in NMP	5.4 ± 0.2	4.5 ± 0.3	4.6 ± 0.2
10% cholestrol, 20% benzyl benzoate , 10% doxycycline hyclate in NMP	5.7 ± 0.3	4.2 ± 0.3	4.7 ± 0.4
10% cholestrol, 30% benzyl benzoate , 10% doxycycline hyclate in NMP	5.3 ± 0.1	4.1 ± 0.3	4.5 ± 0.1

Table 55 Inhibition zone diameter of the *in situ* forming gel prepared from bleached shellac containing doxycycline hyclate against *S. aureus*, *S. mutans* and *P. gingivalis*.

Formula	Clear zone (mean ± SD)		
	Streptococcus mutans	*Porphyromonas gingivalis*	*Staphylococcus aureus*
30% Bleached shellac in NMP	2.0 ± 0.1	1.4 ± 0.3	1.3 ± 0.1
30% Bleached shellac in DMSO	1.2 ± 0.1	1.2 ± 0.1	1.0 ± 0.1
30% Bleached shellac in 2-pyrroridone	1.5 ± 0.4	1.2 ± 0.1	0.7 ± 0.0
30% Bleached shellac in menthol and champhor	0.8 ± 0.1	0.7 ± 0.0	0.9 ± 0.3
30% Bleached shellac, 10% Doxycycline hyclate in NMP	3.7 ± 0.6	4.0 ± 0.2	3.4 ± 0.2
30% Bleached shellac, 10% Doxycycline hyclate in DMSO	4.7 ± 0.3	4.3 ± 0.2	3.7 ± 0.1
30% Bleached shellac, 10% Doxycycline hyclate in 2-pyrroridone	4.0 ± 0.5	4.1 ± 0.0	3.4 ± 0.1
30% Bleached shellac in, 10% Doxycycline hyclate menthol and champhor	3.4 ± 0.1	3.4 ± 0.3	3.2 ± 0.1
Cup doxycycline hyclate 15 mcg	5.6 ± 0.3	5.8 ± 0.1	5.3 ± 0.3

Table 56 Inhibition zone diameter of the ISM prepared from bleached shellac containing doxycycline hyclate against *S. aureus, S. mutans* and *P. gingivalis* (n=3).

Formula	Clear zone (mean ± SD)		
	Streptococcus mutans	*Porphyromonas gingivalis*	*Staphylococcus aureus*
30% Bleached shellac in DMSO (olive oil with 5% GMS)	0.7 ± 0.0	0.7 ± 0.0	0.7 ± 0.0
30% Bleached shellac, 10% Doxycycline hyclate in DMSO (olive oil with 5% GMS)	3.7 ± 0.4	2.8 ± 0.0	3.3 ± 0.2
30% Bleached shellac in 2-pyrroridone (olive oil with 5% GMS)	1.2 ± 0.1	0.7 ± 0.0	0.7 ± 0.0
30% Bleached shellac, 10% Doxycycline hyclate in 2-pyrroridone (olive oil with 5% GMS)	3.4 ± 0.1	2.4 ± 0.4	3.3 ± 0.2
Olive oil with 5% GMS	0.7 ± 0.0	0.7 ± 0.0	1.2 ± 0.5

Cup doxycycline hyclate 7.5 mcg	5.0 ± 0.1	5.4 ± 0.2	5.0 ± 0.5

Output

Presentation

Oral

Orn Setthajindalert and Thawatchai Phaechamud. (2013). Cholesterol *In situ* Forming Gel Loading Metronidazole. Sustainable Technology Devolopment Conference at Faculty of engineering and industrial technology Silpakorn University, Nakhon Pathom, Thailand. (Nov 2-3, 2013)

Scientific Publication

Orn Setthajindalert and Thawatchai Phaechamud. (2013). Cholesterol *in situ* forming gel loading metronidazole. Sustainable Technology Devolopment Conference at Faculty of engineering and industrial technology Silpakorn University. Eng Ind Tech Silpakorn University (proceeding). 2-3 December 2013: pp. 265-272.

Manuscripts (Submitted)

- Doxycycline Hyclate-loaded Bleached Shellac *In Situ* Forming Microparticle for Intraperiodontal Pocket Local Delivery.

- Cholesterol *In Situ* Forming Gel for Intraperiodontal Pocket Delivery of Antimicrobial Agents.

Review Article Publication

Orn Setthajindalert and Thawatchai Phaechamud. (Jul-Sep, 2012). Solvent exchange *in situ* forming gel. Thai Pharm Health Sci J. 7(3): 137-142.

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- Pharmaceutical dry coating
- Kinetics of drug release
- Controlled drug delivery system
- Herbal preparation and development
- Pharmaceutical excipient characterization
- Polymeric film characterization
- Compatibility between dye and polymer

5. Technique Speacialisation

- X-ray powder diffraction
- Tensile testing
- IR spectrometry
- Dissolution test analysis
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