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Solvent exchange-induced *in situ* forming gel comprising ethyl cellulose-antimicrobial drugs

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ABSTRACT

Solvent-exchanged *in situ* forming gel is a drug delivery system which is in sol form before administration. When it contacts with the body fluid, then the water miscible organic solvent dissipates and water penetrates into the system, leading the polymer precipitation as *in situ* gel at the site of injection. The aim of this research was to study the parameters affecting the gel properties, drug release and antimicrobial activities of the *in situ* forming gels prepared from ethyl cellulose (EC) dissolved in *N*-methyl pyrrolidone (NMP) to deliver the antimicrobial agents (doxycycline hyclate, metronidazole and benzyl peroxide) for periodontitis treatment. The gel appearance, pH, viscosity, rheology, syringeability, gel formation, rate of water diffusion into the gels, *in vitro* degradation, drug release behavior and antimicrobial activities against Staphylococcus aureus, *Escherichia coli*,*Candida albicans*, *Streptococcus mutans* and *Porphyrommonas gingivalis* were determined. Increasing the amount of EC increased the viscosity of system while still exhibiting Newtonian flow and increased the work of syringeability whereas decreased the releasing of drug. The system transformed into the rigid gel formation after being injected into the simulated gingival crevicular fluid. The developed systems containing 5% w/w antimicrobial agent showed the antimicrobial activities against all test bacteria. Thus the developed solvent exchange-induced *in situ* forming gels comprising EC-antimicrobial drugs exhibited potential use for periodontitis treatment.

1. Introduction

In situ forming gel is a drug delivery system, which is in a sol form before administration in the body and alters into a gel form after injection into the target site (Jigar, 2011). Nowadays, *in situ* forming gel systems are gaining importance in drug delivery, since the advantages of them possess over the conventional formulations. They could sustain the drug action, localize the drug delivery, reduce the dose and frequency of administration and improve a patient compliance (Hatefi and Amsden, 2002; Nirmal et al., 2010). Injectable systems are particularly attractive for the delivery of drugs into the periodontal pocket. This application can be easily and rapidly performed, without pain, by using the proper needle (Xiong et al., 2011). The treatment of periodontitis by the intra-pocket drug delivery systems is interesting due to the prospect of maintaining effective high level of drug in the gingival crevicular fluid for a prolonged period of time to produce the desirable clinical benefits (Medlicott, 1994). Chitosan gels comprising metronidazole demonstrated the effectiveness in the periodontitis treatment (Akncbay et al., 2007). Tetracycline-loaded bioadhesive semisolid based upon hydroxyethylcellulose and polyvinylpyrrolidone (Jones et al., 1996) and metronidazole-loaded systems based upon Carbopol 974P, hydroxyethylcellulose and polycarbophil have also been mentioned (Jones et al., 1997). *In situ* gels of moxifloxacin loaded into gellan gum and sodium alginate hydrocolloids using ion activated systems were developed for the periodontitis treatment (Kunche et al., 2012). Secnidazole-serratiopeptidase was formulated as a pH-sensitive *in situ* gelling periodontal formulation using alginate combinded with HPMC (Priyanka and Meenakshi, 2011). *In situ* gel implants of ornidazole with mucoadhesive polymers including Pluronic F-127, HPMC K-100, Carbopol 934P and PVP-K-30 were prepared for periodontal treatment (Rawat et al., 2010). HPMC gel matrix was prepared as *in situ* forming gel using biphasic calcium phosphate as filler (Seyedlar et al., 2014). The delivery of articaine for anesthetizing periodontal pockets using poloxamer has been reported (Kulkarni et al., 2012). Owing to the hydrophilic nature of aboved polymers, the loaded active compounds were rarely sustainable.

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For solvent exchange-induced *in situ* forming depot system, the water insoluble polymer is dissolved in a pharmaceutically acceptable organic solvent and is miscible or partially miscible with aqueous environment. Drug is incorporated to this polymeric solution. The precipitation of polymer occurred after exposure with the aqueous at injection site inducing the formation of a depot entrapping the drug with the controlled release manner. Ethylcellulose or Ethocel (EC), a well-known water-insoluble polymer, could dissolve in an organic solvent to produce the water-insoluble films after solvent evaporation (Bodmeier and Paeratakul, 1994; Madan et al., 2009). It is widely used in oral and topical pharmaceutical formulations, because it is nontoxic, non-allergenic and non-irritant. It has been used as a hydrophobic coating agent in oral dosage forms for controlled drug release, moisture protection and taste masking (Sadeghi et al., 2001). It has been used as main matrix polymer for chlorhexidine-loaded strip (Friedman and Golomb, 1982) and metronidazole-loaded film (Golomb et al., 1984) or tetracycline HCl-loaded film (Azoury et al., 1988). The use of EC as *in situ* forming gelling agent has not been reported previously.

The formation mechanism of *in situ* gel forming systems is the precipitation of a polymer by solvent exchange. The 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). The diffusion of solvent from polymer solution into surrounding environment resulted in precipitation or solidification of polymer matrix for controlling the drug release (Nirmal et al., 2010; Parent et al., 2013). NMP is used as solvent in this study. It is thermally stable, biodegradable and biocompatible which can be used as an attractive solubilizer in the pharmaceutical field (Sanghvi et al., 2008).

Doxycycline hyclate (DH) is a bacteriostatic antibiotic used for periodontal therapy. This drug interferes the bacterial protein synthesis (Seymour and Heasman, 1995). Atrigel® is an injectable delivery system for periodontitis treatment. It contains 5 or 10% doxycycline hyclate using poly(DL-lactide) as polymer dissolved in NMP (Schwach et al., 2000). The use of Atrigel® could provide a high level of doxycycline (250 μg/mL) in the gingival crevicular fluid for a period of 7 days and 10–20 μg/mL was still present for 3–5 days after the polymer had been removed (Jain et al., 2008). Metronidazole (MT) is a bactericidal agent against anaerobic bacteria. It has been proposed that MT is intracellularly activated by reduction and the toxic effect of reduced intermediates bind to DNA leading to loss of helical structure, strand breakage and impairment of DNA function and it has been used in the field of periodontal therapy (Rizzo et al., 2010). Benzoyl peroxide (BP) consists of two benzoyl peroxide groups bridged by a peroxide link releasing the free radical oxygen species capable of oxidizing bacterial proteins (Waller et al., 2005). BP in Eudragit RS systems containing peppermint oil has been developed for periodontitis treatment (Mahadlek et al., 2013). These three drugs were used as model drugs in this study.

The aim of this study was to develop the *in situ* gel forming systems of antimicrobial agents for periodontitis treatment. The *in situ* gel formulations containing EC, drugs (DH, MT and BP) and solvent (NMP) were prepared and investigated for their physical properties and biological action as appearance, pH, viscosity, rheology, syringeability, gel formation, rate of water diffusion into the gels, *in vitro* degradation and antimicrobial activities against *Staphylococcus aureus*, *Escherichia coli*, *Candida albicans*, *Streptococcus mutans* and *Porphyrommonas gingivalis*.

2. Materials and methods

2.1. Materials

Ethocel 10 (EC) (The Dow Chemical Company, USA) was used as received. DH (Batch No. 20071121, Huashu Pharmaceutical Corporation, Shijiazhuang, China), BP and MT (kindly supported by T.MAN Pharma Ltd., Part, Bangkok, Thailand) were used as model drugs. Brain Heart Infusion (BHI) (lot no. 0270845, Bacto™, USA), Brain Heart Infusion Agar (BHA) (lot no. 0298038, Bacto™, USA), Mitis Salivarius Agar (MSA) (lot no. 0118681, DifcoTM, USA), Sabouraud Dextrose Agar (SDA) (lot no. 7312647, Difco™, USA), Sabouraud Dextrose Broth (SDB) (lot no. 6345690, Difco™, USA), Tryptic Soy Agar (TSA) (lot no. 7341698, Difco™ , USA), Tryptic Soy Broth (TSB) (lot no. 8091999, Difco™, USA) were used as media for antimicrobial test. *N*-methyl-2-pyrrolidone (NMP) (lot no. A0251390, Fluka, New Jersey, USA) was used as solvent for EC. Potassium dihydrogen orthophosphate (lot no. E23W60, Ajax Finechem, Australia) and sodium hydroxide (lot no. AF 310204, Ajax Finechem, Australia) were used as components in phosphate buffer pH 6.8. Dialysis tube (Spectra/Por®® membrane MWCO: 6,000-8,000, lot no. 32644, Spectrum Laboratories, Inc., CAL, USA) was used as received.

2.2. Preparation of the in situ forming gel systems

The injectable *in situ* forming gel base was prepared by dissolving different amounts of EC (5, 10, 15, 20, 25, 30, 35 and 40% w/w) in NMP. Each dispersion was stirred for 24 h then a clear solution was formed. The 5% w/w antimicrobial agents (DH, MT and BP) were incorporated into the prepared EC solution. Each system was kept for 24 h then a clear solution was formed. The components of the prepared systems are shown in Table 1.

2.3. Evaluation of gel properties

2.3.1. Gel appearance and pH measurement

The appearances of systems, color and homogeneity were observed by visual observation. The pH value of each formulation was measured by pH meter using flat surface probe (Ultra Basic UB-10, Denver Instrument, Bohemia, New York) ($n = 3$).

2.3.2. Viscosity studies

The viscosity of the prepared systems was determined using Brookfield DV-III Ultra programmable rheometer (Brookfield Engineering Laboratories Inc, Middleboro, MA, USA) with spindles (CP-40). Viscosity parameters were collected at different shear rates with 15 s equilibration time at every shear rate. The viscosity measurements were done at 25 °C and 37 °C which were the room and physiological temperatures, respectively. 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 (Sato et al., 2012).

Table 1
Composition formula of various gel systems containing different drugs (5% w/w).

Formula	Amount (% w/w)				
	EC	NMP	DH	MT	BP
EC-1	5	90	5	–	–
EC-2	10	85	5	–	–
EC-3	15	80	5	–	–
EC-4	20	75	5	–	–
EC-5	5	90	–	5	–
EC-6	10	85	–	5	–
EC-7	15	80	–	5	–
EC-8	20	75	–	5	–
EC-9	5	90	–	–	5
EC-10	10	85	–	–	5
EC-11	15	80	–	–	5
EC-12	20	75	–	–	5

2.3.3. Rheological behavior studies

Rheological studies were performed by Brookfield programmable rheometer (Brookfield DV-III Ultra, Brookfield Engineering Laboratories, Middleboro, MA, USA) fitted with CP-40 spindle. The shear stress of samples was measured at various shear rates at 25 °C and 37 °C. The temperature was maintained within ±0.1 °C by water bath (Buchi Heating bath B-490, New Hampshire, USA) connected to the sample cup of rheometer. The samples were equilibrated on the plate to reach the running temperature prior to measurement. The flow parameters were characterized using the exponential formula (Martin, 1993):

$$F^N = \eta^G \quad (1)$$

$$\mathrm{LogG} = \mathrm{NLogF} - \mathrm{Log}\eta \quad (2)$$

where F is shear stress, G is shear rate, N is an exponential constant and η is a viscosity coefficient.

2.3.4. Syringeability test

Syringeability of the dosage form is an important factor to consider for the ease of administration by injection which is the force required to expel the prepared product via a needle. Syringeability of each sample 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 stand. The 18-gauge needle is widely used in the dental field. The upper probe of the texture analyzer moved downwards at constant speed (1.0 mm s^{-1}) until it came in contact with the syringe barrel base. A constant force of 0.1 N was applied to the base and the distance required to expel the contents for a barrel length of 20 mm was measured at room temperature ($n = 3$). Force displacement profiles were determined, which the force at a distance of 10 mm were selected for analysis. The area under the resulting curve was used to determine the work of expulsion.

2.3.5. In vitro *gel formation*

In order to investigate the injectability and gel formation of the prepared solutions, samples (1 mL) were injected into phosphate buffer solution pH 6.8 in test tube (5 mL) with 18-gauge needle. Then the turbid gel formation was observed visually at various times (0, 1, 5 and 30 min).

2.3.6. Rate of water diffusion into the gels

The diffusion of aqueous phase into the prepared systems was studied using the observation of the system change from a sol into a turbid gel phase. Samples were filled in transparent plastic tube (diameter 6 mm) and they were immersed into 15 mL phosphate buffer solution pH 6.8. The system was changed apparently from transparent solution to opaque gel when the water diffused into. The distance of water front diffusion was observed at various times (0, 4 and 24 h). The rate of water diffusion into the gels was calculated as the following formula:

$$\text{Rate of water diffusion into the gels} = \frac{\text{distance of water front diffusion (mm)}}{\text{time(min)}} \quad (3)$$

2.3.7. In vitro *drug release studies*

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

2.3.7.1. Dialysis membrane method. The 1 g prepared system was weighed into dialysis tube (Spectrapor, MW cutoff: 6000-8000) and immersed in 100 mL phosphate buffer pH 6.8 (to simulate the gingival crevicular fluid) (Esposito et al., 1996) at 37 °C with maintained the rotational speed at 50 rpm using shaking incubator Model SI4 (Shel Lab, Cornelius, USA). Aliquots, 10 mL each, were withdrawn from the release medium at time intervals and each aliquot was replaced with 10 mL fresh medium. The amount of samples was determined by UV–vis spectrophotometer at 349, 320 and 275 nm for DH, MT and BP respectively. All of the experiments were triplicately done, and the mean cumulative drug release ± S.D. were calculated.

2.3.7.2. Membrane-less diffusion method. A membrane-less diffusion system was also used for studying drug release from the *in situ* gel systems. Sample (0.4 g) was added into the ceramic cup (10 mm × 12 mm) and then placed in 100 mL of phosphate buffer pH 6.8 at 37 °C and maintained the speed of rotation at 50 rpm using shaking incubator (Model SI4, Shel Lab, Cornelius, USA). Aliquots, 10 mL each, were withdrawn and determined for the drug release with the method described in Section 2.3.7.1.

2.3.7.3. Analysis of drug release data. The data obtained from the *in vitro* release were analyzed by a nonlinear computer programe, Scientist® for Windows, version 2.1 (MicroMath Scientific Software, 1995, SaltLake City, UT, USA). The cumulative 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 high value of coefficient of determination (r^2) or model selection criteria (msc) indicated a superiority of the release profile fitting to mathematical equations.

2.3.8. Determination of surface morphology of gels

Samples were determined for topography after the release study by drying them using the freeze dryer (Triad™ Labconco, Missouri, USA) for 48 h in order to avoid the collapse of porous structures. The dried samples were coated with gold prior to testing by scanning electron microscope (SEM) (Maxim 200Camscan, Cambridge, England). Micrographs were taken with SEM at an accelerating voltage of 15 kV.

2.3.9. In vitro degradation studies

Degradation studies of the prepared gels were studied by incubating in phosphate buffer pH 6.8. The sample was injected into phosphate buffer pH 6.8 (10 mL). Each sample was incubated in shaking bath at 37 °C with 50 rpm. Fresh phosphate buffer solution was replaced every week for 1 month. Then the sample was dried in hot air oven at 65 °C. The percentage of weight loss was carried out as following:

$$\%\text{weight loss} = \frac{\text{Initial weight} - \text{Finalweight}}{\text{Initial weight}} \times 100 \quad (4)$$

2.3.10. Antimicrobial activity studies

Antimicrobial activities of the samples were evaluated for both the standard microbes (*Staphylococcus aureus* ATCC 6538P, E. coli ATCC 25922 and *Candida albicans* ATCC 17110) and anaerobic microbes (*Streptococcus mutans* ATCC 27175 and *Porphyromonas gingivalis* ATCC 33277) using agar-cup diffusion method. The actively growing broth culture of microbes was prepared and the turbidity was approximately 10^8 cells/mL. Then, the swab was spread onto the agar plate and dried. The sterilized cylinder cups were carefully placed on the surface of the swabbed agar. The prepared gels were filled into the cylinder cup (8 mm diameter and 10 mm height) and incubated at 37 °C for 48 h. For the anaerobic bacteria the test was conducted in anaerobic incubator

(Forma Anaerobic System, Thermo Scientific, Ohio, USA). 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. were calculated.

2.3.11. Statistical analysis

Values from experimental data were expressed as mean ± standard deviation (S.D.). Statistical significance of data 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 $p < 0.05$.

3. Results and discussion

3.1. Gel appearance and pH measurement

The appearances of EC formula containing DH, MT and BP were yellowish solutions. The pH values of EC formula containing 5% w/w of drugs DH, MT and BP are shown in Table 2. The pH of EC formula containing DH, MT and BP (5% w/w) were in the range of 3.95 ± 0.05–4.42 ± 0.02, 9.53 ± 0.02–10.22 ± 0.02 and 8.15 ± 0.05–8.63 ± 0.03 respectively. The results indicated that the incorporation of drug affected the pH values of the prepared solutions because the pH of DH solution was between 2.0 and 3.0 (Kogawa and Salgado, 2012), whereas the pH of a saturated aqueous MT solution was 5.8 (Alexander et al., 1997). The pH of BP solution has not been reported because of its low solubility in water.

3.2. Viscosity studies

The viscosities of the EC formulations comprising DH at 25 °C and 37 °C, are shown in Fig. 1. The apparent viscosities of the 5–15% w/w EC formula comprising DH, MT and BP were constant when the shear rate was increased indicating Newtonian behavior, which the viscosity did not change as the shear rate was increased. Data of systems containing MT and BP was not shown however they showed the same trend. While the apparent viscosities of the EC (20% w/w) formula comprising DH, MT and BP at a low shear rate were higher than that at a high shear rate, indicating pseudoplastic behavior. All formula comprising DH, MT and BP exhibited a decrease in viscosity with increasing temperature. The viscosity of systems was increased when drugs were incorporated as some research reported the drug-polymer interaction increased a viscosity of the gel system (Mayol et al., 2008).

3.3. Rheological behavior studies

The rheological behavior of solutions comprising DH, MT and BP was investigated as a function of polymer amount and temperature. The shear stress of all formulae with drugs was higher than that of gel base (data not shown). The shear stress of all formula comprising 5% w/w drugs (DH, MT and BP) was increased as the shear rate and polymer amounts were increased. All formula showed the Newtonian flow, indicating the up curve did coincide

Table 2
pH values of EC systems containing different drugs.

EC (%w/w)	pH ± S.D. (n = 3)			
	Without drug	With drug (5%w/w)		
		DH	MT	BP
5%	10.58 ± 0.04	3.95 ± 0.05	10.22 ± 0.02	8.15 ± 0.05
10%	9.24 ± 0.02	4.05 ± 0.01	9.86 ± 0.02	8.49 ± 0.04
15%	9.19 ± 0.02	4.36 ± 0.01	9.63 ± 0.05	8.56 ± 0.02
20%	9.57 ± 0.06	4.42 ± 0.02	9.53 ± 0.02	8.63 ± 0.03

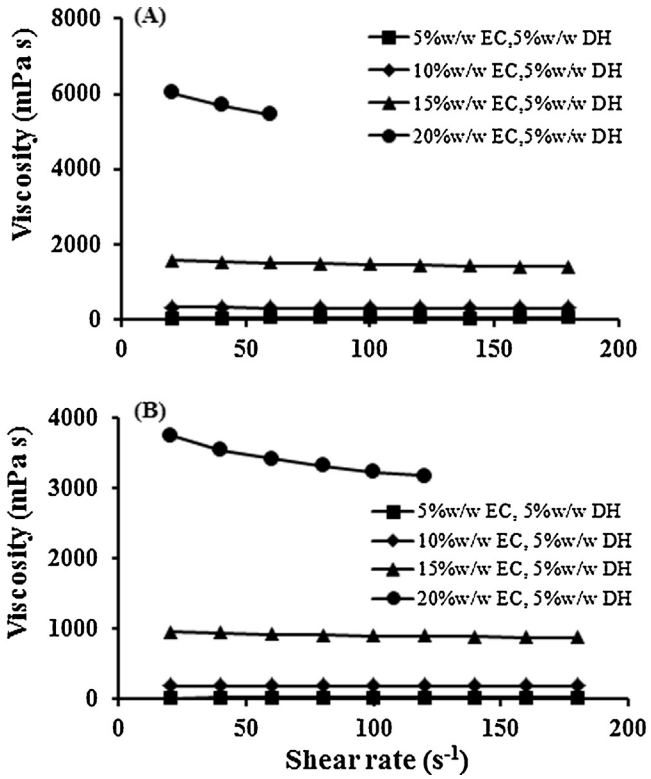

Fig. 1. Viscosity curves of EC systems containing 5% w/w DH at (A) 25 °C and (B) 37 °C.

with the down curve. The curves moved to a higher shear stress value indicating compact structure of the gels. On the other hand, the effect of temperature on rheological study of the formula comprising drugs (DH, MT and BP) was investigated. The shear stress of all formulations at 37 °C was lower than that at 25 °C which the curves of formulations moved to a lower shear stress value indicating loose structure. The viscosity measurement was determined at 25 and 37 °C to know its viscosity and flow behavior during administration by injection at room temperature (25 °C) and after injection into gingival crevicular fluid of periodontal pocket of human body (37 °C). Typically, the viscosity of solution is decreased as the temperature is higher therefore the viscosity of systems at 37 °C was lower than that at 25 °C.

The rheological behavior of solutions comprising the drugs was confirmed by the N value and viscosity coefficient (η) as shown in Fig. 2. The N value of all formula closed to 1, indicating the Newtonian flow similar to that of the gel base and the temperature did not influence the flow types. In the case of the viscosity coefficient (η), the amount of EC in each formula was higher, the viscosity coefficient was also significantly greater ($p < 0.05$). In contrast, the viscosity coefficient significantly decreased when the temperature was increased ($p < 0.05$). The results indicated that the amount of polymer affected the viscosity coefficient of each system. The gel structure could be altered the deformation by changing in the shape of polymer molecules and in the number of molecular entanglements, possibly by shear rate (Fresno et al., 2002).

3.4. Syringeability

Syringeability was evaluated to determine the force required to expel the product. The work of syringeability in all formula significantly increased ($p < 0.05$) as the amount of polymers was increased (Fig. 3). The increasing of polymer content increased the

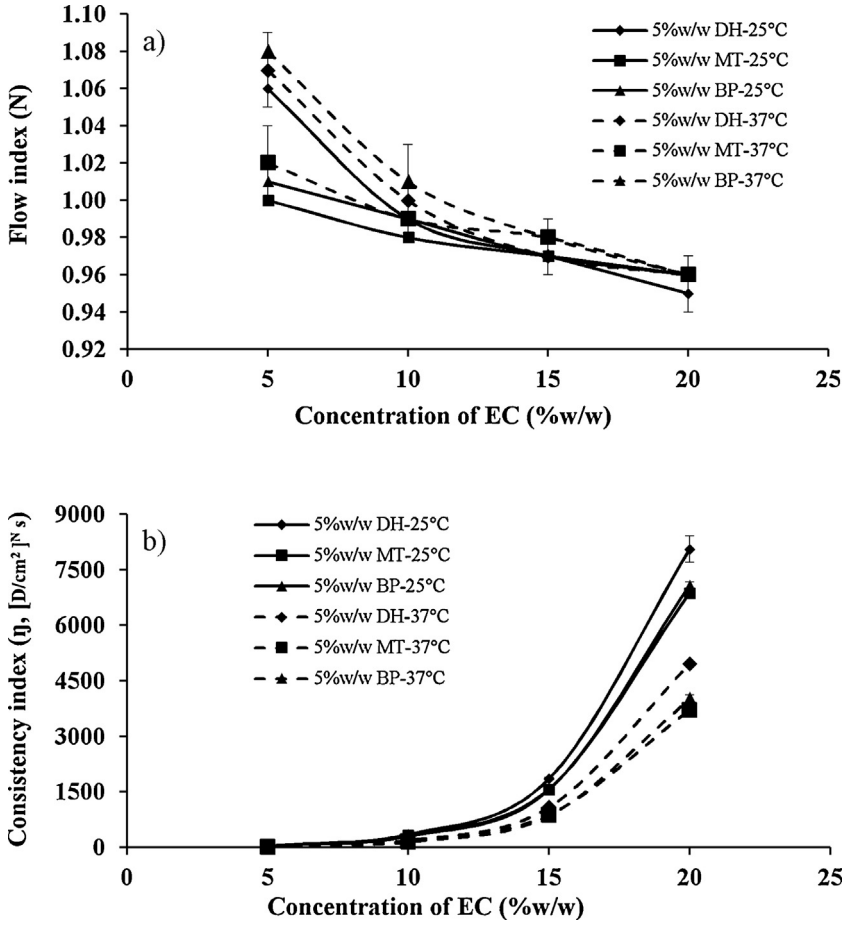

Fig. 2. Flow parameters of EC formula containing different drugs at 25 °C and 37 °C; a) Flow index (N) and b) Consistency index (η) ($n = 3$).

work required for expulsion, indicating a lower syringeability as previously reported (Chang et al., 2002). Typically, the syringeability is also facilitated by solvent affinity to the polymer which for the good solvent the polymer–solvent interaction is dominant over the polymer–polymer therefore lowering the viscosity (Parent et al., 2013). NMP could solubilize the high amount EC which was enough for controlling the drug release and easy for syringeability.

3.5. In vitro gel formation

The *in vitro* gel formations of EC formulae containing different drugs are shown in Figs. 4–6, respectively. The 5% w/w EC formulation formed into gel after injected into phosphate buffer pH 6.8, whereas the gels from 10% w/w EC formulation were stronger than that of 5% w/w. As the EC amount was increased, the obtained gel was much more solid and opaque. The topography as presented in SEM also confirmed this characteristic that would be discussed later. This *in vitro* injection experiment suggested that the polymer solution could be easily injected into the periodontal pocket and formed *in situ* gel immediately. The higher concentrations of polymer promoted the apparent precipitation of polymer. Hence the lower amount of polymer did not result in the gelation of the system owing to the discontinuing of precipitated network of EC, while increasing the polymer amount resulted in the rapid gelation as previously mentioned (Abashzadeh et al., 2011). The contact of drug-loaded EC solution into the buffer or gingival crevicular fluid triggered a phase inversion process because of an exchange between NMP and water from buffer or gingival crevicular fluid which finally promoted an EC precipitation as depot matrix at target site.

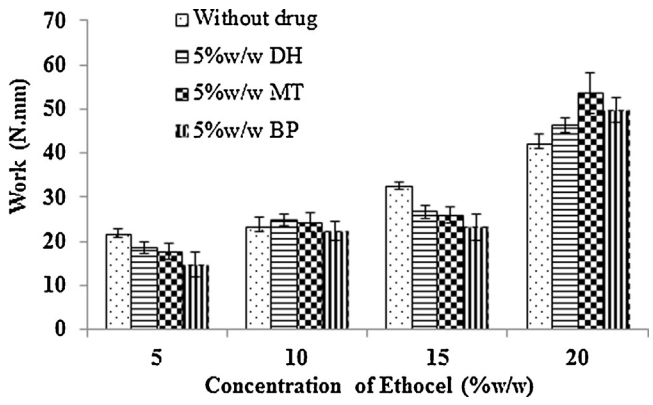

Fig. 3. Syringeability of EC formula containing different drugs.

3.6. Rate of water diffusion into the gels

The rate limiting step of depot formation or structure change into a solid matrix was the diffusion rate of aqueous into the drug-loaded EC solution. The diffusion rate of phosphate buffer pH 6.8 into the prepared solutions decreased with the increasing EC concentration ($p < 0.05$) (Table 3). Increasing polymer amount increased the viscosity of *in situ* gel system and decreased the water diffusion into gels. It has been reported that the fast solvents extraction was often followed by a fast drug release. On the other hand, for the drugs of poor solubility, a fast diffusion of DMSO

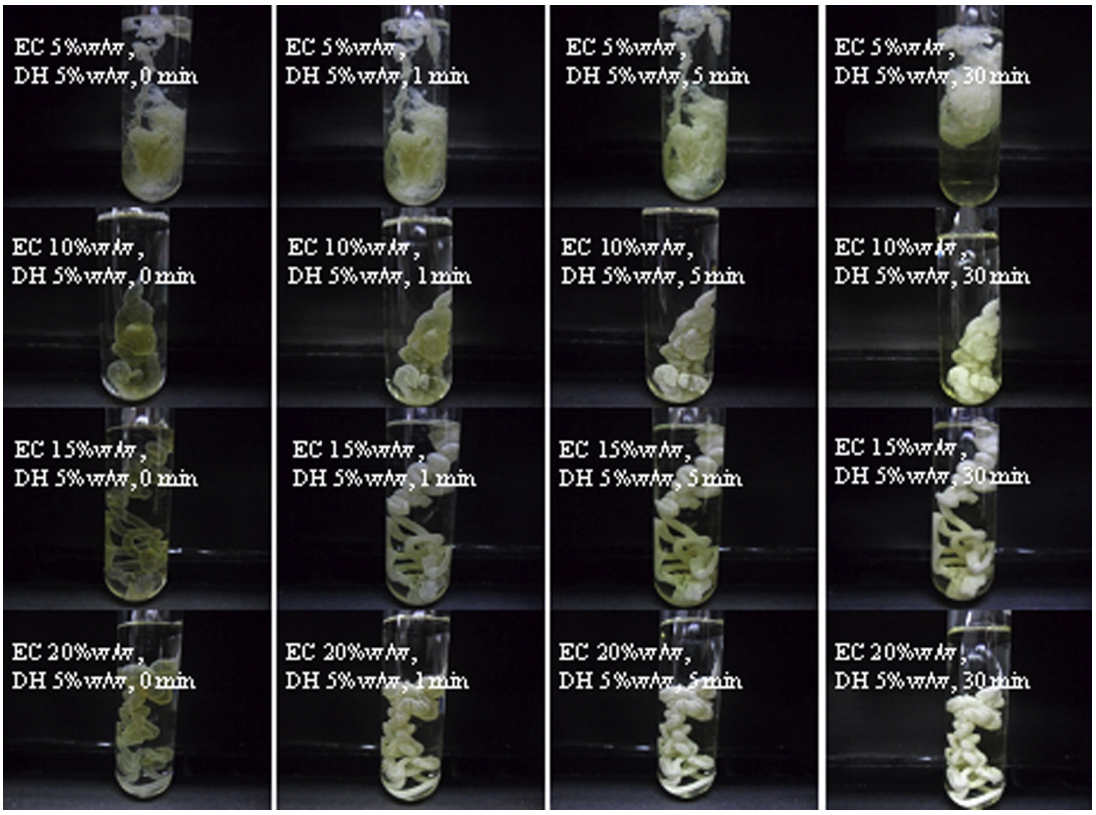

Fig. 4. *In vitro* gel formation of EC systems containing 5% w/w DH at various times (0, 1, 5 and 30 min).

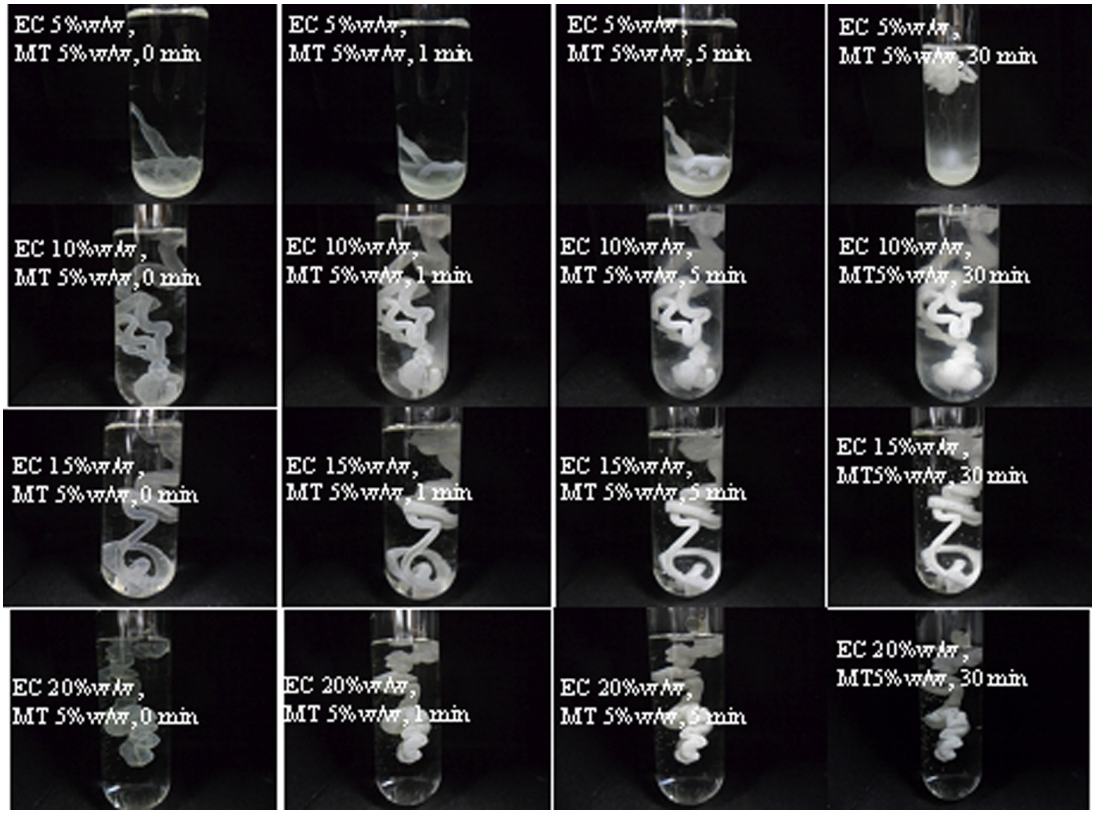

Fig. 5. *In vitro* gel formation of EC systems containing 5% w/w DH w MT at various times (0, 1, 5 and 30 min).

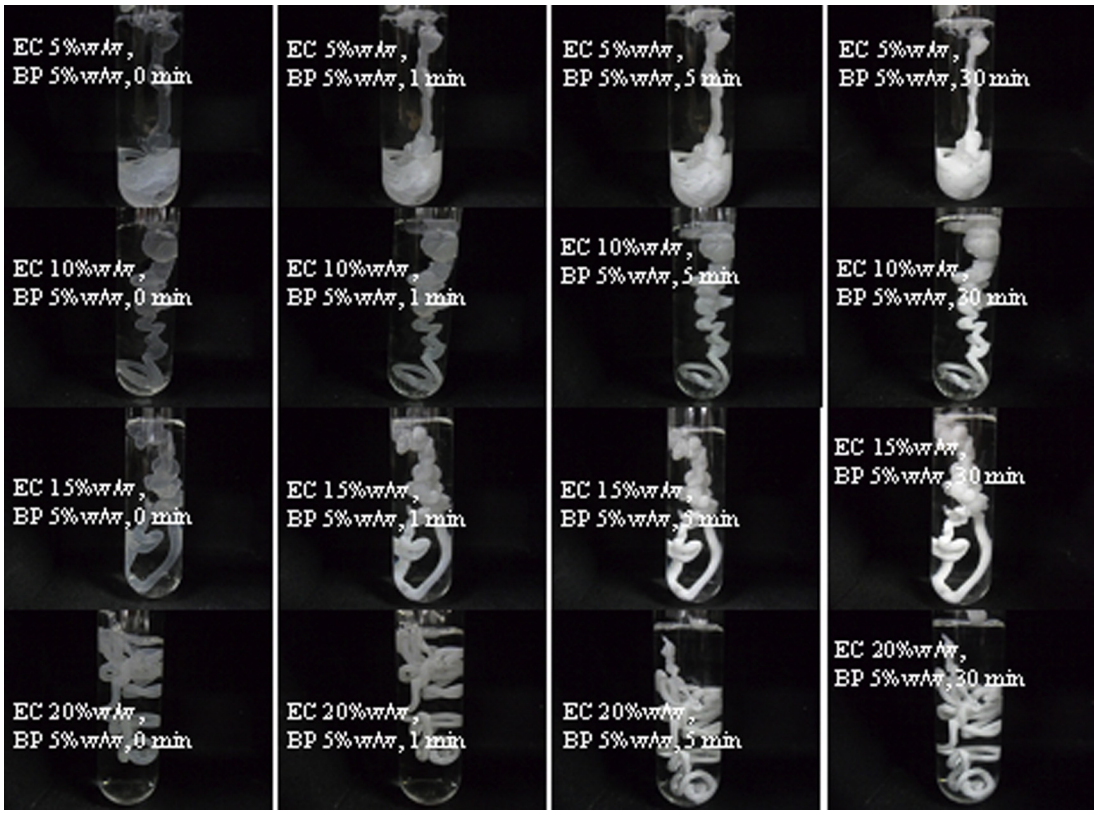

Fig. 6. *In vitro* gel formation of EC systems containing 5% w/w DH BP at various times (0, 1, 5 and 30 min).

Table 3
Effect of EC amounts in the formula containing different drugs on rate of water diffusion into gels.

EC (%w/w)	Rate of water diffusion into gels (mm/min) (mean ± S.D.)					
	With DH (5%w/w)		With MT (5%w/w)		With BP (5%w/w)	
	At 4 h	At 24 h	At 4 h	At 24 h	At 4 h	At 24 h
5	0.0104 ± 0.0013	0.0033 ± 0.0002	0.0097 ± 0.0012	0.0072 ± 0.0004	0.0092 ± 0.0004	0.0019 ± 0.0001
10	0.0101 ± 0.0005	0.0028 ± 0.0000	0.0090 ± 0.0012	0.0047 ± 0.0005	0.0069 ± 0.0012	0.0019 ± 0.0001
15	0.0088 ± 0.0004	0.0028 ± 0.0001	0.0063 ± 0.0000	0.0041 ± 0.0005	0.0049 ± 0.0012	0.0018 ± 0.0002
20	0.0078 ± 0.0005	0.0022 ± 0.0001	0.0049 ± 0.0012	0.0020 ± 0.0002	0.0042 ± 0.0000	0.0017 ± 0.0000

resulted for a fast solidification of the implant, resulting in a high drug retention rate, which reflected by the drug release (Wang et al., 2012).

3.7. In vitro *drug release studies*

3.7.1. *Dialysis membrane method*

The drug release was tested in phosphate buffer pH 6.8 to simulate the environment of periodontitis. The drug release profile of 5% w/w DH in NMP without polymer showed the fastest release rate with about 90% drug release at 3 h whereas the systems containing 5%, 10%, 15% and 20% w/w EC systems containing 5% w/w DH were about 90%, 85%, 84% and 80% drug release at 6 h (Fig. 7) respectively. The drug release from the *in situ* gel systems decreased with increasing the polymer amount. The physical entanglements between polymers, producing a dense matrix controlled the drug diffusion. It has been reported that the initial burst release significantly affected by polymer phase inversion dynamics and the increase in polymer concentrations could reduce the burst release (Liu and Venkatraman, 2012). The solvent type and polymer concentration were the most critical factors influencing the drug release (Brodbeck et al., 1999). The hydrophobic poly (dl-lactide co-caprolactone) (PLC) showed the slowest release of drug, whereas the hydrophilic poly (dl-lactide-co-glycolide) (PLG) exhibited low initial release of drug followed by a more rapid release once the polymer became hydrated (Malik et al., 2010). The diffusion of NMP exhibited a rapid phase inversion of PLGA associated with a high burst release of drug due to the formation of a porous rubbery gel structure whereas other solvents such as triacetin and ethyl benzoate which were the weak solvents for PLGA showed the slow gelation with the reducing of a burst liberation of active compound significantly. The phase inversion of EC gradually occurred and the precipitated EC solid matrix controlled the drug release with the EC concentration dependence.

3.7.2. *Membrane-less diffusion method*

The membrane-less diffusion method was studied to allow the medium solution to directly contact the gel surface and thus eroded the gel. The drug release from the formula decreased with an increased polymer amount (Fig. 8). Higher polymeric content in the matrix decreased the release rate because of the greater tortuosity and minimized porosity (Reza et al., 2003). The specific

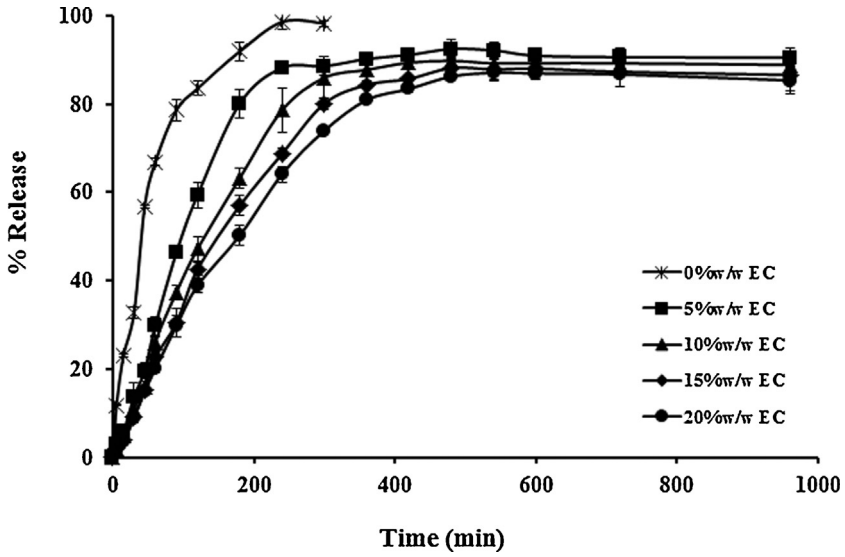

Fig. 7. Effect of EC amount on release of DH using dialysis method.

properties of the network of polymer chains, e.g., chain length, flexibility, mobility, water-uptake and swelling behavior, extent of plasticization, or potential interactions between polymer and drug all potentially affected the rate of solvent diffusion in the polymer matrix and the drug release (Wischke and Schwendeman, 2008). The hydrophobic PLGA dissolved in NMP was used as the solvent exchange-induced hydrophobic depot for sustaining the release of polysaccharide drug (Shi et al., 2015). The hydrophobic depot from EC precipitation was able to effectively prolong the drug release. By comparison with the aboved dialysis membrane method, the drug release from membrane-less diffusion method was apparently slower. The sudden exposure to the aqueous induced the rapid phase inversion from a solution into a hardend depot matrix especially onto its surface which exhibited the higher capacity for controlling the drug release than that from dialysis tube. Therefore the deposited gel at periodontal pocket should effectively prolong the drug release after direct exposure to gingival crevicular fluid..

3.7.3. Analysis of drug release data

The r^2 and msc from curve fitting to the first order, Higuchi's, zero order, and power law equations of drug release data obtained from dialysis membrane and membrane-less methods are shown in Table 4. The DH release from all EC formulae using dislysis membrane method fitted well with a first order model. It has been reported that the drug release from hydrophobic polymer matrix in water provided a first order release kinetic (Khairuzzaman et al., 2006).

The release exponent values (n) for power law of all formulations are shown in Table 5. For dialysis method, the n value of the 5–10% w/w EC formula indicated that the drug released by a Fickian diffusion mechanism. The drug release rate decreased as a function of time due to a decrease in the concentration gradient. The drug releases from system containing 15–20% w/w EC were the anomalous or non-Fickian diffusions indicating the drug release was controlled by both mechanism of diffusion and polymeric chain relaxation (Pahwa et al., 2011). The

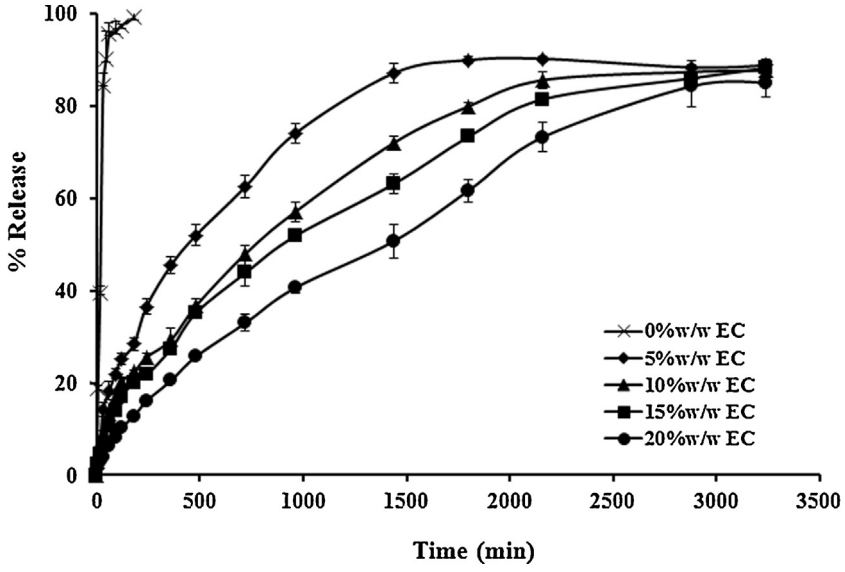

Fig. 8. Effect of EC amount on release of DH using membrane-less method.

Table 4
Comparison of degree of goodness-of-fit from curve fitting of the release profiles of DH in phosphate buffer pH 6.8 using dialysis membrane method (A) and membrane-less method (B) to different release models.

EC (%w/w)	First order		Higuchi's		Zero order		Power law	
	r^2	msc	r^2	msc	r^2	msc	r^2	msc
(A)								
5	0.9926	4.41	0.9621	2.70	0.9545	2.52	0.9774	2.93
10	0.9963	5.17	0.9845	3.60	0.9398	2.37	0.9905	3.91
15	0.9968	5.40	0.9796	3.49	0.9653	2.91	0.9826	3.45
20	0.9980	5.86	0.9900	4.21	0.9780	3.37	0.9900	4.01
(B)								
5	0.9937	4.77	0.9874	3.98	0.9315	2.35	0.9927	4.33
10	0.9956	5.16	0.9857	3.97	0.9673	3.11	0.9876	3.89
15	0.9966	5.41	0.9933	4.71	0.9480	2.65	0.9943	4.74
20	0.9925	4.59	0.9828	3.73	0.9740	3.34	0.9951	4.87

increased polymer amounts significantly decreased the drug release rate (k) ($p < 0.05$) (Table 5) because of the high tortuosity of the system containing higher polymer amount

The DH released from EC (5–15%w/w) using membrane-less method were fitted well with a first order model (Table 4). Whereas the DH released from Ethocel (20% w/w) formula was best explained by a power law model, but a close relationship was also noted with a first order kinetics. The release exponent values (n) for power law from the release using a membrane-less method are shown in Table 5. The n value obtained from power law equation of all formulae (5–20% w/w EC) after release studies using membrane-less method were an anomalous (non-Fickian) diffusion controlled release ($0.45 < n < 0.89$). Considering the drug release rate (k) parameter from a membrane-less method, it indicated that the increased polymer amounts tended to decrease the drug release rate (k) ($p > 0.05$) which was similar to the previous release study using dialysis membrane method. The factors affecting release kinetic were the liquid diffusion rate and polymeric chain relaxation rate. When the liquid diffusion rate was slower than the relaxation rate of the polymeric chains, the diffusion was a Fickian, whereas when the relaxation process was very slow compared with the diffusion, the case II transport occurred. When liquid diffusion rate and polymer relaxation rate were of the same order of magnitude, anomalous or non-Fickian diffusion was observed (Perioli et al., 2004). Solute transport from non-degradable polymeric systems was mainly considered as diffusion driven. Non-degradable polymers could be fabricated into "reservoir-" and "matrix-" type devices, which could be a rate-controlling membrane (Ful and Kao, 2010). For matrix-type devices, drug release was more likely to be a Fickian diffusion driven, which was associated with concentration gradient, diffusion distance, and the degree of swelling (Siepmann and Siepmann, 2008).

Table 5
Estimate parameter from curve fitting of DH release in phosphate buffer pH 6.8 using dialysis membrane method (A) and membrane-less method (B) to power law expression.

EC (%w/w)	k ± S.D.	n ± S.D.	Release mechanism
(A)			
5	0.1015 ± 0.0083	0.40 ± 0.02	Fickian
10	0.0784 ± 0.0101	0.42 ± 0.02	Fickian
15	0.0499 ± 0.0115	0.49 ± 0.04	Anomalous
20	0.0448 ± 0.0059	0.49 ± 0.02	Anomalous
(B)			
5	0.0253 ± 0.0017	0.49 ± 0.01	Anomalous
10	0.0152 ± 0.0034	0.62 ± 0.03	Anomalous
15	0.0093 ± 0.0014	0.58 ± 0.02	Anomalous
20	0.0053 ± 0.0013	0.64 ± 0.03	Anomalous

k = release rate; tl = lag time and n = diffusional exponent.

3.8. Surface morphology

The SEM micrographs of EC formulae with 5% w/w DH containing different amounts of EC after release are shown in Fig. 9. The 15–20% w/w EC formula containing DH showed the porous scaffold and interconnected porous structure. The pore sizes of structure decreased with an increased EC amount. This characteristic corresponed with the *in vitro* gel formation which the obtained gels were much more solid and opaque as the EC concentration was increased as presented in Figs. 4–6. SEM study confirmed the diffusion mechanism during drug release from the *in situ* gel systems. The *in situ* gel system taken after the release test showed that the pores had been formed throughout the matrix. Hence, the formation of both pores and scaffolds structure on *in situ* gel sytems indicated the involvement of both erosion and diffusion mechanisms to be responsible for modulating the drug release from matrix. The results indicated that the solvent diffusion out and water prenetration into the systems promoted the highly porous structures formation. The porosity and pore-connectivity were suggested to ensure the solvent exchange and drug diffusion from *in situ* gel systems and then formed the scaffolds. Many studies showed that the injectable biomaterials formed scaffolds *in situ* were useful as injectable drug delivery (Kranz and Bodmeier, 2008; Rezaei and Mohammadi, 2013). Therefore the drug-loaded EC solution using NMP as solvent could be applied as an *in situ* forming scaffold.

3.9. In vitro degradation studies

The percentage of degradation of EC with and without drug loading significantly decreased with increasing polymer amounts ($p < 0.05$) (Table 6). The degradation of the prepared gel occurred by the diffusion of drug and solvent after exchange with medium. The degradation could be described by weight loss or molecular weight loss (Ren et al., 2006). The injection of EC solution into the aqueous medium led to a rapid solvent/nonsolvent exchange and a formation of porous structure. Therefore the main mass loss from these systems was owing to the diffusion out of NMP and also the drug released. The EC structure does not degrade with enzyme or acidic condition. Therefore, the main mass loss found in this study was from solvent diffusion out of the depot matrix.

3.10. Antimicrobial activity studies

The inhibition zone diameter of the EC formulae containing different drugs against *S. aureus*, *E. coli*, *C. albicans*, *S. mutans* and *P. gingivalis* are shown in Fig. 10. The inhibition zone of all drug-loaded systems was significantly higher than that of the polymer solutions ($p < 0.05$) except that against *C. albicans*. The increased EC amount did not affect the antimicrobial activity against all microbes ($p > 0.05$), however the increased EC of systems containing MT significantly decreased the inhibition zone diameter against *C. albicans* ($p < 0.05$). DH showed the highest activity in broad spectrum against all tested microorganisms. Gram-positive bacteria are more sensitive to the presence of DH than Gram-negative bacteria. Typically, the Gram-negative bacteria possess an outer membrane surrounding the cell wall, which restricts diffusion of hydrophobic compounds through its lipopolysaccharide covering (Beveridge, 1999). In addition, MT-loaded systems displayed a rather high antimicrobial activity against both *S. mutans* and *P. gingivalis* whereas BP-loaded *in situ* forming system also effectively inhibited against *P. gingivalis*. The results suggested that EC systems containing these three drugs showed the

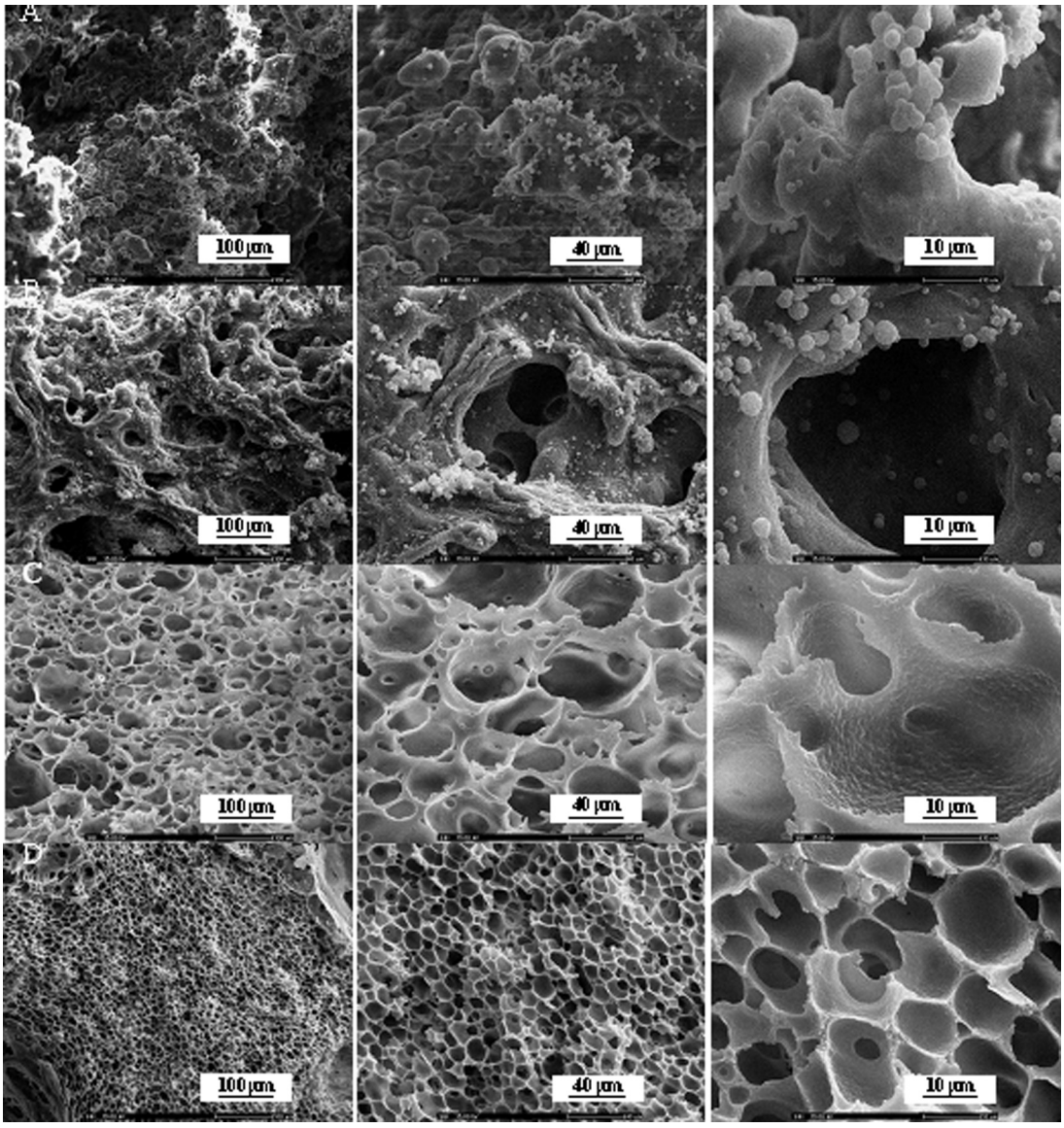

Fig. 9. SEM micrograph of the dried gel obtained from DH solutions containing EC 5% w/w (A); 10% w/w (B); 15% w/w (C) and 20% w/w (D) with different magnifications (200X (left), 500X (middle) and 2000X (right)).

Table 6
Percentage of weight loss of formulae containing different drugs ($n = 3$).

EC (%w/w)	% Weight loss ($n = 3$)			
	Without drug	With drug (5%w/w)		
		DH	MT	BP
5	94.05 ± 0.44	99.72 ± 0.31	99.33 ± 0.26	95.30 ± 0.39
10	89.14 ± 0.37	89.12 ± 0.31	89.30 ± 0.22	89.04 ± 0.46
15	84.22 ± 0.18	84.95 ± 0.20	84.94 ± 0.22	83.93 ± 0.57
20	79.09 ± 0.10	79.47 ± 0.54	79.74 ± 0.14	78.68 ± 0.16

antimicrobial activities against *S. mutans* and *P. gingivalis*. In some case, the antimicrobial activities tended to decrease as the amount of polymer was increased. The drug gradually released and prolonged regarding the increasing tortuosity and decreasing porosity owing to the higher polymeric content in the matrix (Reza et al., 2003). This dense topography could slow down the entry of water and the drug diffusion therefore it minimized the burst release as well as delayed the drug release. The polymer solutions which were used as negative control could inhibit all microbes because NMP exhibited antimicrobial activities against various bacteria and *Candida albicans* with the NMP dose dependent manner as reported in previous research works (Phaechamud et al., 2013a,b). The thermosensitive gel containing ethylene oxide-propylene oxide block copolymer using NMP as co-solvent exhibited the antimicrobial activities against *S. aureus* and *E. coli* while antifungal activity against *C. albicans* was enhanced by increasing the NMP amount. The inhibition zones of pure NMP against *S. aureus*, *E. coli* and *C. albicans* were also tested which the inhibition zone for *C. albicans* was notably larger that that of *E. coli* and *S. aureus* respectively (Phaechamud et al., 2013b). The solubility parameter of NMP is similar to those of ethanol and DMSO (Hansen and Just, 2001), thus it should solubilize the lipid in cell membrane and promote the leakage of microbial cell membranes. Fig. 10 indicates that the EC solutions comprising NMP could also inhibit the anaeerobic bacteria such as *S. mutans* and *P. gingivalis* however the the activity was lower than that containing MT and BP for *P. gingivalis* and and lower than that containing MT for *S. mutans*. Therefore the DH-loaded *in situ*

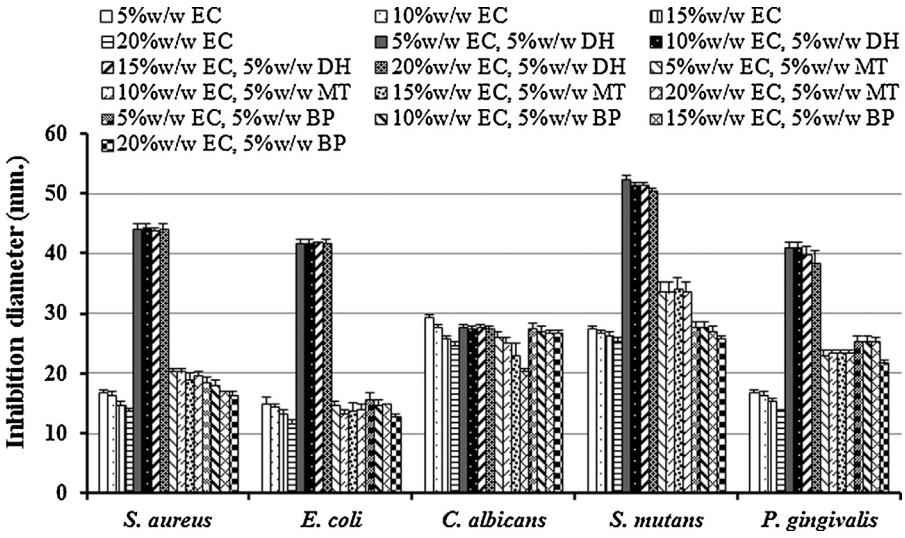

Fig. 10. Inhibition zone diameter of the EC formulae containing different drugs against *S. aureus, E. coli, C. albicans, S. mutans* and *P. gingivalis*.

forming gel using EC as matrix former was interesting as intraperiodontal pocket drug delivery system for periodontitis treatment due to its highest antimicrobial activities. NMP used as solvent in this system also effectively inhibited *C. albicans* which has been found in oral cavity as an opportunistic human fungal pathogen.

4. Conclusion

The antimicrobial *in situ* forming gel for periodontitis was developed using EC as polymeric matrix material. EC solutions using NMP as solvent exhibited a Newtonian flow behavior and formed into the solid-like depot in phosphate buffer pH 6.8 when the solvent exchange-polymer precipitation occurred. The transformation capacity and syringeability were EC concentration dependent. DH, MT and BP loaded-EC solutions exhibited a Newtonian flow which transformed from solution into matrix in PBS pH 6.8 which the drug release was more sustainable with increased polymer concentration. Surface topography of dried structure revealed the continuous phase, which the pore sizes of scaffold topography was decreased with the enhanced EC amount therefore the obtained denser matrix sustained the drug release effectively. This developed DH, MT and BP loaded-EC systems effectively inhibited *S. aureus*, *E. coli*, *S. mutans* and *P. gingivalis* and exhibited the potential use *as in situ* forming gel for periodontitis treatment.

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