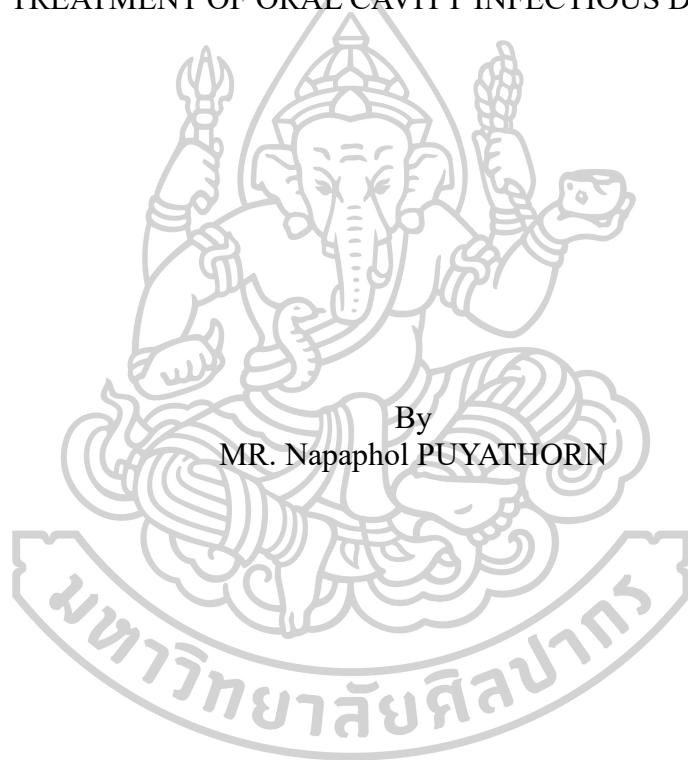




MECHANISTIC PHASE TRANSITION AND COMPUTERIZED MODELING OF
SOLVENT EXCHANGE-BASED *IN SITU* FORMING MATRIX SYSTEMS FOR
TREATMENT OF ORAL CAVITY INFECTIOUS DISEASES



By
MR. Napaphol PUYATHORN

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

Silpakorn University

Academic Year 2023

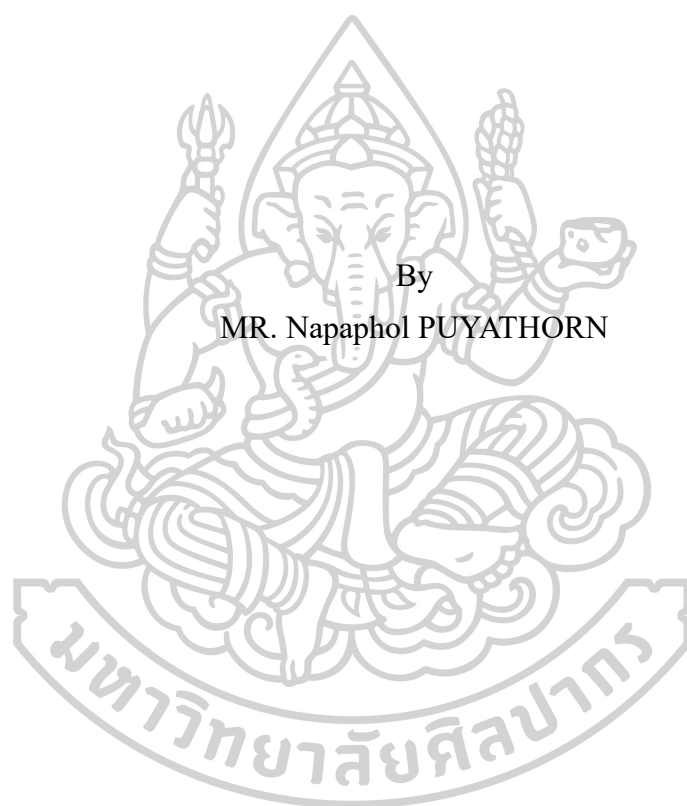
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กลไกการเปลี่ยนวิวัฒนาการและแบบจำลองคอมพิวเตอร์ของเมทริกซ์ก่อตัวเองจากการ
แลกเปลี่ยนตัวทำละลายสำหรับรักษาโรคติดเชื้อในช่องปาก



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Title	Mechanistic phase transition and computerized modeling of solvent exchange-based <i>in situ</i> forming matrix systems for treatment of oral cavity infectious diseases
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MR. Napaphol PUYATHORN : Mechanistic phase transition and computerized modeling of solvent exchange-based *in situ* forming matrix systems for treatment of oral cavity infectious diseases Thesis advisor : Professor Thawatchai Phaechamud, Ph.D.

In situ matrix-forming systems possess a unique capability for spontaneous transformation into a solid matrix upon administration to the target site. This approach offers less invasive and prolonged drug release at the local site. The transformation of liquid formulations into a solid matrix after delivery is attributed to solvent exchange-induced mechanisms. This thesis focuses on *in situ* forming matrices (ISMs), with particular emphasis on the small molecule including borneol (BOR) and ibuprofen (IBU) as matrix formers. The selected ISM formulation maintains a matrix concentration of 40% w/w. These ISMs were characterized by low viscosity (< 20 cP) and high injectability (< 3 N). They also exhibited excellent spreadability and wettability with a low surface tension (< 45 mN/m) and low contact angle ($< 25^\circ$), respectively. The BOR-based ISM, when incorporated with lincomycin HCl (LH), demonstrated an exceptionally low water tolerance value (8.29%) and exhibited statistically significant antimicrobial activity at LH loadings of 5% (NBL5) and 7.5% (NBL7.5) against *Staphylococcus aureus* ATCC 25923 and *Porphyromonas gingivalis* ATCC 33277, enabling precise drug release over 8 days. In the case of 5% doxycycline hyclate (DH) loaded IBU-based ISMs (DID40 and DIN40), a unique phase transformation is observed, characterized by IBU droplet movement before transitioning to a solid matrix. The tortuous structure of the IBU matrix effectively retarded drug release over a week (% drug release on the seventh day were $35.17 \pm 2.23\%$ for DID40 and $53.19 \pm 2.66\%$ for DIN40). Furthermore, these formulations displayed a strongly statistically significant antimicrobial activity against periodontitis pathogens like *P. gingivalis* ATCC 33277 and *Aggregatibacter actinomycetemcomitans* ATCC 29522. Additionally, the DH-loaded IBU-based ISMs demonstrated superior anti-inflammatory activity compared to the control. To gain a deeper understanding of the mechanistic phase transition attributed to solvent exchange at the molecular level, molecular dynamics (MD) simulations using the Amber20 software were conducted. These simulations unveiled profound insights into the molecular mechanisms governing solvent exchange. In LH-loaded BOR-based ISMs, an increase in BOR content reduced the mobility and fluctuation of LH molecules, as indicated by a decrease in diffusion constant (from 0.56 to 0.21) and root mean square deviation (RMSD) values (from around 50 to 35 Å), respectively. The MD simulation revealed that solvent exchange process was interfered by BOR, as evidenced by a decreased in the diffusion coefficient and RMSD of both organic solvents and water molecules within the simulation box. In IBU-based ISMs, DH's diffusion constant decreased from 1.2452 to 0.3372, and in the NMP series, it decreased from 0.3703 to 0.2245. The RMSD values of DH also decreased from over 140 to 40 Å following the addition of IBU. This indicated that IBU retarded the mobility of both solvent and drug molecules. Additionally, intermolecular H-bonding interactions between the hydroxyl group of IBU and DH were observed in the final state of the simulation. This comprehensive investigation shed light on the multifaceted nature of solvent exchange-based ISM systems and their potential in addressing oral cavity infectious diseases. It leveraged state-of-the-art molecular dynamics simulations to unravel the intricacies of their phase transformation phenomena.

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TABLE OF CONTENTS

	Page
ABSTRACT.....	D
ACKNOWLEDGEMENTS.....	E
TABLE OF CONTENTS.....	F
LIST OF TABLES	K
LIST OF FIGURES	M
LIST OF ABBREVIATION.....	1
CHAPTER 1 INTRODUCTION.....	4
Rational and problem statement	4
Objective of research	5
Research hypothesis.....	6
CHAPTER 2 LITERATURE REVIEW.....	7
Binary mixture	7
<i>In situ</i> forming matrix (ISM)	7
Solvent and additive in ISM	10
Matrix former material	12
Antimicrobial agent	15
Periodontitis.....	17
Computational Molecular dynamics simulation.....	19
CHAPTER 3 METHODOLOGY	23
Equipment and instrument	23
Materials	24
Experimental set-up	26
3.1. Binary mixture of ISM evaluation.....	26
3.1.1. Preparation of binary mixture formulas	26
3.1.2. Physicochemical characterization of ISM-solutions	26

3.1.2.1. Appearance of prepared formulation	26
3.1.2.2. Determination of density	26
3.1.2.3. Apparent viscosity and rheological behavior.....	27
3.1.2.4. Determination of contact angle and surface tension.....	27
3.1.2.5. Injectability and mechanical properties	27
3.1.3. Characterization of matrix forming behavior	28
3.1.3.1. Matrix formation after injection	28
3.1.3.2. Morphology of matrix formation and matrix formation rate in an artificial crevicular pocket.....	28
3.1.3.3. Phase transformation tolerability property of binary mixtures against water addition.	28
3.1.3.4. Interfacial interaction of binary mixture and aqueous.....	28
3.2. Drug-loaded ISMs preparation and their physicochemical properties, drug release and bioactivities.....	29
3.2.1 Preparation of drug-loaded ISMs preparation	29
3.2.2. Physicochemical characterization of drug-loaded ISM-solutions..	30
3.2.3. Characterization of drug-loaded matrix forming behavior.....	30
3.2.4. <i>In vitro</i> drug release.....	30
3.2.4.1. IBU/DH amounts determination.....	30
3.2.4.2. LH concentration determination.....	31
3.2.4.3. Kinetic of drug release study	31
3.2.5. Antimicrobial activities	31
3.2.6. Anti-inflammation activity	32
3.3. Statistical analysis	32
3.4. Characterization of dried matrix precipitate.....	32
3.4.1. Preparation of dried selected matrix.....	32
3.4.2. Fourier-transform infrared (FT-IR) spectroscopy	32
3.4.3. Powder X-ray diffractometer (PXRD)	33
3.4.4. Differential scanning calorimetry (DSC)	33

3.4.5. Thermogravimetric analysis (TGA) and differential thermogravimetric analysis (DTGA).....	33
3.4.6 Water induced crystallization study <i>via</i> Wide Angle X-ray Scattering (WAXS)	33
3.4.7. Topographic observation of surface matrix under scanning electron microscope (SEM)	34
3.5. <i>In silico</i> molecular interaction and diffusion study of drug loaded ISM.	34
3.5.1. Study of molecular interaction	34
3.5.2. Computer dynamics modelling of mechanistic phase inversion and drug movement	35
Force field generation step of each molecule	35
The design of model box for MD simulation	35
Step 1. Minimization of solvent molecule	37
Step 2. Minimization of all molecule	37
Step 3. Heating molecule in system	37
Step 4. Equilibrating system	37
Step 5. Production MD simulation.....	37
MD simulation for induced phase transformation of ISM system	37
Analysis of MD simulation result.....	38
H-bonding and density profile of molecule	38
Root-mean-squared deviation (RMSD)/Diffusion constant/Radius of gyration	38
CHAPTER 4 Result and discussion.....	40
Part 1: 4.1. LH -Loaded BOR-Based <i>In Situ</i> Matrix for Periodontitis Treatment ..	40
4.1.1. Physicochemical Properties.....	40
4.1.1.1. Physical Appearance, Density, and Viscosity	40
4.1.1.2. Surface Tension and Contact Angle.....	41
4.1.1.3. Water Tolerance	42
4.1.1.4. <i>In Situ</i> Matrix Formation	44
4.1.1.5. Mechanical Properties	47

4.1.1.5 Water induced crystallization study through Wide Angle X-ray Scattering (WAXS) analysis	48
4.1.1.6. Microscopic Interface Interaction.....	49
4.1.1.7. Drug Content and <i>In Vitro</i> Drug Release.....	53
4.1.2. Antimicrobial Activities	57
4.1.3. <i>In silico</i> molecular study of drug loaded ISM.....	60
4.1.3.1. RMSD and Diffusion constant	67
4.1.3.2. Radius of gyration	69
4.1.3.3. H-bonding amount.....	70
Summary	72
Part 2: 4.2. DH-loaded Solvent Removal-induced IBU-based <i>In Situ</i> Forming Matrix	73
4.2.1. Physicochemical properties	73
4.2.1.1. Physical appearance.....	73
4.2.1.2. Density, pH, and viscosity	73
4.2.1.3. Surface tension and contact angle.....	74
4.2.1.4. Water tolerance	76
4.2.1.5. <i>In situ</i> matrix transformation	79
4.2.1.6. Injectability properties.....	83
4.2.1.7. Mechanical properties	84
4.2.1.8. <i>In vitro</i> drug release	85
4.2.1.9. Surface topography.....	89
4.2.1.10. Differential scanning calorimeter (DSC), thermogravimetric analysis (TGA), powder X-ray diffraction (PXRD), Wide Angle X-ray Scattering (WAXS) and Fourier transform infrared spectroscopy (FTIR)	92
4.2.2. Bioactivities studies.....	95
4.2.2.1. Antimicrobial activities	95
4.2.2.2. Anti-inflammatory activities.....	98

4.2.3. <i>In silico</i> simulation results.....	100
4.2.3.1 Binding energy (effect of organic solvent).....	100
4.2.3.2. MD simulation of phase transformation.....	100
4.2.3.2.1. RMSD and Diffusion constant.....	109
4.2.3.2.2. Radius of gyration.....	111
4.2.3.2.3. Matrix-drug interaction (No. of H-bonding interaction and DH-IBU complex configuration)	111
Summary	113
Chapter 5 Conclusion.....	115
Benefits of this study	117
Suggestions	117
REFERENCES	118
Appendices.....	136
Appendix I chromatogram and calibration curve of LH release from LH-loaded BOR-based ISM	136
Appendix II chromatogram and calibration curves of DH and IBU release from DH-loaded IBU-based ISM	137
Appendix III script for running MD simulation through Amber20 software package.....	138
VITA	145

LIST OF TABLES

	Page
Table 1 The composition of BOR-based binary mixtures.....	26
Table 2 The composition of IBU-based binary mixtures.	26
Table 3 Composition of DH-loaded IBU-based ISM formulations.	29
Table 4 Composition of LH-loaded BOR-based ISM formulations.	30
Table 5 Residue name of molecule component	35
Table 6 Number of different molecules of LH-loaded BOR-based ISM (A) and DH-loaded IBU-based ISG (B) formulations.	36
Table 7 Physical properties of LH-loaded BOR ISM formulations (n = 3).....	44
Table 8 Degrees of goodness-of-fit and estimated parameters from drug release profile fittings of LH-loaded BOR-based ISMs in PBS pH 6.8 using the cup method.....	56
Table 9 Clear zone diameter of the LH-loaded BOR-based ISM systems against <i>S. aureus</i> ATCC 25923, <i>E. coli</i> ATCC 8739, <i>C. albicans</i> ATCC 10231 and <i>P. gingivalis</i> ATCC 33277 (n = 3).	59
Table 10 Diffusion constant of each substance in formulations box contact with water.	68
Table 11 Physical properties of IBU in DMSO or NMP and DH-loaded solvent removal-induced IBU-based ISM systems (n=3).....	76
Table 12 Water tolerance value of IBU in NMP or DMSO and DH-loaded solvent removal-induced IBU-based ISM systems after titration with deionized water at temperatures of 25°C and 37°C (n=3).	78
Table 13 Summary of the injectability and mechanical parameters of DH, IBU, and remnants from DH-loaded IBU-based ISM formulations (n = 3; mean ± SD).	84
Table 14 Degree of goodness-of-fit and estimated parameters from the curve fittings of the DH release profiles of different ISMs in PBS pH 6.8 using the cup method (n=3).....	88
Table 15 Degree of goodness-of-fit and estimated parameters from the curve fittings of the IBU release profiles of different ISMs in PBS pH 6.8 using the cup method (n=3).....	89

Table 16 Summary of the thermal parameters of DH, IBU, and remnants from DH-loaded IBU-based ISM formulations after drug release from DSC and TGA.....94

Table 17 Clear zone diameters of DH-loaded IBU-based ISM systems against *S. aureus* (ATCC 6538, 6532, and 25923), MRSA (*S. aureus* ATCC 4430), *E. coli* ATCC 8739, *C. albicans* ATCC 10231, *P. gingivalis* ATCC 33277, and *A. actinomycetemcomitans* ATCC 29522 (n = 3). 98

Table 18 Diffusion constant of each substance in formulations box contact with water 110



LIST OF FIGURES

	Page
Figure 1 Schematic diagram of drug-loaded ISM administered into the crevicular pocket and its phase transformation [17].	8
Figure 2 Chemical structure of solvent DMSO (A) and NMP (B).	11
Figure 3 BOR chemical structure.	13
Figure 4 IBU chemical structure.	14
Figure 5 Chemical structure of LH.....	16
Figure 6 Chemical structure of DH.....	17
Figure 7 Stages of gum disease from healthy gums to advanced periodontitis [129].	18
Figure 8 Schematic of ISM solution self-transformation to solid matrix after administration and controlled drug release to target site [130, 131].	19
Figure 9 The matrix formation MD simulation under difference water ratio between low and high molecular weight fatty acid [143].	21
Figure 10 The transfer of surfactant across the water-oil interface simulation (left) and free energy result (right) [145].	22
Figure 11 MD simulation of surfactant orientation in the interfacial region of organic-water and comparison between protonate (left) and deprotonate laurate surfactant (right) [146].	22
Figure 12 Completed simulation box of ISM system.	36
Figure 13 experimental design of MD simulation around interface region between formula and aqueous.	38
Figure 14 Basic information flow of Amber20.	39
Figure 15 <i>In vitro</i> matrix formation behaviors of LH -loaded BOR-based ISMs in PBS pH 6.8.	46
Figure 16 Cross-sectional view of matrix formation behaviors of LH -loaded BOR-based ISMs in agarose wells under a stereo microscope at a magnification of $\times 12$	46
Figure 17 Mechanical properties of LH-loaded BOR-based ISMs after phase alteration for 24 h (n=3).	47
Figure 18 WAXS diffractogram of water-induced ISM system.	48

Figure 19 Matrix formation at the interface of the aqueous phase and ISMs under visible light (A) and fluorescence light (B) at magnification $\times 100$ under an inverted microscope. (The red line is the interface between agarose (left side) and formula (right side)).....	51
Figure 20 LH release from BOR-based ISMs in PBS pH 6.8 using the cup method (n = 6).....	55
Figure 21 Inhibition zone of LH -loaded BOR-based ISM systems against <i>S. aureus</i> ATCC 25923, <i>E. coli</i> ATCC 8739, <i>C. albicans</i> ATCC 10231 and <i>P. gingivalis</i> ATCC 33277.....	59
Figure 22 Molecular dynamic modeling of solvent exchange mechanism focusses at interface of each ISMs against aqueous, (DB10 (A), DB40 (B), NB10 (C), NB40 (D), NL5 (E) and NBL (F) after running in MD simulation in 200 ns.....	61
Figure 23 Root mean square deviation (RMSD) of LH (A), BOR (B), organic solvent (C) and water (D) conformation relative to the starting point of the MD simulations box contact with water.....	68
Figure 24 Radius of gyration of LH and BOR conformation of the MD simulations box contact with water.....	69
Figure 25 hydrogen bonds amount of DMSO series (A) and NMP series (B) ISM formulations box contact with water.....	71
Figure 26 <i>In vitro</i> matrix formation behavior of IBU-based ISM and DH-loaded IBU-based ISM in PBS (A) and cross-sectional view of matrix formation of IBU-loaded ISM systems using DMSO and NMP as the solvents in the agarose well under a stereo microscope at a magnification of $\times 12$ (B).	81
Figure 27 Matrix formation at the interface of the aqueous phase and ISM systems during the initial period (i.e.; 1, 3, and 10 min) at a magnification of $\times 60$ under an inverted microscope. Abbreviations: G = matrix layers, D = droplet fluctuation layers, C = clear ISM layers.....	82
Figure 28 D-layer of IBU-based ISM for monitoring droplet movement behavior at a magnification of $\times 100$ under an inverted microscope.	83
Figure 29 Comparison of the release profiles of DH and IBU from DH-loaded 40%IBU ISM systems (n=3).....	87
Figure 30 SEM photographs of DH and IBU powders and remnants from DH-loaded IBU ISM systems using DMSO and NMP as the solvents and IBU in DMSO (ID40) after drug release test in PBS pH 6.8 for 7 days.	90

Figure 31 TGA (A), dTGA (B) and DSC (C) thermograms, and XRD diffractograms (D) of DH, IBU, and remnants from DH-loaded IBU-based ISM formulations after drug release.	93
Figure 32 FTIR spectra of DH, IBU, and remnants from DH-loaded IBU-based ISM formulations after drug release.	94
Figure 33 WAXS diffractogram of water-induced ISM system.	94
Figure 34 Inhibition zones of DH-loaded IBU-based ISM systems against <i>S. aureus</i> (ATCC 6538, 6532, and 25923), methicillin-resistant <i>S. aureus</i> (MRSA) (<i>S. aureus</i> ATCC 4430), <i>E. coli</i> ATCC 8739, <i>C. albicans</i> ATCC 10231, <i>P. gingivalis</i> ATCC 33277, and <i>A. actinomycetemcomitans</i> ATCC 29522 (n = 3).....	97
Figure 35 Percentage of inhibition of protein denaturation of DH-loaded IBU-based ISM (n=3). The asterisk symbol indicates no significant difference ($p \geq 0.05$) using one-way ANOVA followed by the LSD posthoc test.....	99
Figure 36 Binding energies and binding location of optimized molecules.....	100
Figure 37 Molecular dynamic modeling of solvent exchange mechanism focusses at interface of each ISMs against aqueous, (DD (A), DN (B), ID40 (C), IN40 (D), DID40 (E) and DIN40 (F) after running in MD simulation in 200 ns.....	102
Figure 38 The density profile of IBU and DH molecules in coordinate of different formulation box after contact with water box.....	108
Figure 39 Root mean square deviation (RMSD) of DH (A), IBU (B), organic solvent (C), and (D) water conformation relative to the starting point of the MD simulations box contact with water.	110
Figure 40 Radius of gyration of DH and IBU conformation of the MD simulations box contact with water.	111
Figure 41 Change in number of hydrogen bonds of IBU-IBU (A) and DH-IBU (B) molecule of formulations box contact with water.....	112
Figure 42 Configuration between DH and IBU at final stage of MD simulation in DID40 systems. (DH and IBU molecules were demonstrated as stick and rough surface mass structure, respectively. Blue, white, red and dark blue structure or surface are represented C, H, O and N atom, respectively. The yellow bond is intermolecular H - bonding of DH and IBU). A 2D-chemical structure represents C atom numbering of DH molecule.	113

Figure 43 chromatogram of LH release from LH-loaded BOR-based ISM (NBL) (HPLC-UV-vis at 214nm).....	136
Figure 44 Calibration curve of LH in phosphate buffer pH 6.8 for drug release study.	136
Figure 45 chromatogram of DH and IBU release from DH -loaded IBU-based ISM (DIN40) (HPLC-UV-vis at 264nm).....	137
Figure 46 Calibration curve of DH in phosphate buffer pH 6.8 for drug release study.	137
Figure 47 Calibration curve of IBU in phosphate buffer pH 6.8 for drug release study.	138



LIST OF ABBREVIATION

%	percentage (s)
%w/w	percent weight by weight
°C	degree Celsius
<	less than
>	more than
±	plus-minus sign
®	Registered sign
Å	Angstrom
µg	microgram (s)
µL	microliter (s)
µm	micrometer (s)
AIC	Akaike information criterion
ATCC	American Type Culture Collection
ANOVA	analysis of variance
BBB	blood brain barrier
BOR	borneol
cm	centimeter (s)
C18	18 carbon atoms
CCDC	Cambridge Crystallographic Data Centre
DFT	density field theory
DH	doxycycline hyclate
DMSO	dimethyl sulfoxide
DSC	Differential scanning calorimeter
e.g.	(Latin); for example
Eq.	Equation
FE-SEM	field emission-scanning electron microscope
FTIR	Fourier transform infrared spectroscopy

G	(needle)gauge
g	gram (s)
h	hour (s)
HPLC	high-performance liquid chromatography
IBU	ibuprofen
ICH	International Conference on Harmonization
ISM	<i>in situ</i> forming matrix
k_0	zero order's rate constant
k_1	first order's rate constant / Peppas-Sahlin's rate constant No.1
k_2	Peppas-Sahlin's rate constant No.2
k_H	Higuchi's rate constant
k_{KP}	Korsmeyer-Peppas's rate constant
k_{HC}	Hixson's rate constant
k_{HB}	Hopfenberg's rate constant
L	liter (s)
LD ₅₀	50% lethal dose
LH	lincomycin HCl
MD	molecular dynamic
mg	milligram (s)
min	minute (s)
MIC	The minimum inhibitory concentration
ml	milliliter (s)
MRSA	methicillin-resistant <i>Staphylococcus aureus</i>
MSC	model selection criterion
N	newton
Nm	nanometer (s)
NMP	<i>N</i> -methyl pyrrolidone
ns	nanosecond
No.	number

ND	not determined
NSAIDs	non-steroidal anti-inflammatory drugs
PBS	phosphate buffer solution
PLGA	poly (lactic-co-glycolic acid)
pH	potentia hydrogenii (Latin); power of hydrogen
PXRD	powder X-ray diffraction
rpm	revolutions per minute
R ²	coefficient of determination
RMSE	root mean square deviation
sec	second (s)
SD	standard deviation
SS	sum of squares
SPSS	statistical package for social science
TGA	thermogravimetric analysis
UV	ultraviolet
UK	United Kingdom
USA	United States of America
WAXS	wide angle X-ray scattering

CHAPTER 1 INTRODUCTION

Rational and problem statement

The research of *in situ* forming system has grown exponentially in last few years. *In situ* forming systems are the one of useful pharmaceutical delivery systems due to their self-transformation into an expected state such as gel, solid, and microparticle after administrating into the target site for less invasive and painful manner modes compared to conventional pharmaceutical implants, which require local anesthesia and a small surgical intervention are necessary for localized or systemic drug delivery with prolonged periods of time liquid formulations generating a semisolid-like or solid matrices after delivered *in situ* forming system occurs due to one or combination of different stimuli like pH change, temperature modulation and solvent exchange. The solvent exchange-induced *in situ* forming matrix (ISM) contains a water-insoluble and biodegradable matrix former material dissolved in a biocompatible organic solvent to which a drug is added for forming into the solution or the suspension after mixing. When this formulation is administrated into the body, the water miscible organic solvent dissipates and water penetrates into the organic phase. This leads to phase separation and precipitation of the matrix former at the site of injection [1, 2]. In this study, binary mixtures comprising various matrix former materials and organic solvents are prepared for altering of their physicochemical properties and elucidation the interaction between components in binary mixtures. The candidate matrix former material is including borneol (BOR) and ibuprofen (IBU) that dissolved with biocompatible organic solvent such as dimethyl sulfoxide (DMSO) and *N*-methyl pyrrolidone (NMP).

The basic requirements of matrix former material in solvent exchange-based ISM should be hydrophobic, biocompatible, and biodegradable. The emergence of small molecule-based *in situ* forming matrix systems has provided an alternative to the conventional polymeric-based ISM systems. Additionally, in recent years, the formulation concept of "using active chemicals as excipients" has sparked considerable interest. The matrix-forming materials that align with the aforementioned properties include BOR and IBU. BOR has been used as the food flavouring, air care products, decorative cosmetics, traditional Chinese medicine. The United States Food and Drug Administration (U.S. FDA) has accepted and considered this generally recognized as safe (GRAS) compound as food flavor due to its non-toxicity. Minimal skin irritation in human volunteers and mice/mouse has been reported due to the low toxicity of BOR [3]. In the pharmaceutical field, it has antibacterial effect and antinociceptive effect and it has been developed as a carrier of a drug delivery system [4]. Another candidate material is Ibuprofen. Ibuprofen is a non-steroidal anti-inflammatory drug for mild to moderate pain and inflammation. It's a hydrophobic drug and insoluble in water. Ibuprofen is used for relief inflammation or effect from inflammation such as bone loss in periodontitis. Chlorhexidine-ibuprofen loaded-*in situ* gel-forming system presented an antibacterial activity against

periodontal pathogens. These four compounds are of interest to apply as matrix forming agent of solvent exchange-based ISM systems dissolved in organic solvents comprising DMSO and NMP in this research work. These solvents have been reported for their safety and applying as vehicles of *in situ* matrix-forming systems. Nevertheless, the molecular aspect understanding these solvent exchange-induced ISM has not been reported with computational chemistry techniques previously. In addition, more grateful data about water tolerance under influence of different temperature and matrix forming behavior in different ionic strength environments should be revealed.

Thus, the binary mixtures of the above matrix forming agents and solvents are interesting for preparing drug-loaded ISM. The developed binary mixtures are of interest for applying as a drug delivery vehicle for various antimicrobial agents for the treatment of periodontitis, which are important diseases affecting oral health. The interesting antimicrobial agents against periodontitis pathogens including lincomycin HCl (LH) and doxycycline hyclate (DH) are of interest to incorporate into these binary mixtures. Their profound physicochemical characteristics of solvent exchange-induced ISM are investigated as a product for periodontitis treatment. Physicochemical properties of both the solution and the transformed state of prepared solvent exchange-induced ISM were investigated including viscosity, rheology, matrix formation behavior, solvent exchange mechanism, ability to prolong drug release, and surface topography. Their water tolerance under influence of different temperature is of interest to determine. Moreover, for more understanding of molecular aspects, computational chemistry techniques are conducted in parallel with empirical experiment studies to explain physicochemical property alteration and mechanistic phase transition owing to solvent-exchange.

Objective of research

1. To investigate the physicochemical properties and matrix forming behavior of different solvent exchange-induced binary mixture-based ISM systems.
2. To understanding the mechanistic phase transition of solvent exchange-based ISM systems for treatment of oral cavity infectious diseases.
3. To develop and evaluate LH-loaded solvent exchange-induced BOR-based ISM systems for periodontitis treatment.
4. To develop and evaluate DH-loaded solvent exchange-induced IBU-based ISM systems for periodontitis treatment.
5. To apply computerized molecular simulation for fulfill understanding the physicochemical properties and mechanistic phase transition of solvent exchange-induced antimicrobial agent-loaded binary mixture-based ISM systems.

Research hypothesis

1. Type of solvents and matrix formers and their concentration influence the physicochemical properties and transformation behaviors of solvent exchange-induced binary mixture-based ISM systems.
2. Mechanistic phase transition can be employed for explaining matrices morphology and drug release property.
3. LH-loaded solvent exchange-induced BOR-based ISM systems can be used for periodontitis treatment.
4. DH-loaded solvent exchange-induced IBU-based ISM systems can be used for periodontitis treatment.
5. Dynamic computerized modelling can explain and support the mechanistic phase inversion of solvent exchange-induced antimicrobial agent-loaded binary mixture-based ISM systems for treatment of oral cavity infectious diseases.



CHAPTER 2

LITERATURE REVIEW

Binary mixture

Solutions are homogeneous mixtures of two or more than two components. For homogenous mixture, this means that its composition and properties are uniform throughout the mixture. Generally, the component that is present in the largest quantity is known as solvent. One or more components present in the solution other than solvent are called solutes. A solution, depending upon the number of components present, is classified as binary, tertiary, quaternary and so on. A binary solution consists of two components. Each component of a solution may be solid, liquid or in gaseous state. Solvent determines the physical state in which solution exists. A binary mixture consists of two types of molecules, A and B [5]. It has been prepared for design experiment altering of mixture properties in various field. The physiochemical properties of should be investigated for binary mixture including viscosity, density, contact angle, surface tension, speed of sound and etc. for designing processes in chemical, pharmaceutical, petrochemical fields. Many of studies have been set of binary mixture for mole fraction component, temperature or etc. as independent variable [6-9].

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

Controlled drug delivery systems are capable for regulating rate of drug delivery, maintaining drug concentration within the therapeutic range with prolonged duration of drug release, and/or targeting drug to a specific tissue [10]. Of the many controlled drug release technologies, *in situ* forming matrix (ISM) systems have risen popularity over decades due to a range of advantages which include the site-specification, less invasive application, prolonged drug release, reduction in side effects associated with systemic delivery and also improvement of the patient compliance and comfort [11, 12]. Importantly, an administration by this method allows the injection of a relatively low viscosity material which then solidifies to a solid-like matrix form that controls the drug delivery to improve long-term therapeutic action [12]. The mechanism of *in situ* forming systems can be classified into different types such as phase separation systems (*via* thermo-responsive, solvent exchange and pH change), crosslinked systems (*via* photo-initiated, chemical and physical) and solidifying organogels (*via* solubility change) [10]. The solvent exchange-based ISM systems technology has attracted worldwide interest among pharmaceutical companies, which led to the development of commercial therapeutic products for a wide range of clinical applications [10]. This system comprises of a water insoluble polymer that is dissolved in an organic, water-miscible, biocompatible solvent, into which a drug is incorporated by dissolving. When this solution is injected into an aqueous environment, such as saliva, the organic solvent diffuses outward into the surrounding environment (being miscible with water), while water diffuses into the system. Since the polymer is water insoluble, it gradually or rapidly solidified and transforms into the solid-like matrix [13-15]. The process where a

single-phase homogenous solution is critically quenched, causing separation into a two phases can call “phase inversion” or “phase separation” [16]. The drug molecules or particles trapped within the matrix slowly released over time. This mechanism results in solvent exchange causing a phase inversion that leads to matrix formation, which in turn controls the rate of drug release [14]. To clearly understanding, the schematic diagram of drug-loaded BOR-based ISM administered into the crevicular pocket and its transformation from the solution state into a solid-like matrix *via* phase inversion mechanism to modulate the drug release is shown in Figure 1.

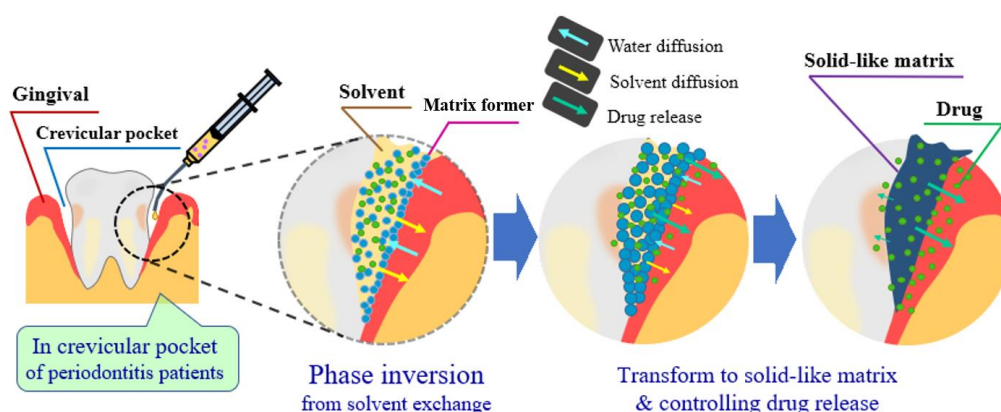


Figure 1 Schematic diagram of drug-loaded ISM administered into the crevicular pocket and its phase transformation [17].

Type of phase inversion is classified in 2 types, according to their rate of phase inversion [10]. First, fast forming phase inversion undergo phase inversion at a rapid rate (from seconds to minutes) resulting in formation of a thin membrane with an inner porous matrix structure [15]. This change is a result of formulation using solvents that are strong and hydrophilic in nature (e.g. NMP, DMSO, benzyl alcohol) [18]. This system is of a lower viscosity and it requires lower force of injection [19]. In contrast, a slow forming phase inversion undergoes the phase inversion at a slow rate (from hours to days). Solvents used in this system are weaker and are hydrophobic in nature, therefore a matrix formation occurs at a slow rate [18]. The viscosities of this solutions system is usually of an order that makes an injection difficult unless the system is pre-emulsified or preheated to 37 °C [19]. Moreover, the matrix conformation can be described as being uniformly dense with a limited number of pores [12, 18]. Limited pores result in slower drug release from these implants than that from fast forming phase inversion matrix but burst release is notably reduced. Thus, this system is not suitable to be used for sustainable dosage form due to the extended period of time for solidification of matrix formation [10].

Nowadays, ISM showed a great promise in the field of drug delivery, which led to commercial products are recently approved by the FDA, whilst some are currently undergoing clinical trials. Most attracting feature of ISM systems has been its applicability to a wide range of clinical indications such as Atridox[®] containing DH for treatment of a chronic periodontal disease, Eligard[®] containing leuprolide

acetate for treatment of an advanced prostate cancer, Sandostatin[®] containing octreotide for acromegaly treatment and Relday[™] containing risperidone for schizophrenia and bipolar disorder treatments [10]. In 1990s, the first ISM commercial product was released onto the market, namely Atridox[®], and approved by U.S.FDA in the late 1998. It consists of DH as active ingredient by using poly DL-lactide as matrix former dissolved in NMP. It is licensed for the treatment of chronic periodontal disease and it utilizes the initially liquid state of the polymer solution to allow injection of the antibiotic into the periodontal pocket. This system has been reported to allow the release of doxycycline over 7 days [20]. Atridox[®] allows local treatment of the periodontal disease *via* direct application of the antibiotic and also that removal is not required as the system is bioabsorbable. Clinical research determined that the local delivery of doxycycline resulted in a gain in clinical attachment and also a reduction in probing depths [21]. Atridox[®] is designed as the two-syringe system to ensure that the drug stability is maintained for the shelf life, that based on Atrigel[®] technology [22]. One syringe contains the dried drug powder, whilst the other contains the required amount of polymer solution. Before injection, the syringes are coupled and both components are mixed thoroughly in a cyclical fashion between the two syringes for nearly 100 times to form a homogeneous mixture prior to injection [23]. Nevertheless, this method can be a lengthy process and is obviously not ideal. A two-component system could therefore be detrimental in terms of progress towards a patient self-administered formulation [10].

The most ISM formulations, matrix-forming materials are required which are considered as a key substance of this system. The matrix-forming materials should be a water insoluble, biocompatible, biodegradable, safety (non-toxic) and miscible with solvent and drug [10]. Recently, aqueous insoluble materials have been employed as matrix forming components for ISM of such as poly-(lactic-co-glycolic acid) or PLGA, hydroxypropyl methylcellulose or HPMC, Eudragit[®] RS, bleached shellac, ethyl cellulose and cholesterol [24-28]. For example, thermosensitive *in situ* forming gel was developed by using poloxamer 407 as a gelling agent. The anti-solvent mechanism through solvent exchange-based is the migration of solvent outward from the systems to the environment while the aqueous phase of environment migrates inward resulting in the phase inversion of the matrix component which is a poorly water-soluble material [29, 30]. PLGA was used as a matrix former for *in situ* forming implants to carry typical antipsychotics such as risperidone and paliperidone, which required a long-term drug delivery system. The application with this delivery system showed a good result clinically since it could load the 50% antipsychotics and minimize the burst release together with sustainable drug release over 3 weeks [30]. Ethylcellulose, bleached shellac, and eudragit RS were used as the aqueous insoluble materials of solvent exchange-induced *in situ* forming matrix systems. These polymers were dissolved in NMP then the prepared solutions were tested on viscosity, rheology, rate of water penetration, injectability, matrix formation behavior and matrix degradation. The rapid matrix formation behavior was achieved after injected their solutions into the aqueous phase [31]. Thereafter, DH, metronidazole, and benzoyl

peroxide were used as the antimicrobial agents for the antimicrobial-loaded *in situ* forming gel systems and they were evaluated similarly as previously mentioned. The systems exhibited a sustainable drug release pattern together with the efficient antimicrobial activities against *S. aureus*, *Escherichia coli*, *Candida albicans*, *Streptococcus mutans*, and *Porphyromonas gingivalis* [24]. The *in situ* forming matrix of buprenorphine was formulated by using PLGA-PEG-PLGA (triblock) as a matrix former together with NMP as a solvent for minimizing the burst release. The thermosensitive property of the triblock and H-bonding between the solvent and the PEG of the triblock minimized the rapidly outward diffusion of the solvent resulted in a limited burst release [32]. The PLGA/HPMC-based *in situ* forming implants was developed to improve the physical key properties such as hardness, springiness, resilience and stickiness. The developed system exhibits a sticking to teeth' surfaces and greater mechanical strength than the commercial products. Chlo-site® showed less adhesion and quickly shrank while Parocline® showed less mechanical strength [33]. Alginate microspheres encapsulated with *in situ*-formed bismuth sulfide nanoparticles was developed for tumor therapy *via* embolization, hyperthermia and sustained doxorubicin release ability [34]. Recently, not only polymers but also lipids that were used for *in situ* forming system development as a matrix component. Cholesterol was used as a matrix former for *in situ* forming gel to deliver DH at a periodontal pocket for periodontitis treatment [28]. Phospholipid-based phase transition gel was developed for prolonging the release of ropivacaine. The prepared solution containing phospholipid, medium chain triglyceride dissolved in ethanol transformed into matrix state rapidly after subcutaneous injection and prolonged drug release subsequently [35]. Solvent exchange *in situ* forming system was also developed for the macromolecule delivery such as insulin in which the phospholipid was used as a matrix former [36]. Drug-loaded *in situ* forming systems *via* anti-solvent exchange mechanism, which self transforms into a solid-like matrix after being exposed to an aqueous environment of the target site, is one of the useful systems for many purposes since there is the biological fluid located in almost every part of the creatures.

Solvent and additive in ISM

Practically, the solvents or vehicles of the ISM system are water miscible, biocompatible and organic in nature. Importantly, solvents should efficiently dissolve the matrix forming material and active compound, and be miscible with water and bodily fluids. Polarity of the solvent should be such that at least 10% should be soluble in water [10, 37]. The viscosity of solvent should be within the range that is injectable. Solvent strength and its affinity for water directly influence the nature of phase inversion and matrix formation. For example, solvents having a high water affinity such as NMP and DMSO provoke a fast forming phase inversion [30]. Whereas, the hydrophobic solvents such as triacetin and ethyl benzoate promote a slow forming phase inversion [38]. Solvents that possess a water solubility of

below 7% w/w have been shown to result in a slower drug release due to a reduction in water uptake [39].

Nowadays, ISM systems most commonly consist of DMSO (Figure 2 (A)) and NMP (Figure 2 (B)) as the solvents preferentially due to their pharmaceutical precedence over other solvents [10]. NMP, molecular weight of 99.13, is a very strong solubilizing agent with low viscosity. It has known as a solubilizing excipient used in parenteral and oral medications. Many commercial pharmaceutical products containing NMP are currently available in the market. The European Commission Scientific Committee on Consumer Safety concluded that NMP has low acute toxicity by oral, dermal, and inhalation routes of administration [40-42]. DMSO, molecular weight of 78.13 is a non-aqueous dipolar aprotic solvent with colorless, oyster-like odor and relatively low toxicity (LD₅₀: 2.5 to 8.9 g/kg) [43, 44]. It is safe if used less than 100 mg/day [43]. It is miscible with most common solvents such as alcohol, ether, aromatics and water [45]. It readily penetrates into the cellular membranes; thus, it has been employed as a skin penetration enhancer [43, 46, 47]. It is approved by US-FDA to treat the interstitial cystitis [46]. In addition, it shows various pharmacological actions including analgesic, antioxidant and anti-inflammatory activities [47, 48]. In results of many studies found that DMSO is soluble in water which can breaks down the water structure and forms the strong hydrogen bond complexes with water molecules due to stronger interaction between DMSO-water [43, 46].

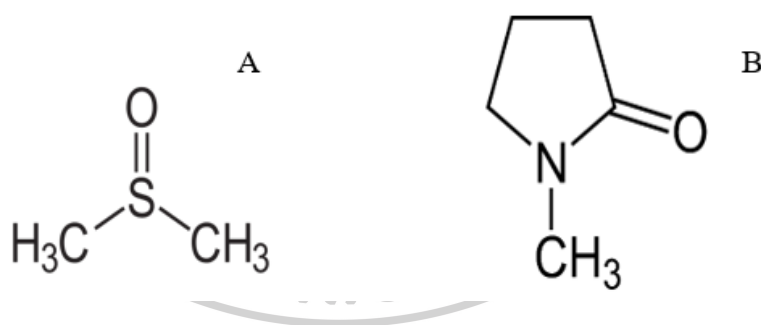


Figure 2 Chemical structure of solvent DMSO (A) and NMP (B).

Normally, slow phase inversion systems may have a limited burst release but they take a lengthier period of time to form a matrix, compared to that of fast inversion systems. As a way of compromising, the hydrophilicity of main solvents such as NMP and DMSO is reduced by the addition of more hydrophobic solvents (e.g., ethyl benzoate, triacetin, and benzyl benzoate) to act as a co-solvent. Because the solvent quality and water miscibility are reduced, the drug release progresses from a profile that involves a high burst release to one that follows a zero-order release kinetics. This can be attributed to a change in the implant/depot morphology from a porous structure to a more uniformly formed dense structure [10, 18]. Similarly, the modification of the release rate is possible by selecting appropriate ratio of hydrophilic solvent and hydrophobic solvent [10]. Ethanol has been reported as a solvent of *in situ* forming gel comprising Carbopol[®] (poly(acrylic acid)) and HPMC

combination, and water-soluble polymers polymethacrylic acid (PMA) and polyethylene glycol (PEG) for delivery plasmid DNA in their role to provide protection from DNase degradation [49]. Ethanol was also a solvent in preparation *in situ* gel prepared from HPMC K100 / carbopol934 for loading zolmitriptan and gellan gum/ Lutrol 127-based for loading rivastigmine for treatment migraine and nasal congestion, respectively[50-52]. Moreover, ethyl acetate is one of organic solvents for preparing PLGA-based *in situ* forming drug delivery system for sustained release of montelukast [53]. For preparation of oil in-water *in situ* forming microparticle system of risperidone prolonged release, ethyl acetate was employed as a solvent of the internal polymer solution phase for dissolving PLGA [54].

Matrix former material

In recent years, the formulation concept of "using active chemicals as excipients" has sparked significant interest. This innovative approach harnesses the physical and chemical properties of active ingredients to facilitate preparation or enhance the efficacy of the primary pharmaceutical ingredient. Within the realm of ISM systems, the matrix-forming material necessitates being a hydrophobic substance capable of dissolving in selected organic solvents (DMSO and NMP). This material should also possess pharmacological activity that proves beneficial for periodontitis treatment or displays synergistic effects when loaded with a therapeutic drug [55]. Moreover, the emergence of small molecule-based *in situ* forming systems presents a viable alternative to conventional polymeric-based systems, addressing certain limitations associated with the latter. Consequently, the matrix-forming materials chosen for this thesis study are BOR and IBU, as they align aptly with the innovative formulation concept and the distinctive properties of ISM systems.

Borneol (BOR)

BOR ($C_{10}H_{18}O$, molecular weight 154.25) (Figure 3) is a highly lipid-soluble ($\text{Log } K_{o/w}=2.85$) bicyclic monoterpene alcohol with fragrant odor and bitter tastes. It occurs naturally in over 260 plants and found in citrus peel oils, spices such as nutmeg, ginger and thyme. It has melting point and boiling point at 202°C and 212°C , respectively [3, 4]. It's slightly denser than water, insoluble in water (1,186 mg/L), soluble in alcohol and ether and slightly soluble in propylene glycol [56, 57]. It can be analyzed by microwave-assisted extraction (MAE) followed by gas chromatography with flame ionization detector (GC-FID) or gas chromatography-mass spectrometry (GC-MS) method. Nowadays, it has been used as the food flavoring, air care products, decorative cosmetics and etc. [3]. BOR has been approved by the Food and Drug Administration as a flavor because of its non-toxicity [3]. However, it can be metabolized to camphor; meanwhile, camphor could be absorbed rapidly and reported to cause the seizures, respiratory failure, myocarditis and hepatotoxicity; thus, BOR should not be applied in children and pregnant women without caution [58].

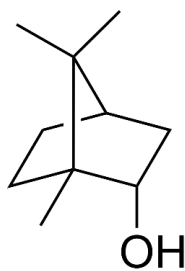


Figure 3 BOR chemical structure.

BOR is classified as natural BOR and synthetic BOR. Traditional natural BOR originated from the resin of *Dipterocarpus aromaticus* Gaertn.f. in the forest of Southeast Asia. Because of its rare resource and expensive price, the essential oil extracted from the fresh branches and leaves of *Cinnamomum camphora* (L.) Presl. is widely used as a substitute of natural BOR in recent years. In contrast, synthetic BOR is obtained by simple chemical transformation of camphor and turpentine oil [59].

In the pharmaceutical field, BOR has been reported to treat cancer, ischemic stroke, heal of skin wound. Additionally, it could relieve the local itching, minor aches and pains of muscles and joints. Furthermore, it has antibacterial effect, antinociceptive effect and anti-inflammatory activity. In traditional Chinese medicine, it has been used for treatment of ocular disease, preventing and curing cardiovascular and cerebrovascular diseases [59]. BOR has been claimed to co-administer with an active compound for enhancing their delivery manner to the brain; thus, it is a promising new agent that deserves further use as a blood brain barrier (BBB) permeation enhancer for drug deliver to central nervous system [59-62]. BOR can enhance the BBB permeability and improve the transportation of kaempferol in rats by *in vivo* microdialysis sampling to brain which the brain bioavailability evidently increased with the co-administration with BOR of 15 and 30 mg/kg [59]. BOR-modified solid lipid nanoparticles were effective for transporting drugs to the brain [62]. It was considered as a safe and effective penetration enhancer for ocular drug administration because of improving the transcorneal penetration of both hydrophilic and lipophilic compounds without causing toxic reactions [63]. It has also reported to enhance an intestinal absorption of drug [64]. Electrospun BOR-polyvinylpyrrolidone (PVP) nanocomposites enhanced the BOR dissolution and increased its physical stability against sublimation for long-time storage by immobilization of BOR molecules with PVP through hydrogen bonding; however, BOR weakened the tensile properties of nanofibers [65]. BOR significantly reduced the nociceptive behavior at the early and late phases of mice paw licking and reduced the writhing reflex in mice and BOR-treated mice reduced the carrageenan-induced leukocytes migration to the peritoneal cavity without impair motor coordination [66]. High dose of BOR could cause a stomach irritation [67]; thus, the sustained-release solid dispersions (SRSDs) for co-loading gastrodin and BOR were prepared as a sustained release matrix [61]. Compared with the free mixture of gastrodin and BOR, brain targeting of SRSDs was slightly weaker, but stomach irritation caused by BOR was obviously minimized. Combined bismuth subgallate and BOR evenly applied to cheesecloth and placed over the lesion areas on the back of Sprague–Dawley rats had a synergistic effect on the skin wound restoration by decreasing lesion area, while increasing granulation tissue

formation, re-epithelialization, eating behavior and reconstitution of skin appendages [68]. BOR showed an anti-fibrosis activity which its mechanism of action might partly be relevant to inhibiting effects on fibroblasts mitosis, collagen and TIMP-1 production; thus, it is recommended for safe to use as a penetration enhancer for treatment of oral submucous fibrosis [69]. BOR has been used as sublimable agent for highly porous and low-density non-effervescent floating tablets [70]. BOR at concentrations of 0.5 and 5 $\mu\text{g/ml}$ did not induce a significant toxicity on the immune system cells. A significant increase of cell viability was detected when the thymocytes were cultivated with BOR at concentrations of 0.5 and 5 $\mu\text{g/ml}$ [71]. Low toxicity of BOR has been reported for skin irritation on human volunteer and mice/mouse [3]. Owing to its longed term use with safety and apparent low aqueous solubility but miscible in organic solvent, BOR may serve as a matrix forming component of *in situ* forming matrixes for oral cavity and crevicular pocket targeting.

Ibuprofen (IBU)

IBU ($\text{C}_{13}\text{H}_{18}\text{O}_2$ molecular weight: 206.28) (Figure 4) is a monocarboxylic acid that is propionic acid in which one of the hydrogens at position 2 is substituted by a 4-(2-methylpropyl) phenyl group. It has a role as a non-steroidal anti-inflammatory drug, a non-narcotic analgesic, a cyclooxygenase 2 inhibitor, a cyclooxygenase 1 inhibitor. This drug belongs to class II in The Biopharmaceutical Classification System, which means that despite the good permeability, this drug has poor aqueous solubility (21 mg/L at 25°C) and hydrophobic as high logP value (3.97) [72]. This drug is a mostly common over the counter (OTC) drug for mild to moderate pain and inflammation in conditions such as dysmenorrhea, headache, postoperative pain, dental pain, musculoskeletal and joint disorders, osteoarthritis, rheumatoid arthritis, peri-articular disorders such as bursitis and tenosynovitis, and soft tissue disorders such as sprains and strains. It is also used as an antipyretic [73]. IBU have a common GI adverse events as dyspepsia, nausea, heartburn, diarrhea, constipation and vomiting. In rarely event may found case related with cardio vascular system or respiratory system or liver and kidney function such as asthma, agranulocytosis, neutropenia, aplastic anemia, hemolytic anemia, eosinophilia and coagulation defects [73, 74].

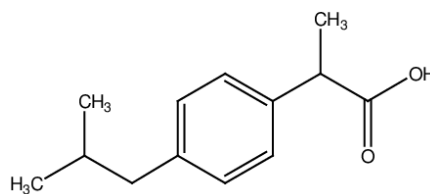


Figure 4 IBU chemical structure.

Interestingly, numerous studies have reported an antimicrobial activity of IBU. the effect of IBU on an inhibited growth of the *gram*-positive bacteria *Bacillus subtilis* and *S. aureus*, as evidenced by an MIC (Minimum Inhibitory concentration) of 1.25–2.5 mg/mL, respectively [75, 76]. It has shown equipotent antibacterial activity against three *gram*-negative human pathogens such as *E. coli*, *Salmonella*

typhi, and *Enterobacter sp.* with an MIC range of 2.5–5 mg/ml [76]. For *in vivo* study, IBU was demonstrated a significant reduction in bacterial burdens in lung and spleen in mice that infected with a *P. aeruginosa*. Moreover, IBU enhances the treatment and exhibits synergism with pyrazinamide in murine infection model [77, 78].

For treatment oral cavity disease, IBU treatment for both oral and topical route enable to reduce the sign and severity of gingivitis is including gingival color, bleeding, pocket depth and bone loss [79]. IBU and other nonsteroidal anti-inflammatory drugs (NSAIDs) used to treat periodontitis have also been shown to reduce bone loss caused by inflammatory responses [80, 81]. IBU loaded drug delivery system for local administration in oral cavity is fabricated for controlled release rate and delivery drug to target site such as *in situ* matrix-forming system [82, 83] and hydrogel system (Carbopol®) [84]. The IBU release kinetic from *in situ* matrix and hydrogel was presented as first order kinetic and Korsmeyer-Peppas release kinetic, respectively. Furthermore, chlorhexidine-IBU loaded-*in situ* matrix-forming system presented an antibacterial activity against periodontal pathogens such as *P. gingivalis* [82].

Antimicrobial agent

Lincomycin HCl (LH)

LH (C₁₈H₃₄N₂O₆S molecular weight: 443) (Figure 5) is the first lincosamide that has found use in clinical practice that consists of an unusual amino acid (propylhygric acid) linked by a peptide bond with the sugar (6-amino-6,8-dideoxy-1-thio-D55 erythro- α -D-galactopyranoside) as presented in Figure 5 [85-87]. The solubility data present this drug as very low hydrophobic drug (logP : 0.2) and quite high soluble in water (927 mg/L at 25 °C). LH is bacteriostatic antibiotic that bind with the 50 S ribosomal subunit and inhibit protein synthesis sensitive microorganisms. LH is highly active against *gram*-positive micro-organisms infections (Staphylococci, Streptococci, Pneumococci, *Diphtheria bacillus*, and Clostridia) and anaerobic infections (Peptococcus, Peptostreptococcus, Actinomyces, Propionibacterium, and *Clostridium fringens*, a few types of Peptococcus and Clostridium). It is used for serious bacterial infections: sepsis, osteomyelitis, septic endocarditis, pneumonia, pulmonary abscess, infected wounds, and purulent meningitis. LH is a reserve drug for infections caused by strains of staphylococci and other Gram-positive microorganisms that are resistant to penicillin or patients allergic to penicillin. Commonly adverse effect of LH is including nausea, vomiting, diarrhea and stomach pain. In the field of dentistry LH is effective in treating acute and chronic bone infections and soft-tissue infections of the face [86, 88]. LH was shown to be susceptible to an anaerobe bacterium found in the mouth is *P. gingivalis*. [89]. Drug delivery system of this antibiotic was fabricated for controlled drug release *via* several method such as attaching drug with albumin nanoparticles and loading drug into hydrogels for topical applications on wounds [90-92].

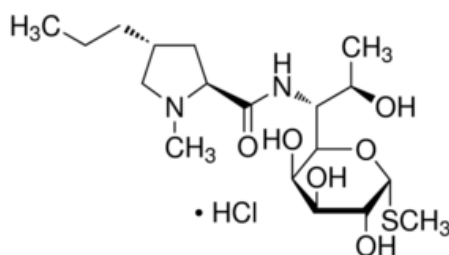


Figure 5 Chemical structure of LH

Doxycycline hyclate (DH)

DH ($2C_{22}H_{24}N_2O_8 \cdot 2HCl \cdot H_2O \cdot C_2H_6O$; molecular weight: 545) This is broad-spectrum tetracycline antibiotic exhibiting antimicrobial activity. It is soluble >76.9 ug/ml in water, act as hydrophilic compound ($\log P$: -0.25) and decomposes by photolysis, hydrolysis and thermal degradation. The structure of doxycycline is shown in Figure 6. It's a bacteriostatic drug that binds reversibly to the 30S ribosomal subunit, and perhaps the 50S subunit as well, preventing aminoacyl-tRNA from attaching to the mRNA-ribosome complex. This leads to an inhibition of protein synthesis. It is given orally or parenterally and is absorbed by gastrointestinal tract and excreted by kidneys. DH can cause the undesired side effects such as nausea, diarrhea, rash, and photosensitivity. Furthermore, this agent has exhibited an inhibition of collagenase activity [93]. It also causes the growth retardation and enamel hypoplasia. Furthermore, this drug is contraindicated from second half of the pregnancy to seven years in children because this drug have the adverse effects against the bone and tooth development [93].

DH is one of the most commonly used for periodontitis treatment. the oral regimen administration for periodontitis is 250 mg of drug four time a day for up to 9 months whereas the dose of local treatment depends on the severity of symptom (the shape, size and number of pockets) [94]. Sustainable drug delivery system od DH has been developed to reduce the frequency of drug administration and prevent the side effect occurred by systemic administration. Atridox[®] is a commercial product containing 5 or 10% of DH using poly (dl-lactide) as polymer dissolved in NMP prepared as an *in situ* forming gel for controlled release of DH for 7 days [95, 96]. The use of Atridox[®] could provide a high level of drug (250 mg/mL) in the gingival crevicular fluid for a period of 7 days and 10–20 mg/ml was still present for 3–5 days after the polymer had been removed [97]. Furthermore, Atrisorb-D[®] FreeFlow[™] which containing 4% doxycycline is licensed for local bacterial growth inhibition and healing with controlled release manner for a period of 7 days [10]. As previous studies, ISM formulation containing DH as model drug with various types of polymers such as Eudragit RS, ethyl cellulose and bleached shellac exhibited an effective inhibition of *S. aureus*, *E. coli*, *S. mutans* and *P. gingivalis* [24, 25, 27, 28]. Moreover, this drug released from different polymeric-based ISM containing 20% ethyl cellulose, 40% Eudragit RS, 30% bleached shellac, 10% cholesterol with 10%

benzyl benzoate for 9 h, 16 h, 2 days and 10 days, respectively [25, 27, 28, 98]. All these formulations were easy to inject into the target site because of their minimal force of injectability and could transform from solution into matrix-like structures for drug retention.

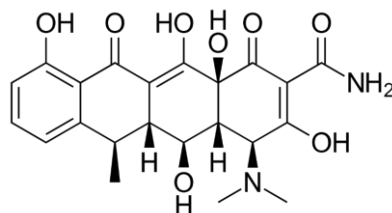


Figure 6 Chemical structure of DH.

Periodontitis

Nowadays, periodontal diseases affect about 20-50% of global population [99]. The 25.9% of an adult and 36.3% of an elder in Thailand have a health problem on the periodontitis with 4-5 mm depth of periodontal pocket. Over 19.8% and 12.2% of those adult and elder have the severe periodontitis (>6mm deep of periodontal pocket) which can cause more risk of infection, inflammatory, losing teeth and also affect the quality of life [100]. Moreover, it is a risk factor for cardiovascular disease such as coronary heart disease and stroke [101]. The main causes of periodontal disease are poor oral hygiene and tobacco use [102]. This disease is characterized by bleeding or swollen gums (gingivitis), pain, bad breath and destruction of surrounding structures of teeth resulting in alveolar bone loss, periodontal ligament destruction, attachment loss, gingival recession, bacteria accumulation, increased tooth mobility and eventually leading to tooth loss [102, 103]. In its early stage, called “gingivitis”, the gums can become swollen and red, and they may bleed. In its more serious form, called “periodontitis”, the gum can come away from the tooth and supporting bone, causing teeth to become loose and sometimes fall out [104].

Stages of gum disease from healthy gums to advanced periodontitis are shown in Figure 7. Most of the pathogen of this disease are gram-negative bacteria and some of them are anaerobe bacteria, for example, *Aggregatibacter actinomycetemcomitans*, *P. gingivalis*, *Bacteroides forsythus*, *Prevotella intermedia*, *Campylobacter rectus*, *Eubacterium nodatum*, *Streptococcus intermedius*, and *Treponema denticola* [105]. These bacterial infection can cause the periodontium (gingiva, periodontal ligament, dental cementum, and alveolar bone) damages by the bacterial by-product and immune activation [106]. Some of microbes is related to the inflammation and tissue loss including enterobacteria (such as *E. coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*), *C. albicans*, *Neisseria* spp., *Olsenella uli*, *Hafnia alvei*, *Serratia marcescens*, and *Filifactor alocis* [107, 108]. Moreover, *staphylococcus aureus* could be found in periodontal pocket [109]. In healthy man, the gap between gingival and tooth is around 1 mm. However, the periodontal pocket of periodontitis patient is usually exceeding 5 mm [110]. In general, the pH of the normal oral cavity

of a human is maintained near neutrality (~ 7.06) by saliva, whereas the average pH of the normal oral cavity of a patient with chronic periodontitis is 6.85, indicating slight acidemia [111].

The treatment techniques for these diseases are recommended as following: mechanical (scaling and root planning), systemic and local antibiotics treatment [112]. Several antimicrobial agents have been evaluated for their clinical and microbiological efficacy in periodontal diseases such as chlorhexidine, tetracyclines (tetracycline, doxycycline and minocycline), metronidazole, ciprofloxacin, vancomycin and etc. [113-118]. The large doses of systemic antibiotic must be taken in order to achieve sufficient concentrations in the gingival crevicular fluid of the periodontal pockets [119, 120], which may be caused side effects of antibiotics [121, 122] and antibiotic resistance [123, 124]. The local antibiotics delivery systems are interesting to keep a high and effective drug concentration at the target site with minimizing the side effect [125]. Nowadays, several dosage form such as films [126, 127], gel [128] including *in situ* forming systems [14, 25] have been reported for carrying antibiotics as the local drug delivery systems for periodontitis treatment. In the present study, various antimicrobial and anti-inflammatory agents are chosen as the model drugs for local drug administration.



Figure 7 Stages of gum disease from healthy gums to advanced periodontitis [129].

ISM is an interesting drug delivery system for the delivery of antimicrobial agents to treat periodontitis and oropharyngeal candidiasis. ISM is a low viscosity liquid system that makes it easy to deliver to difficult administration sites in such a small cavity. As demonstrated in Figure 8[130, 131], after self-transformation to a solid-like matrix phase in the cavity, ISM is able to attach at the administration site and sustain drug release from the system. In this study, the aim is to select the appropriate biosafety material for solvent exchange-induced ISM. The interesting materials include fatty acid, rosin, BOR, and IBU as matrix formers, whereas DMSO, NMP, and ethanol are applied as the solvents. These compound both of matrix forming agent and solvents have been mentioned to have beneficial bioactivity for treatment against these diseases. Their antimicrobial and anti-inflammatory activities

might promote the efficiency of treatment. All candidate antimicrobial agents have shown interesting inhibitory activity against periodontitis pathogen microorganisms.

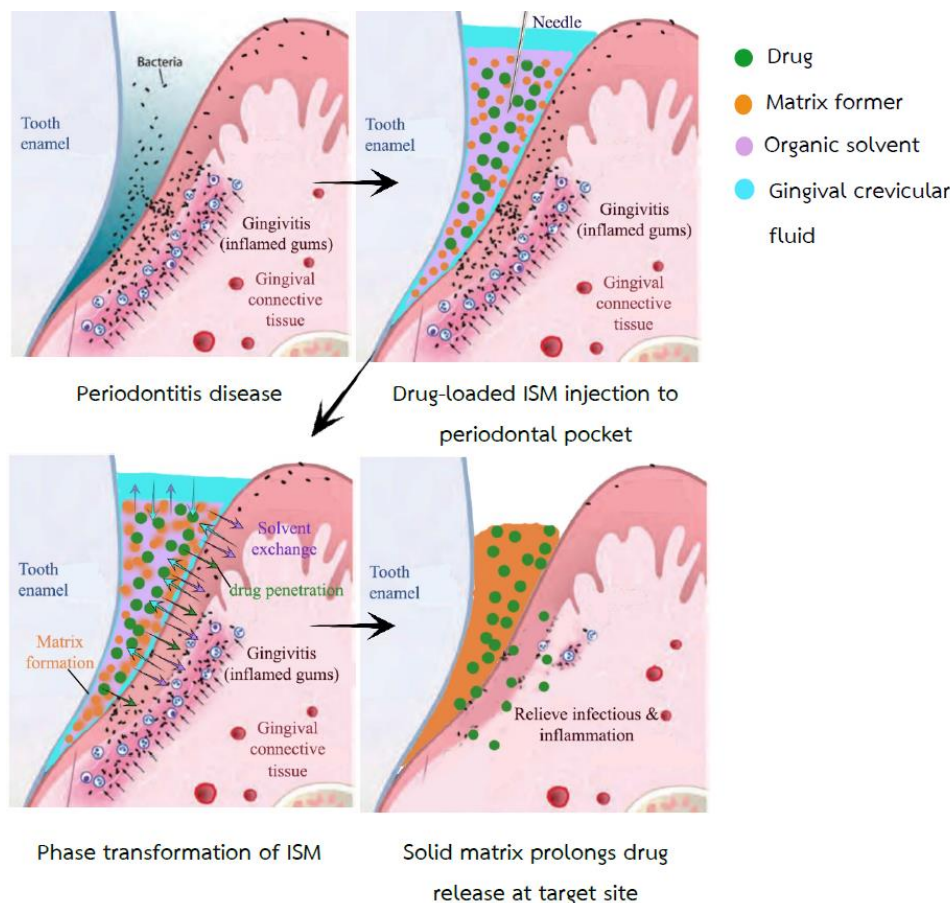


Figure 8 Schematic of ISM solution self-transformation to solid matrix after administration and controlled drug release to target site [130, 131].

Computational Molecular dynamics simulation

Molecular dynamics (MD) has been classified as a computer simulation method for determining the physical movements of atoms and molecules over time, femtoseconds to milliseconds. The atoms and molecules are permitted to interact for a fixed period of time, giving a view of the dynamic "evolution" of the system. For a briefly MD flow, an initial atom coordinate is received from both experimental structures or similar modeling data [132]. The forces acting on every atom (force fields) are calculated subsequently when the initial model is built. The force fields could reveal various molecular features for example springs for bond length and angles, periodic functions for bond rotations and the Coulomb's law for van der Waals and electrostatic interactions [133]. Subsequently, accelerations and velocities and to rearrange the atom positions is calculated using the equations of motion (classical Newton's law of motion) through the force field. The most popular simulation codes including CHARMM [134], NAMD [135] and AMBER [136] have long been compatible with the messaging passing interface (MPI). The term Amber refers to the

various programs that work together to setup, perform, and analyze the MD simulations. The result of this program is two text files including a coordinate (prmcrd) file that contains the Cartesian coordinates of all atoms, and a parameter-topology (prm-top) file that contains all other information required to compute energies and forces (includes atom names and masses, force field parameters, lists of bonds, angles, and dihedrals, and additional book keeping information) [137]. Additionally, MD calculates the properties of the system containing several atoms or molecules by tracking atom motion dynamics with the equations of motion through the force field, resulting in a large number of calculations. Dynamics tracking can be achieved using an integrator such as Verlet method, Rangegatta method, and Gear predictor corrector [133]. Although MD is a computation of properties systems based on very small numbers of atoms or molecules, recent studies found that the results from MD simulations were not much different from the macro-level experiments [133]. Recently, MD simulation has been employed as a necessary tool in the biological and chemical systems, investigating the structure, dynamics, and thermodynamics of biomaterials [132].

In the pharmaceutical field, the practical application of the MD is docking strategies which useful in phase of the drug design [138, 139]. MD was used to explain and explore various phenomenon such as mechanism for hydrophobic gating in ion channels [140] and water permeation through aquaporin [141]. MD has also used to simulate the nucleation and crystallization of materials in various conditions. The microscopic mechanism of crystal nucleation and growth behavior of methane hydrate in the porous media was revealed using a couple of simulations [142]. Focusing on drug delivery study, MD has been performed for study possibility of material or drug for formulation and explanation of drug delivery property and phenomenon such as miscibility of material, drug diffusion and mobility, drug permeability through membrane, drug loading efficiently and drug release behavior. Molecular simulations can provide a detailed description of the mechanism of drug loading/release. However, unlike continuum approaches. A useful approach is to obtain the drug diffusivity through the carrier, which can either be correlated with the experimental data or used as a model parameter in the continuum model. For example, Chantadee *et al.* applied MD to explain matrix formation of fatty acid-based ISM system, to compare the crystallization of fatty acid in system under difference water ratio and movement of vancomycin HCl from fatty acid crystal to environment and correlation the MD output result with the experimental drug release data. This research work revealed the more retardation of drug release behavior by lower molecular weight fatty acid than higher molecular weight. MD results presented the matrix formation of low molecular weight fatty acid was influenced with effect of water ratio more than high molecular weight fatty acid (Figure 9). In addition, the molecular weight of fatty acid influenced on tortuosity of the fatty acid matrix structure and correlated with drug release pattern [143]. Mahdavi *et al.* attempted to study the influence of pH of aqueous environment against doxorubicin loading and drug releasing efficiency from graphene oxide nanocarriers system and pristine graphene system. The computational results presented in this work revealed the underlying mechanisms of doxorubicin loading and release from graphene oxide and pristine graphene surfaces in which the drug diffusion coefficient increased with decreasing pH of aqueous environment [144].

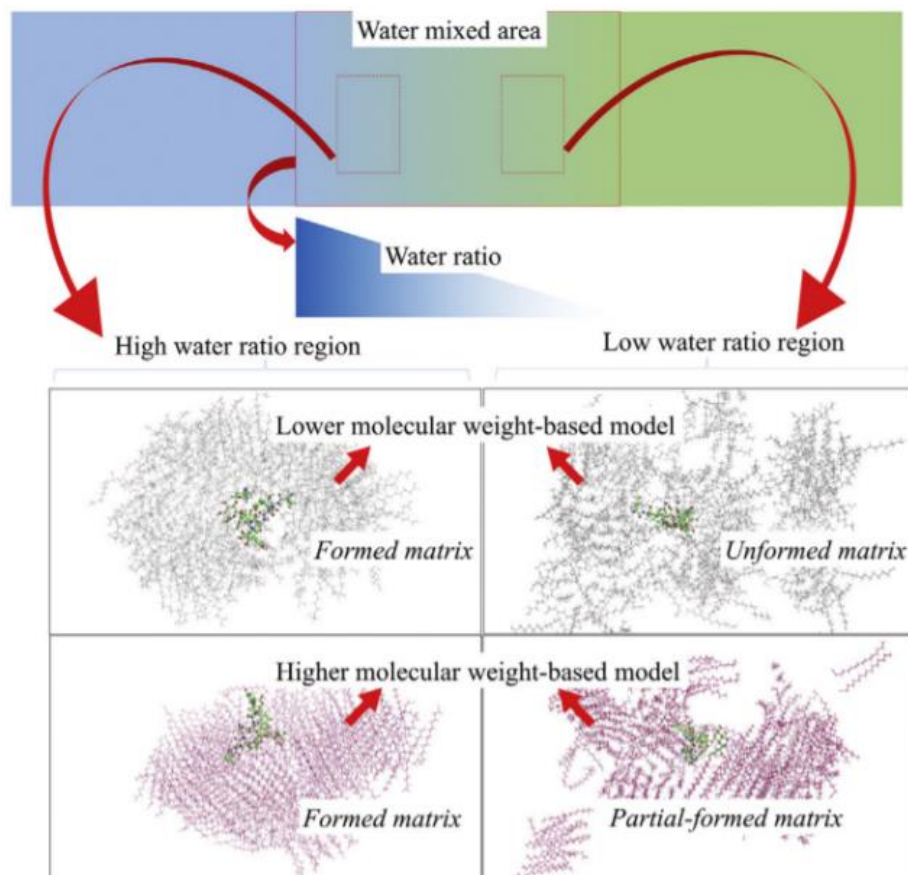


Figure 9 The matrix formation MD simulation under difference water ratio between low and high molecular weight fatty acid [143].

MD was also applied for understanding the 2-phase system behavior and investigating the phenomenon among interfacial plane of them, which could explain the dynamic changing property of system. Zahariev *et al.* studied the adsorption and transfer of simple non-ionic amphiphiles surfactant (alkanols and the alkyl-oligoethylene glycol ethers) across the water-oil (pentane, hexane and heptane) interface (Figure 10 (left)) to correlate the free energy change to thermodynamic (Figure 10 right) and structural parameters of the model with empirical experiment [145]. Moreover, Holte *et al.* used MD to investigate the behavior of surfactant molecules in the interfacial region of organic–water for comparison between protonate and deprotonate laurate surfactant. The simulation presented the deprotonated laurate surfactant molecules causing a larger increase in interfacial width than their protonated interfacial width. The broad distribution of protonated lauric acid molecules might move into the organic layer while the tails of the deprotonated surfactants were observed to align more nearly normal to the interfacial plane than their protonated and the deprotonated surfactant headgroups penetrated farther into the water layer than the protonated (Figure 11) [146]. Herein, computer simulation techniques are applied to correlate with the macro-level properties of the *in situ* forming process to fulfill and complete understanding mechanistic underpinning phase transformation of solvent exchange-induced ISM for treatment of oral cavity infectious diseases.

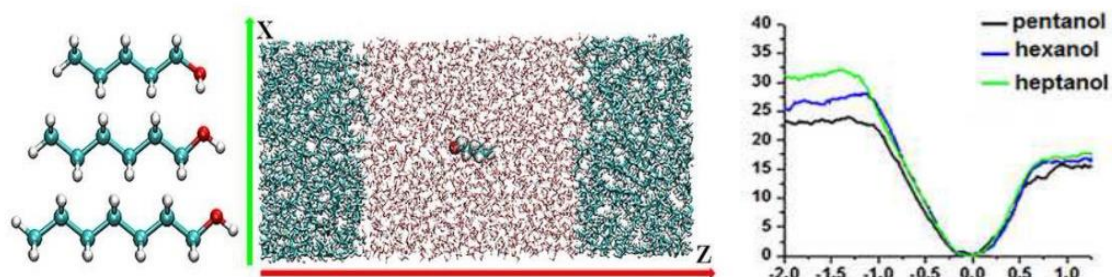


Figure 10 The transfer of surfactant across the water-oil interface simulation (left) and free energy result (right) [145].

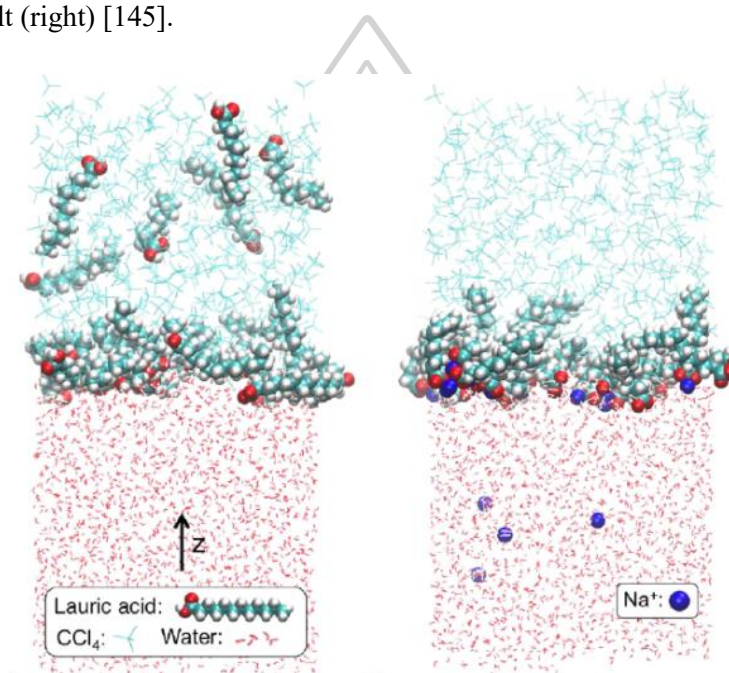


Figure 11 MD simulation of surfactant orientation in the interfacial region of organic-water and comparison between protonate (left) and deprotonate laurate surfactant (right) [146].

CHAPTER 3 METHODOLOGY

Equipment and instrument

1. Aluminum pans and covers (Perkin Elmer, Shelton, USA)
2. Amber 20 software package (University of California, San Francisco, California, United States)
3. Anaerobic incubator (Forma Anaerobic System, Thermo Scientific, Ohio, USA)
4. Analytical balance (CP224S, Sartorius, Germany)
5. Analytical balance (PA4102, Ohaus, USA)
6. BIOVIA Discovery Studio Visualizer 23 programs (3DS Boston Campus, WALTHAM, Massachusetts, USA)
7. Brookfield rheometer (HADV-III U CP, Brookfield Engineering, USA)
8. Chimera v. 1.17.3 (the University of California, San Francisco, USA)
9. Desiccators (Biologix Research Company, USA)
10. Differential scanning calorimeter (DSC 8000, Perkin Elmer, USA)
11. Filter paper (Whatman no.1, United Kingdom)
12. Filter set (Nylon membrane, 47 mm, 0.45 μ m, Lot 43920100, ANPEL Laboratory Technologies (Shanghai) Inc., China)
13. Fourier-transform infrared spectrophotometer (Nicolet 4700, Madison, USA)
14. Gauss View program (GaussView 6.0.16, Semichem Inc., Shawnee Mission KS, USA)
15. Gaussain program (Gaussain 16, Gaussian Inc., Wallingford CT, USA)
16. Glass slide
17. Goniometer (FTA 1000, First Ten Angstroms, USA)
18. High-performance liquid chromatography (Agilent 1260 Infinity II, Agilent Tech, USA)
19. Incubator (BPH-9042, Shenzhen Kingdak, China)
20. Inverted stereomicroscope (TE2000S, Nikon Instruments Inc., New York, USA)
21. LC Column (Eclipse XDB-C18, 4.6 x 150mm, 5 μ m, Dr. Maisch, Germany)
22. Magnetic stirrer (C-MAG-HS7, IKA, Germany)
23. Microbiological safety cabinet class II (safe 2010, Holten, EU)
24. Micropipette 2-20 μ l, 20-200 μ l, 100-1000 μ l and micropipette tip
25. Microplate reader (Victor Nivo, Perkin Elmer, USA)
26. Needle (27 guage)
27. Notepad++ v. 8.5.7
28. Nylon membrane 0.45 μ m. 47 mm.
29. Petri dish glass (9 cm, 15cm) & petri dish plastic (15 cm)
30. pH meter (Seven compact, Mettler Toledo, Japan)
31. Plastic syringe (1 mL, 3 mL, 5 ml, 10 mL)
32. Powder X-ray diffractometer (Mini Flex II, Rigaku cop, Japan)
33. Pycnometer (Densito 30PX, Mettler Toledo, Japan)
34. Quartz cuvette
35. Shaking incubator (NB-205, N-Biotek, Korea)
36. Sonicator (ELMA Transsonic Cleaning Baths 890/H, Germany)

37. Stainless cylinder cup (6 mm)
38. Stereo-microscope (SZX10, Olympus corp., Japan)
39. Syringe filter (Preclean™ 13 mm, Nylon membrane, 0.45 µm, Lot 41890480, ANPEL Laboratory Technologies (Shanghai) Inc., China)
40. Texture analyzer (TA.XT plus, Stable Micro Systems, UK)
41. Thermal gravimetric analysis (Pyris TGA, Perkin Elmer, USA)
42. UV-Visible spectrophotometer (Cary 60 UV-Vis G6860A, Agilent Technologies, Malaysia)
43. VMD program (VMD 1.9.4, the Theoretical and Computational Biophysics Group, University of Illinois at Urbana-Champaign, USA)
44. Vortex mixer (KMC-1300V, Vision scientific, Korea)

Materials

1. Acetonitrile, HPLC grade (lot no. K3H111, Fisher Chemical, Korea)
2. Agarose (lot H7014714, Vivantis, Malaysia)
3. *A. actinomycetemcomitans* ATCC 29522 (MicroBiologics Inc., U.S.A.)
4. BOR (Chareorsuk-Osot, Nakhon Pathom, Thailand)
5. Brain heart infusion (BHI) (lot no. 0270845, Bacto, USA)
6. Brain heart infusion agar (BHA) (lot no. 0298038, Bacto, USA)
7. *C. albicans* ATCC 17110 (Department of Medical Sciences, Ministry of Public Health, Nonthaburi, Thailand)
8. Dimethyl sulphoxide (DMSO) (lot no. 1862992, Fisher Chemical, UK)
9. DH (lot no. 20071121, Huashu Pharmaceutical Corporation, China)
10. *E. coli* ATCC 8739, *E. coli* ATCC 25922 (Department of Medical Sciences, Ministry of Public Health, Nonthaburi, Thailand)
11. Ethanol, AR grade (lot no. 195125-1223, QReC, New Zealand)
12. Fully refined paraffin wax (lot no. 7119K18, Nippon Seiro, Japan)
13. IBU (lot no. 4000/1101/18/A-0150B, P.C. drug center CO.LTD, Thailand)
14. LH (lot no. 2005012, Nova medicine CO.LTD, Thailand)
15. Methanol, HPLC grade (lot no. 2040269, Fisher Chemical, UK)
16. Monobasic sodium phosphate (lot no. 1310152834, Ajax Finechem, Australia)
17. Nile red (lot no. BCBP8959V, Sigma, USA)
18. *N*-methyl pyrrolidone (NMP) (lot no. 144560-118, QReC, New Zealand)
19. Phosphoric acid (lot no. AA606020, Ajax Finechem, Australia)
20. *P. gingivalis* ATCC 33277 (MicroBiologics Inc., U.S.A.)
21. Potassium bromide, For IR spectroscopy (lot no. I297362002, Loba Chemie PVT, India)
22. Potassium dihydrogen orthophosphate (lot no. E23W60, Ajax Finechem, Australia)
23. Sabouraud dextrose agar (SDA) (lot no. 7312647, Difco, USA)
24. Sabouraud dextrose broth (SDB) (lot no. 6345690, Difco, USA)
25. Salicylic acid (lot no. RAS1101900, P.C. drug center CO.LTD, Thailand)
26. Sheep blood agar (Department of medical science, Ministry of public health, Thailand)
27. Sodium chloride (lot no. B302511906, Loba chemie PVT. LTD, India)
28. Sodium fluorescein (lot no. SHBL6563, Sigma, USA)
29. Sodium hydroxide (lot no. AF310204, Ajax Finechem, Australia)

30. *S. aureus* ATCC 25923, *S. aureus* ATCC 6923 and *S. aureus* ATCC 6538
(Department of Medical Sciences, Ministry of Public Health, Nonthaburi,
Thailand)
31. Tryptic soy agar (TSA) (lot no. 7341698, Difco, USA),
32. Tryptic soy broth (TSB) (lot no. 8091999, Difco, USA),



Experimental set-up

3.1. Binary mixture of ISM evaluation

3.1.1. Preparation of binary mixture formulas

Matrix forming agents, including BOR and IBU at concentrations of 10-40% w/w, were dissolved in DMSO and NMP by continuous mixing overnight with a magnetic stirrer until clear solutions were obtained. The component of each binary mixtures ISM is shown in Table 1 and 2.

Table 1 The composition of BOR-based binary mixtures.

Formulation	BOR (%w/w)	Solvent (%w/w)	
		DMSO	NMP
DB10	10	90	
DB20	20	80	
DB30	30	70	
DB40	40	60	
NB10	10		90
NB20	20		80
NB30	30		70
NB40	40		60

Table 2 The composition of IBU-based binary mixtures.

Formulation	IBU (%w/w)	Solvent (%w/w)	
		DMSO	NMP
ID10	10	90	
ID20	20	80	
ID30	30	70	
ID40	40	60	
IN10	10		90
IN20	20		80
IN30	30		70
IN40	40		60

3.1.2. Physicochemical characterization of ISM-solutions

3.1.2.1. Appearance of prepared formulation

The appearances of formulations such as clarity, homogeneity and precipitation were recorded by visual observation.

3.1.2.2. Determination of density

The densities of the solvent and formulations were measured with a density meter (Densito™ 30PX, Mettler Toledo, Japan) (n=3) at room temperature.

3.1.2.3. Apparent viscosity and rheological behavior

The viscosities of all formulations were measured using a Brookfield rheometer (HADV-III U CP, Brookfield Engineering, USA) with spindle number 40 at 1500 rpm (n=3). Furthermore, the rheological behavior of the formulation was evaluated from a relation between shear stress versus shear rate.

3.1.2.4. Determination of contact angle and surface tension

The contact angle of formula on glass, paraffin and agarose gel surfaces was measured using the goniometer (FTA 1000, First Ten Angstroms, USA) with a pump out rate of 1.9 $\mu\text{L/s}$. The contact angle at time point of 5 s was recorded and estimated from the first automatic image of droplet (n=3). Moreover, surface tension was determined the change in shape of a pendant drop of a formulation suspended in air after injection using a goniometer with a pump out rate of 1.9 $\mu\text{L/s}$ (n=3).

3.1.2.5. Injectability and mechanical properties

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

The mechanical properties of the obtained matrix after complete solvent exchange were determined with the same instrument. A 0.6% agarose solution was prepared by dissolving in PBS (pH6.8) was poured onto petri dishes (agarose gel height of 1 cm). After gel setting, it was cut with a cylinder cup into a 300 μL hole (diameter of 7 mm.) at the center of the petri dish. A 150 μL aliquot of the sample is injected into this agarose hole and placed for 3 days to ensure complete phase transformation and matrix formation. A stainless spherical probe (diameter = 5 mm) of the texture analyzer is gradually pressed into an acquired matrix at a constant speed of 0.5 mm/s. When the penetration depth is 1.5 mm, this probe is held for 60 s and then moved backward at a speed of 10 mm/s. The relationship between the applied force and the displacement of the probe is plotted as a function of time and recorded for the maximum penetration force. The remaining force is measured at 60 s after the probe is held, whereas the adhesion force is the maximum force measured during the backward movement. The mechanical property is presented as a ratio of remaining force/maximum penetration force; high values indicate high elasticity, whereas low values indicate high

plasticity [13]. All formulations are determined in triplicate at room temperature.

3.1.3. Characterization of matrix forming behavior

3.1.3.1. Matrix formation after injection

A 1 ml aliquot of the binary mixture is injected directly through the 27-gauge stainless needle into 5 ml of PBS (pH 6.8) in a glass test tube to investigate matrix formation with the time of formulation by photographing at various times (0, 5, 10, 15, 20 and 30 min).

3.1.3.2. Morphology of matrix formation and matrix formation rate in an artificial crevicular pocket

The agarose gel is prepared and cut with a cylinder cup using the same method as mentioned above (topic number 3.1.2.5. mechanical properties). This agarose gel represents artificial crevicular pocket in human. A 150 μ L aliquot of the ISM formulation is added into the cylindrical hole or well of the above agarose gel compare the rate of matrix formation between formulations. After being in contact with the aqueous phase from the agarose gel, the formulation caused the solvent exchange and changed to an opaque solid-like matrix with phase inversion mechanism. The morphology of the matrix formation is photographed under a stereo microscope (SZX10, Olympus corp., Japan) at 1, 3, 5, 10, 15, 20 and 30 min. Matrix formation is determined under a stereoscope with SZX10 Series software.

3.1.3.3. Phase transformation tolerability property of binary mixtures against water addition.

The capability of binary mixture to maintain its solution state against a phase transformation once water is added is determined by %water tolerance value. The experiment is performed by filling 2.5 g of sample into glass test tube then each 20 μ L of aqueous titrant is gradually added through micropipette and stirred using a vortex mixture and repeat water adding until the clear solution became turbid owing to phase transformation of mixture from prepared samples. The environmental temperature of this experiment is controlled under 2 conditions are room temperature (25 °C) and body temperature (37 °C). The amount of total added deionized water was recorded then the water tolerance was calculated with following equation (eq. 1) (n= 3). The water tolerance change was plot between percentage of water tolerance value and matrix former concentration in different solvent systems [147].

$$\% \text{water tolerance} = \frac{\text{water amount (g)}}{\text{Sample amount (g)} + \text{water amount (g)}} \times 100 \quad (\text{eq.1})$$

3.1.3.4. Interfacial interaction of binary mixture and aqueous

The interaction at the interface between the formulation and the agarose gel is observed to investigate phase transition under various environment factors such as ionic strength and matrix forming agent

concentration. A 0.6 % agarose gel is prepared by dissolving in PBS (pH 6.8). For monitoring the diffusion of aqueous and solvent during phase transformation the fluorescent dyes as sodium fluorescein (150 µg/ml) is added into agarose gel. Then, it was poured on the glass slide to obtain a 2 mm thick agarose gel, which is then cut into a direct smooth edge (2 cm away from one edge of the glass slide). It is placed near the sample solution of 20 µL and close to the edge of the incision. After the formulations are exposed to PBS in agarose gel, their morphological changes owing to phase inversion at the interface are photographed under an inverted microscope (TE2000S, Nikon Instruments Inc., New York, USA) at different time intervals.

For tracking hydrophilic and hydrophobic compartment mobility among the interface of ISM and environment, fluorescence dyes including sodium fluorescein (150 µg/ml) (as hydrophilic compartment) and Nile red (100 µg/ml) (as hydrophobic compartment) are loaded into ISM solutions and contact against agarose gel. Their interfacial interaction was also checked using the previous mentioned method.

3.2. Drug-loaded ISMs preparation and their physicochemical properties, drug release and bioactivities

3.2.1 Preparation of drug-loaded ISMs preparation

For preparation of drug-loaded ISMs, the drugs including DH and LH were individually added into the IBU and BOR system, respectively. Each drug-loaded ISMs series was prepared using previous mentioned method for binary mixture preparation (3.1.1. Preparation of binary mixture formulas). The component of each drug-loaded ISMs is shown in Table 3 and 4.

Table 3 Composition of DH-loaded IBU-based ISM formulations.

Formulation	IBU (%w/w)	Solvent (%w/w)		DH (%w/w)
		DMSO	NMP	
DN	-	-	95	5
DIN40	40	-	55	5
DD	-	95	-	5
DID40	40	55	-	5

Table 4 Composition of LH-loaded BOR-based ISM formulations.

Formulation Code	Lincomycin HCl (% w/w)	Borneol (% w/w)	NMP
NL1	1	-	99
NL5	5	-	95
NL7.5	7.5	-	92.5
NBL1	1	40	59
NBL5	5	40	55
NBL7.5	7.5	40	52.5

3.2.2. Physicochemical characterization of drug-loaded ISM-solutions

Physicochemical properties of these formulations were evaluated using the same method as mentioned above (topic 3.1.2.1-3.1.2.5) for their appearance, density, viscosity, rheological behavior, contact angles, surface tension, injectability and mechanical properties.

3.2.3. Characterization of drug-loaded matrix forming behavior

The matrix-forming behavior of these formulations were evaluated using the same method as mentioned above (topic 3.1.3.1– 3.1.3.4) for their matrix formation after injection, interfacial interaction and morphology of matrix formation in an artificial crevicular pocket.

3.2.4. *In vitro* drug release

The drug release from each formula was tested and compared with non-matrix forming agent formula for evaluation of the drug-entrapped properties of interested matrix former. A cylindrical-shape porcelain cup (diameter of 1 cm and higher of 1.2 cm) containing 0.4 g of the formulation is immersed in 80 ml of PBS (pH 6.8) at 37 °C, and the speed of the rotation shaker was maintained at 50 rpm to simulate the drug release behavior from the crevicular pocket. The release medium was sampled (5 mL) and replaced with 5 ml of fresh PBS.

3.2.4.1. IBU/DH amounts determination

The concentration of drug release was determined using high-performance liquid chromatography (HPLC). Agilent 1260 HPLC system with a photodiode array detector (Infinity, USA) and C₁₈ column (150 × 4.6 mm, 5 µm particle size, Dr. Maisch, Germany) were used. The analyte was analyzed using gradient elution with the mobile phase that consisted of three solvents,

i.e., 0.1% formic acid/acetonitrile/methanol with a flow rate of 1 mL/min. The gradient was applied according to the following program: 90:1:9 to 10:40:50 for 8 min, held at 50:20:30 for 5 min, and changed to 90:1:9 for 2 min. The intact DH and IBU were employed as the standard by dissolving in the release medium to obtain the calibration curve. The detection wavelength was at 264 nm. The cumulative mean drug release $\pm$ S.D. in percentage was calculated (n=3).

3.2.4.2. LH concentration determination

The concentration of drug release is determined using high-performance liquid chromatography (HPLC). Agilent 1260 HPLC system with a photodiode array detector (Infinity, USA) and C₁₈ column (150 $\times$ 4.6 mm, 5 μ m particle size, Dr. Maisch, Germany) were used. The mobile phase consisting of 8% acetonitrile in 50 mM aqueous phosphoric acid adjusted to pH 3 and a flow rate of 1.5 mL/min. The retention time for LH hydrochloride was approximately 6.5 minutes. The column temperature was processed at ambient temperature. The sample injection volume was 20 μ l. The detection wavelength was at 214 nm. The cumulative mean drug release $\pm$ S.D. in percentage is calculated (n=3).

3.2.4.3. Kinetic of drug release study

To analyze the *in vitro* drug release data in each formulation, least square fitting the experimental dissolution data (cumulative drug release >10% and up to 80%) to the mathematical equations (zero order, first order, Higuchi's and Korsmeyer-Peppas model) was performed using DDSolver which is a menu-driven add-in program for Microsoft Excel [148]. The coefficient of determination (r^2) was used to indicate the degree of curve fitting. Goodness-of-fit is also evaluated using the model selection criterion (MSC).

3.2.5. Antimicrobial activities

The microbe strains used in this experiment were *S. aureus* ATCC 25923, *S. aureus* ATCC 6923, MRSA ATCC 43300, *E. coli* ATCC 8739, *C. albicans* ATCC 17110, *P. gingivalis* ATCC 33277 and *A. actinomycetemcomitans* ATCC 33384. The antimicrobial activities of the ISM formulation were tested using the cylinder plate method. This technique was performed based on the diffusion of the formulation from a stainless-steel cylinder cup (inside diameter of 6 mm and height of 10 mm) through agar gel inoculated with the test microorganism. The bacterial and fungal inoculum approximately closed to the 0.5 McFarland turbidity standard were spread on TSA and SDA, respectively. A 100 μ l aliquot of each formulation was dropped into the cylinder cap, which was placed on the surface of an inoculated medium plate. For *P. gingivalis*, the test was performed in an anaerobic incubator (Forma Anaerobic System, Thermo Scientific, Ohio, USA). After

incubation at 37 °C for 18 h, the diameter of inhibition zone against the tested organism was measured using a ruler (n=3). The interested binary mixture and DH and LH loaded ISM were selected for test antimicrobial activities and using normal saline solution as negative control. (n=3).

3.2.6. Anti-inflammation activity

The anti-inflammation activities of the doxycycline HCl-loaded IBU-based ISM formulations were tested using the inhibition of albumin denaturation [149]. Briefly, a 5 ml mixture solution composed of 2.8 ml of phosphate buffered saline pH 6.4, 0.2 of egg albumin (from fresh hen's egg), 2 ml of sample from *in vitro* drug release test (topic 3.2.4. *In vitro* drug release) was prepared. Double distilled water and diclofenac sodium were used as negative and positive control, respectively. The mixtures were incubated at 37 °C for 15 min in the incubator and then heated in the water bath at 70 °C for 5 min. After cooling down to the room temperature, the absorbance of the mixtures was measured at 660 nm using a spectrophotometer (Hitachi U-2000, COAX Group Corporation Ltd.). Percentage inhibition of protein denaturation was calculated as equation (eq.2).

$$\%inhibition = \frac{absorbance\ of\ negative\ control - absorbance\ of\ sample}{absorbance\ of\ negative\ control} \times 100 \quad (eq.2)$$

3.3. Statistical analysis

Data were displayed as mean and $\pm$ standard deviation (mean $\pm$ S.D.). SPSS for Windows was used for the statistical analysis. The statistical significance for the measurement was examined and considered at $p < 0.05$ and 0.01 using one-way analysis of variance (ANOVA) followed by an LSD post-hoc test.

3.4. Characterization of dried matrix precipitate.

DH-loaded IBU-base ISM was interested in matrix precipitated study to monitoring the crystallinity of hydrophobic drug and matrix former after phase transition completely in ISM.

3.4.1. Preparation of dried selected matrix

Dried matrix of these formulations was prepared with similar method of mechanical property study (topic 3.1.2.5. Injectability and mechanical properties) and dried into the solid matrix in desiccator for 3 days.

3.4.2. Fourier-transform infrared (FT-IR) spectroscopy

The precipitated samples were blended with potassium bromide (AR grade for IR) and plunged with plunger and die to made a pellet using hydraulic press at compression force of 4 tons. The % FT-IR transmittance of the sample was recorded using FT-IR (Nicolet 4700, Madison, USA).

3.4.3. Powder X-ray diffractometer (PXRD)

PXRD (Mini Flex II, Rigaku corp, Japan) was used to investigate the crystalline properties of the raw BOR and the ISM precipitates. The test samples were grinded into fine particle by mortar and pestle and loaded into aluminum holder. All samples were scanned in the degree of 2θ in the range of 5 to $30^\circ 2\theta$.

3.4.4. Differential scanning calorimetry (DSC)

All precipitated samples were weighed at 2.5 - 4 mg then placed in a non-hermitically aluminum pan. The pan was sealed then the thermal property was determined by DSC (DSC 8000, Perkin Elmer, USA). The samples were investigated under $10^\circ\text{C}/\text{min}$ of heating rate and the heating range is $30\text{-}100^\circ\text{C}$ under $40\text{ mL}/\text{min}$ of nitrogen gas flow.

3.4.5. Thermogravimetric analysis (TGA) and differential thermogravimetric analysis (DTGA)

The 5 mg of each compound was weighed and placed in non-hermitically aluminum pan and then sealed then thermogravimetric analysis was tested with TGA (Pyris TGA, Perkin Elmer, USA). Heating rate is $10^\circ\text{C}/\text{min}$ with heating range is $30\text{-}300^\circ\text{C}$ under $200\text{ mL}/\text{min}$ of nitrogen gas flow. While, DTGA was calculated by rate of %weight change at each time.

3.4.6 Water induced crystallization study *via* Wide Angle X-ray Scattering (WAXS)

The crystallization of ISM was achieved using varying levels of deionized water to replicate different stages of the crystallization process. Specifically, mixed ISM formulas were prepared with three water levels corresponding to 10%, 80%, and 100% of their respective water tolerance values. These formulas were coded as formula-W1, -W8, and -matrix, representing the initial stage, late stage, and crystal stage of ISM crystallization, respectively. Comparative analysis was conducted on the crystal properties of these three water-containing formulas, along with a formula to which no water was added, as well as with the raw matrix former material. To investigate the crystallinity properties of the samples, WAXS data were collected at Beamline 1.3 W at the Synchrotron Light Research Institute (SLRI) in Thailand. In the WAXS conditions, the radiation source utilized a multipole wiggler, and the Q range employed for this study ranged from 0.00 to 32.93 nm^{-1} (corresponding to a 2θ range of $0.00 - 47.59^\circ$). The incident X-rays had an energy corresponding to a wavelength of 0.13776 nm . A sample-detector distance of 182.097 mm , which covered a range of measurable characteristic d-spacing from 1 to 33 nm , was used for these experiments.

3.4.7. Topographic observation of surface matrix under scanning electron microscope (SEM)

The dried solid matrixes were coated with gold and then examined under a field emission-scanning electron microscope (FE-SEM) (TESCAN MIRA3, Czech Republic) at an accelerating voltage of 15 kV.

3.5. *In silico* molecular interaction and diffusion study of drug loaded ISM.

3.5.1. Study of molecular interaction

The computational work like molecular modelling of each component in formulation had been carried out using density field theory (DFT) to ascertain a molecular interaction between each pair molecules.

First, the structure of each substance was built by GaussView program (GaussView 6.0.16, Semichem Inc., Shawnee Mission KS, USA). Moreover, the substances in each pair (e.g., matrix former surrounded by solvent, and solvent with itself) were rearranged and built the new file using the same program. All structures are optimized at the B3LYP with 6-311G** basis set level *via* Gaussain program (Gaussain 09, Gaussian Inc., Wallingford CT, USA). The molecular modelling of each formulation was created following by binary mixture ISM. Each binding intermolecular energy between molecule in formula was calculated by electronic energy of each molecule following equation (eq. 3).

$$BE_{AB} = (EE_A + EE_B) - EE_{AB} \quad (\text{eq. 3})$$

Where BE_{AB} denotes the binding energy of binary molecule A and B, EE_A , EE_B , and EE_{AB} are the electro-energy values of molecule A, B, and of binary molecule between A and B respectively

3.5.2. Computer dynamics modelling of mechanistic phase inversion and drug movement

The computer dynamics modeling technique was employed to gain insight into the phase transformation and molecular movement of each individual component within the ISMs formula when exposed to an aqueous environment. Atom coordinates for each substance were obtained from databases such as Pubchem and Cambridge Crystallographic Data Centre (CCDC). For substances not available in the database, molecular structure was created *via* Gausview programme.

Force field generation step of each molecule

The MD simulations were conducted using the Amber 20 software package, and the force field parameters for the molecules were generated utilizing the Antechamber module with the general AMBER force field [150]. The output files from force field generation step was including .prep and .frmod files. Residue name of molecule for using in MD simulation running in this thesis coding as Table 5.

Table 5 Residue name of molecule component

Molecule	Residue name
IBU	IBU
BOR	BOR
DH	DOX
LH	LIN
Water	WAT
DMSO	DMS
NMP	NMP

The design of model box for MD simulation

Simulation models were established based on the molar ratios of each formulation's components, including the loaded drug (LH or DH), the matrix former (BOR or IBU), and the organic solvent (DMSO or NMP), as depicted in Table 6. Simulation boxes for each formula were created using Chimera ver. 1.17.3 and BIOVIA Discovery Studio Visualizer 23 programs to organize each single molecule.pdb file into a simulation box.pdb file, as shown in Figure 12. Then, certain details in the script of the simulation box.pdb file were adjusted using the Notepad++ v8.5.7 program to ensure compatibility with running in a Linux terminal in the next step.

Table 6 Number of different molecules of LH-loaded BOR-based ISM (A) and DH-loaded IBU-based ISG (B) formulations.

(A)

Molecular dynamic box details	DD	DN	ID40	IN40	DID40	DIN40
Amount of DH molecules	80	80	0	0	80	80
Amount of IBU molecules	0	0	1600	1600	1440	1440
Amount of DMS molecules	8640	0	6400	0	5040	0
Amount of NMP molecules	0	6880	0	4800	0	4000
Amount of WAT molecules	7896	7973	8837	10029	7458	9074
Mole ratio of DH:IBU:(DMSO/NMP):WAT	1:0:108:98.7	1:0:86:99.7	0:18:72:99.5	0:18:54:112.8	1:18:63:93.2	1:18:50:113.4
Total amount of molecule in system	16616	14933	16837	16429	14018	14594
Total amount of atom in system	166408	138490	143311	159687	124774	143222

(B)

Molecular dynamic box details	DB10	DB40	NB10	NB40	NL5	NBL
Amount of LH molecules	0	0	0	0	80	100
Amount of BOR molecules	400	1600	400	1600	0	2100
Amount of NMP molecules	0	0	5600	4800	6240	4500
Amount of DMSO molecules	7200	4800	0	0	0	0
Amount of WAT molecules	6700	1400	15000	5812	4600	5815
Mole ratio of LH:BOR:(DMSO/NMP):WAT	0:4:72:67	0:16:48:14	0:4:56:150	0:16:48:58.2	1:0:78:57.5	1:21:45:58.15
Total amount of molecule in system	14300	7800	21000	12212	10920	12515
Total amount of atom in system	157700	170534	149012	147036	118520	156445

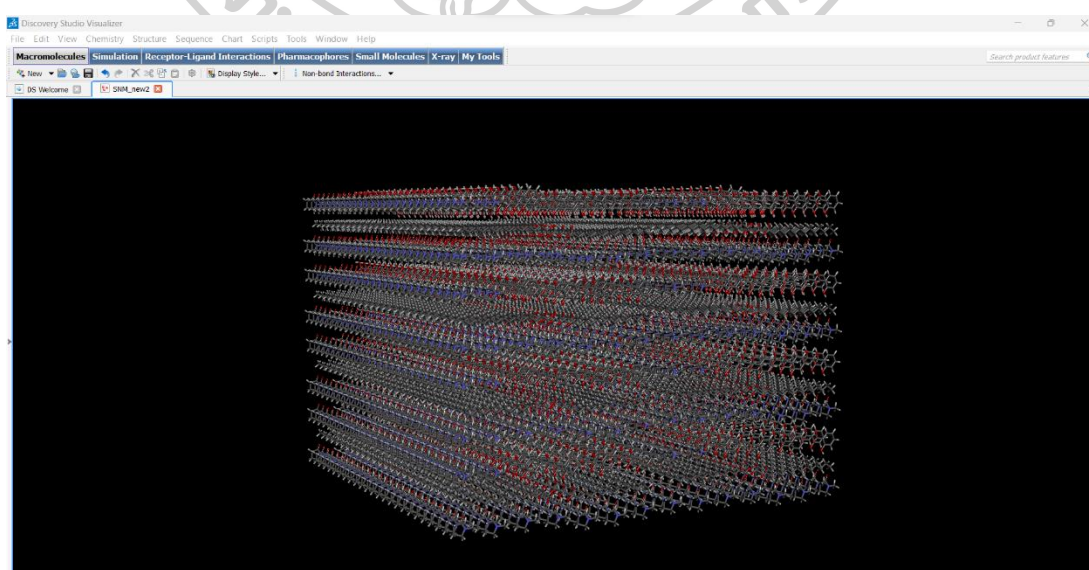


Figure 12 Completed simulation box of ISM system.

The parameters for the simulation box were generated prior to the MD simulation using the leap module. This step required the force field files (.mol2 and .frcmod files) and the simulation box file for the generation of the output parameter file, which included coordinate (.crd) and topology (.top) files.

In the simulation run, the sander module of the Amber20 software package was used to calculate the dynamic motion of the molecular components using the parameter file generated in the previous step. The MD simulation run consisted of five steps, including

Step 1. Minimization of solvent molecule

This step aims to use potential energy minimization (PEM) techniques to eliminate high energies in the predicted model and achieve local minima closer to the native structure of solvent molecules in the system [151].

Step 2. Minimization of all molecule

To perform the minimization of all molecules in the system.

Step 3. Heating molecule in system

This step involves raising the temperature of the system to reach the predetermined temperature of 310 K during the simulation.

Step 4. Equilibrating system

Once the simulation system reaches the temperature of 310 K, the simulation box needs to be equilibrated at a constant temperature before starting the production step

Step 5. Production MD simulation

After preparing the simulation system by completing steps 1-4, the production step involves generating simulation results under the conditions of a 200ns simulation time at 310 K.

MD simulation for induced phase transformation of ISM system

The MD simulation for inducing phase transformation in the ISM system aims to understand the phenomenon of phase transformation and the molecular motion of each substance in the drug-loaded ISM around the interface with the aqueous environment. In the previous step, the solvated ISM simulation box was combined with water on one side, as illustrated in the Figure 13. The water box was generated using TIP3P to solvate the ISM simulation box with a specific number of water molecules determined based on the water tolerance values provided in the Table 6. Subsequently, parameter files (.top and .crd) for the system were generated by “Generation parameter of model system” method, and the MD simulation was run, following a similar process to “Runing MD simulation for solvation system” steps 1-5.

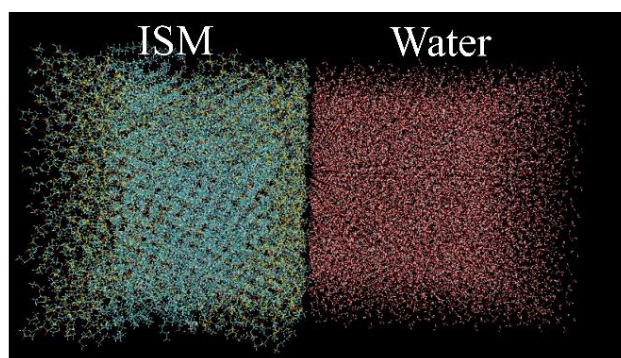


Figure 13 experimental design of MD simulation around interface region between formula and aqueous.

Analysis of MD simulation result

The MD simulation result was analysed for explanation phase transformation in molecular aspect such as mobility, intermolecular interaction and agglomeration of each molecule. Configuration of molecule in system was monitored *via* Visual Molecular Dynamics (VMD) software (The Theoretical and Computational Biophysics group, The Beckman Institute, University of Illinois at Urbana-Champaign, Illinois, United States). The output parameter was comprising H-bonding amount, molecule density profile, root mean square deviation (RMSD), diffusion constant and radius of gyration parameter.

H-bonding and density profile of molecule

Simulation analysis involved the use of VMD software to calculate hydrogen bond (H-bond) occupancy and density of molecules. H-bond formation was determined based on criteria where the distance between the acceptor and donor atoms was less than 3.5 Å, and the angle formed by the acceptor, donor, and hydrogen atoms was less than 60°. Density profiles of drug and matrix former molecules were probed using VMD's density profile tool to observe the density distribution of molecules in the simulation box coordinates [152].

Root-mean-squared deviation (RMSD)/Diffusion constant/Radius of gyration

All RMSD, diffusion constants, and radius of gyration values for components were obtained using the cpptraj module [150].

RMSD measures the deviation of a target set of coordinates from a reference set of coordinates to determine changes in atom locations, and the results were plotted in Å/ns to interpret the fluctuation of observed molecules in the simulation box. Diffusion constants, which indicate the migration distance from the initial position, were calculated using the cpptraj module in conjunction with the time-evolved trajectory of the simulation [150]. The diffusion constant is automatically calculated from the mean square displacement datasets, which were calculated based on the initial positions. The radius of gyration is a fundamental parameter that offers valuable insights into the compactness and structural arrangement of molecular entities within the

system. This metric is critical for understanding how molecules are organized and distributed within the system's spatial confines. When the radius of gyration value is smaller, it signifies a tighter molecular compactness, suggesting more condensed and highly organized configuration. This level of compactness reflects to the physical arrangement of molecules and matrix formation ability.

In summary, Molecular Dynamics (MD) simulations have emerged as a pivotal tool for unveiling the intricate molecular-level details of phase transformation within *in situ* forming matrix systems. Throughout this thesis, the Amber20 software package played a central role in facilitating MD simulations, guiding each step of the process through a series of meticulously designed modules, as illustrated in Figure 14. MD simulations have provided a comprehensive understanding of the structural changes, compactness, and intermolecular forces that govern the phase transformation process. These simulations not only illuminate the mechanisms underlying matrix formation and drug release but also yield invaluable insights into how various factors, such as solvent type and drug presence, influence the behaviour of the system.

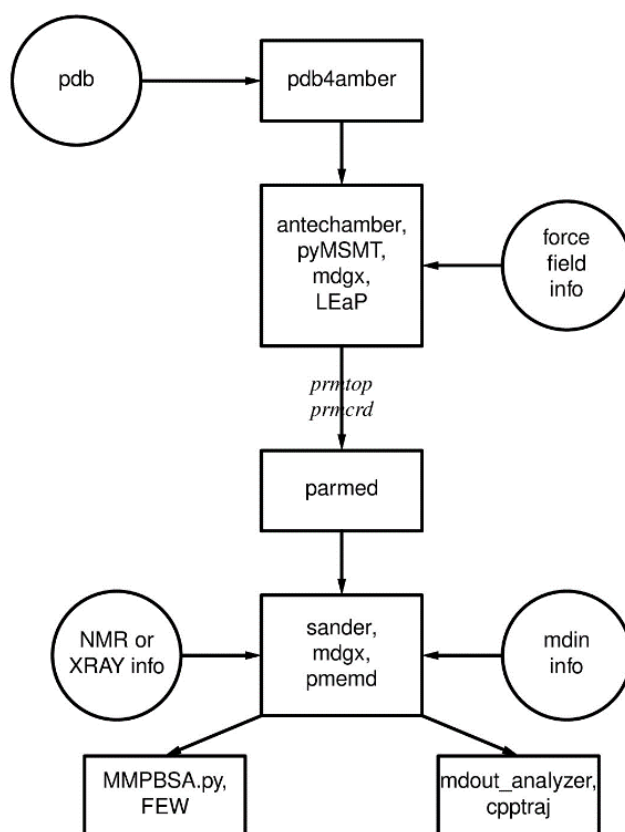


Figure 14 Basic information flow of Amber20.

CHAPTER 4

Result and discussion

Part 1: 4.1. LH -Loaded BOR-Based *In Situ* Matrix for Periodontitis Treatment

4.1.1. Physicochemical Properties

4.1.1.1. Physical Appearance, Density, and Viscosity

The drug-free and LH-loaded BOR-based ISMs were prepared into clear solutions by mixing for a short period using NMP as a solvent. Increasing the concentration of LH turned its color from a colorless to a clear yellowish owing to the yellowish characteristic of the drug. The density values of the drug-free and LH-loaded BOR-based ISMs were significantly ($p < 0.05$) less than that of pure solvent (Table 7). The addition of BOR significantly ($p < 0.05$) decreased the density of the systems both of DMSO and NMP series due to the low-density characteristic of BOR as reported in the literature as 1.01 g/cm³ [153]. Additional loading of LH increased the density of the ISMs; nevertheless, the density of NBL7.5 was slightly less than NMP. Meanwhile, the density values of other formulations were significantly less than that of NMP ($p < 0.05$). Typically, the density value of a mixture is the summation of the characteristic of each intact compound under the mole fraction ratio [154]. Overall, the density of the ISMs was higher than that of the aqueous phase, indicating that they would settle gradually into the crevicular fluid in the periodontal pocket.

The viscosity of the ISMs exhibited a slight increase compared to that of NMP, and this trend was observed in both DMSO and NMP series. It showed a minor upward trend with the inclusion of BOR and LH, as indicated in Table 7. This inherent viscosity change could be attributed to the straightforward structure and relatively diminutive molecular size of BOR, which effectively minimized molecular interactions with NMP. This remarkable feature not only resulted in a small increase in viscosity but also facilitated easy injectability, making these formulations highly advantageous for *in situ* forming systems designed for precise localized drug delivery.

Importantly, the use of small molecular matrix-forming materials like BOR offers even more compelling benefits. This approach presents a significant contrast to systems based on resins and polymers, which are frequently plagued by notably high viscosities as previously reported [25, 98, 155]. For instance, ISM solutions formulated with 25% w/w ethyl cellulose, 35% w/w bleach shellac, and 40% w/w Eudragit RS were observed to be excessively viscous to flow [156]. In stark contrast, due to their composition of uncomplicated molecules, ISMs incorporating high levels of loaded BOR or short-chain saturated fatty acids exhibited remarkably low viscosities and superior injectability [143, 157-159]. This highlights that the use of such small molecular

matrix-forming materials not only ensures easy administration but also offers a viable solution to the challenges associated with high viscosity in traditional polymer-based systems.

4.1.1.2. Surface Tension and Contact Angle

Surface tension, a property arising from cohesive molecular forces that strive to minimize surface area, is a commonplace phenomenon in liquids [160]. The surface tension value of the 40% BOR solution exhibited a significant reduction ($p < 0.05$) compared to that of both the pure organic solvent and the drug-loaded ISMs, as presented in Table 7. This reduction in surface tension can be attributed to the introduction of BOR, which seems to disrupt molecular interactions at the liquid surface. Interestingly, the surface tension of the BOR-dimethyl sulfoxide binary mixture displayed a decline with increasing BOR loading. This trend reached a saturation point when the BOR concentration reached 30% w/w [17], signifying that BOR's effect on surface tension plateaus at this threshold. The observed decrease in surface tension of the ISMs is advantageous, as it enhances their ability to spread and attach to the target site, thereby facilitating their effective application within the periodontal pocket.

The contact angle, generally defined as the angle between a tangent to a liquid droplet and the surface of a solid, is a parameter often used to gauge surface hydrophilicity. In this study, the contact angles of NMP and ISMs on various surfaces, including glass slides, agarose gels, and paraffin, were investigated and are presented in Table 7.

Specifically, on a glass slide, the contact angle of organic solvent (both of DMSO and NMP series) exhibited a significantly higher value ($p < 0.05$) compared to both BOR-based and the drug-loaded ISMs. This disparity suggests that the inclusion of BOR reduced the hydrophilicity of the formulation, leading to a decrease in the contact angle. It's noteworthy that BOR, with a log K_o/w value of 2.85, is a lipophilic bicyclic monoterpene alcohol [161]. The increase in viscosity due to higher drug loading led to reduced spreadability, slightly elevating the contact angle on the glass surface. Conversely, NMP and DMSO displayed good spreadability on the agarose gel surface owing to their inherent hydrophilic characteristics, enabling them to interact miscible with the aqueous phase.

On agarose gel surfaces, NB40 solution exhibited a significantly lower contact angle compared to drug-loaded ISMs, although it remained higher than that of NMP. The transition from liquids to gels or ISM matrices was linked to solvent exchange upon contact with the aqueous agarose gel. This exchange impeded the spreadability of NB40 and drug-loaded ISMs, resulting in a higher contact angle. Interestingly, higher drug concentrations tended to decrease the contact angle due to the increased hydrophilicity of the salt form of the drug on the agarose gel.

In contrast, organic solvent and ISMs displayed a relatively high contact angle on paraffin surfaces, indicating their hydrophilic behavior on the inherently hydrophobic paraffin surface. Notably, NMP, with a molecular weight of 99.13 daltons and a log P of -0.38, is a potent solubilizing agent. It is commonly employed as a solubilizing excipient in various medications due to its low acute toxicity *via* oral, dermal, and inhalation routes of administration [40, 41]. Importantly, all contact angle values in this study were less than 90°, indicative of good wettability [43]. Adequate wettability is crucial for effective adhesion between the ISM and the target surface, such as the periodontal pocket, preventing undesired slippage of the transformed ISM [162].

4.1.1.3. Water Tolerance

The initial phase separation of a hydrophobic compound, such as BOR, from the ISMs through NMP-solvent exchange became evident through turbidity observed when titrating ISMs with distilled water [143]. The extent of water tolerance directly reflects the quantity of the aqueous phase that initiates a phase transition, revealing the ISM's capacity to withstand water. The water tolerances of both drug-free and drug-loaded BOR-based ISMs are detailed in Table 7. Notably, the comparison of water tolerance between the NMP and DMSO series highlights a slight but discernible reduction in water tolerance in the DMSO series. This observation underscores the intriguing role of the solvent type in influencing the water tolerance characteristics of the ISMs.

As the LH content in the ISMs increased, the water tolerance significantly decreased, indicating heightened sensitivity to aqueous environments and an increased likelihood of phase separation. This trend was more pronounced in the DMSO series compared to the NMP series. This difference can be attributed to the distinct solvation behaviors of DMSO and NMP. DMSO, with a higher dielectric constant of 47.24, tends to solvate ions and polar compounds more effectively than NMP with a dielectric constant of 32. Thus, the enhanced solvation effect of DMSO could lead to more efficient penetration of water molecules into the ISM matrix, triggering phase separation at lower water concentrations.

Furthermore, the gradual introduction of water into the ISM system elevated its polarity, as water's polarity surpasses that of NMP and DMSO within ISMs. It is noteworthy that NMP and DMSO possess dielectric constant values of 32 and 47.24, respectively, at 20 °C [163]. This polarity-driven phenomenon further accentuates the differences in water tolerance between the NMP and DMSO series, with DMSO-based ISMs displaying a more pronounced sensitivity to water.

A similar reduction in water tolerance has been documented in fatty acid-based ISMs with higher levels of fatty acids [143, 158, 159]. Moreover,

comparing the water tolerances of all ISMs at body temperature with those at room temperature (Table 7) aligns with the thermodynamics of endothermic solubility. This concept posits that the solubility of a crystalline solute in a liquid solvent increases proportionally with temperature elevation or increased system entropy [164]. Consequently, the alteration of LH-loaded BOR-based ISMs during solvent exchange within the periodontal pocket at body temperature is anticipated to be more pronounced than at room temperature.

It is crucial to acknowledge that the water tolerances of the ISMs were below 14% w/w (Table 7), implying that the system could swiftly approach its saturation point due to the growing hydrophilicity of the system and the low aqueous solubility of the matrix-forming agent [143]. Hence, meticulous consideration of the physicochemical attributes of the components and temperature becomes imperative to ensure effective solvent exchange and proper *in situ* matrix formation.

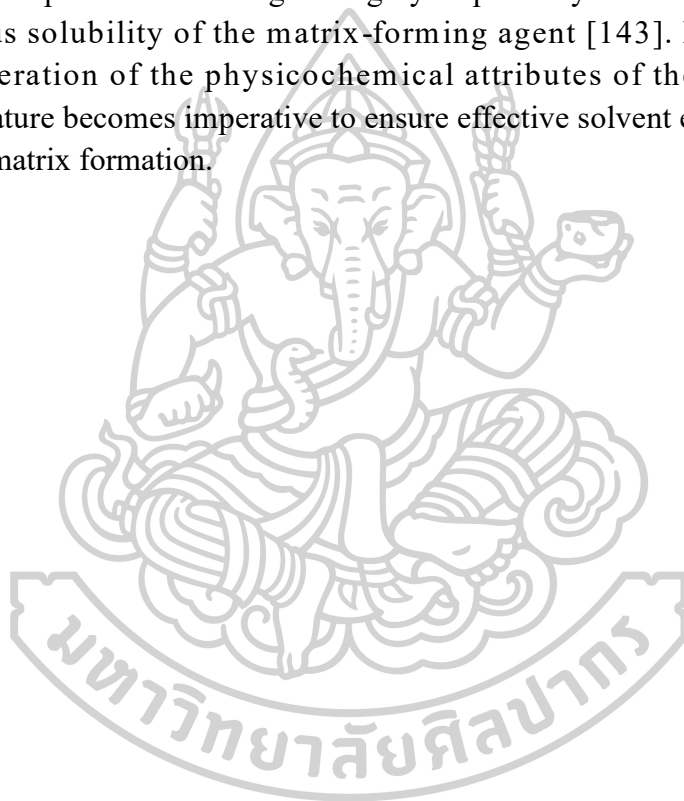


Table 7 Physical properties of LH -loaded BOR ISM formulations (n = 3).

Formul a	Density (g/cm ³)	Viscosity (cP)	Surface tension (mN/m)	Contact angle (Degree)			% Water tolerance (% w/w)	
				Glass Slide	Agarose gel	Paraffin	25 °C	37 °C
DMSO	1.094 ± 0.007	1.98 ± 0.09	43.95 ± 0.13	33.98 ± 1.25	4.30 ± 1.19 ^c	64.70 ± 0.81	-	-
DB10	1.079 ± 0.003	4.07 ± 0.04	36.99 ± 0.22	14.70 ± 0.50	14.02 ± 0.74	54.26 ± 0.06	16.25 ± 0.33	21.23 ± 0.22
DB20	1.065 ± 0.001	4.86 ± 0.10	32.90 ± 0.02	12.37 ± 0.11	25.16 ± 0.52	39.77 ± 0.02	8.73 ± 0.13	11.77 ± 0.23
DB30	1.052 ± 0.000	6.27 ± 0.00	32.44 ± 0.04	11.67 ± 0.30 ^c	25.75 ± 0.18	39.02 ± 0.32	6.23 ± 0.34	8.84 ± 0.22
DB40	1.039 ± 0.001	7.78 ± 0.04	31.45 ± 0.01	11.39 ± 0.70 ^c	41.76 ± 0.55	34.93 ± 0.17	3.84 ± 0.40	5.22 ± 0.16
NMP	1.027 ± 0.001 ^a	2.04 ± 0.13 ^b	49.31 ± 0.28	31.08 ± 0.40	7.12 ± 1.51	44.68 ± 2.15	-	-
NB10	1.022 ± 0.001 ^a	2.19 ± 0.07 ^b	46.41 ± 0.23	34.12 ± 0.13	29.38 ± 0.13	52.44 ± 0.40	31.87 ± 0.60	32.03 ± 0.49
NB20	1.066 ± 0.003	2.62 ± 0.05	46.78 ± 0.07	23.68 ± 0.39	28.39 ± 0.40	50.86 ± 0.28	21.82 ± 0.13	24.03 ± 0.29
NB30	1.011 ± 0.001	3.32 ± 0.10	45.95 ± 0.03	17.49 ± 0.50 ^d	25.90 ± 0.26	48.17 ± 0.13	16.99 ± 0.06	18.00 ± 0.40
NB40	1.010 ± 0.000	3.76 ± 0.06	44.70 ± 0.25	17.16 ± 1.20 ^d	26.98 ± 1.64 ^e	46.56 ± 0.53	12.76 ± 0.29	13.16 ± 0.00
NBL1	1.011 ± 0.001	4.01 ± 0.01	40.18 ± 0.05	9.15 ± 1.98	37.75 ± 1.60	45.82 ± 0.39	11.52 ± 0.36	13.16 ± 0.00
NBL5	1.019 ± 0.000	5.51 ± 0.02	40.64 ± 0.20	10.37 ± 0.46	35.01 ± 1.56	46.84 ± 0.93	9.39 ± 0.00	11.32 ± 0.00
NBL7.5	1.025 ± 0.000	6.78 ± 0.02 ^b	40.12 ± 0.32	13.58 ± 0.38	32.54 ± 1.28	39.16 ± 1.08	8.29 ± 0.39	9.39 ± 0.65

The superscripts ^{a-e} in the columns represent a not significant difference ($p \geq 0.05$) within the tested formulations.

4.1.1.4. *In Situ* Matrix Formation

The phases transition observed from the ISM solutions into gels or matrices upon injection into PBS pH 6.8 and agarose wells are depicted in Figure 15 and Figure 16, respectively. The transformation occurred abruptly upon exposure to PBS, resulting in a cloudy mass, with some initial transparent matrices persisting, gradually transitioning into cloudier matrices over time (as depicted in Figure 15). Notably, NB40 exhibited rapid agglomeration into an opaque mass and settled faster in the test-tube compared to NBL1, NBL5, and NBL7.5, aligning with their respective density

values as previously described. Additional loading of LH slightly retarded the matrix formation, owing to an increase in the hydrophilicity of the salt form of this drug. This matrix formation was also evident and confirmed when using an agarose well instead of PBS (Figure 16). The cross-sectional observation of matrix formation within agarose wells illustrated the gradual penetration of the BOR matrix from the rim towards the center of the agarose hole. Within the agarose wells, incomplete matrix formation was observed for NB40, contrasting with the complete matrix formation witnessed in drug-loaded ISMs within 120 minutes. The introduction of LH appeared to facilitate solvent exchange at a later stage due to the drug's hydrophilic nature in salt form. Conversely, the hydrophobic BOR matrix in NB40 impeded the solvent exchange process, resulting in reduced matrix formation. Interestingly, an increased drug amount marginally prolonged the matrix formation, akin to the earlier observation in PBS pH 6.8. However, the inclusion of 1% vancomycin HCl did not notably impact the rate of BOR matrix formation, potentially due to its minimal quantity, while higher BOR concentrations facilitated the creation of a denser matrix, consequently prolonging the drug's release [17]. The transition from a solution state to a semisolid and eventually to a solid-like state was observed in lauric acid-based ISMs upon contact with simulated crevicular fluid [157, 159]. Matrix formation in ISM systems hinges on solvent exchange—a gradual process where a dissolved matrix-forming agent undergoes phase inversion, transitioning into a matrix state and eventually solidifying, while the solvent diffuses outward and is replaced by the surrounding aqueous phase [10, 143]. Despite the reduction in water tolerance caused by drug addition in the ISMs, the rate of matrix formation did not appear directly linked to water tolerance. The addition of a drug increased both the density and viscosity (as indicated in Table 7) of the ISMs, leading to a slight delay in the formulation's solvent exchange. Therefore, it's essential to consider patient compliance and the effectiveness of the phase alteration post-injection by a dentist in the periodontal treatment process [165]. The application of a small amount of ISM dosage into a tiny periodontal pocket with a large surface area can effectively expose crevicular fluid, inducing its phase inversion.

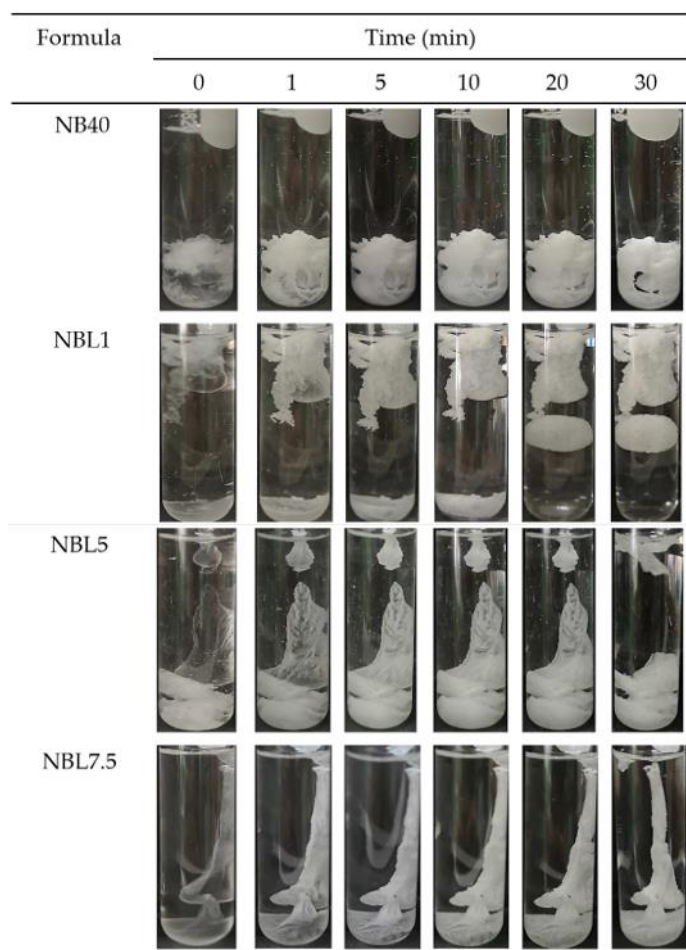


Figure 15 *In vitro* matrix formation behaviors of LH -loaded BOR-based ISMs in PBS pH 6.8.

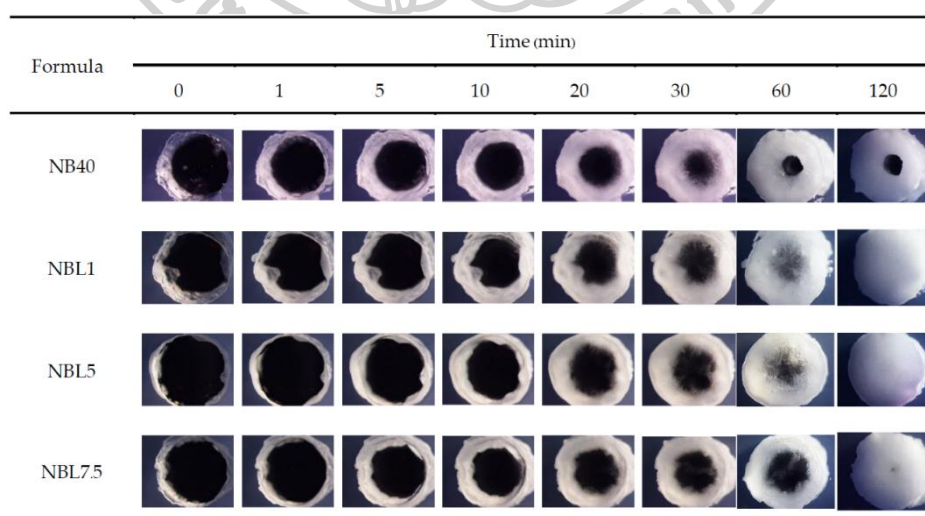


Figure 16 Cross-sectional view of matrix formation behaviors of LH -loaded BOR-based ISMs in agarose wells under a stereo microscope at a magnification of $\times 12$.

4.1.1.5. Mechanical Properties

The mechanical attributes of ISMs are pivotal in their capacity to undergo phase inversion, withstand jaw movements, and maintain their structure within the periodontal pocket [166]. To assess these properties, the ISMs were placed within agarose wells to facilitate complete phase alteration. Subsequently, a texture analyzer was employed to measure the matrix's hardness, along with the ratio of $F_{\text{remaining}}/F_{\text{max}}$ deformation. This ratio serves as a metric for assessing plastic deformation (indicated by low values) versus elasticity (indicated by high values) [167-169].

The analysis of Figure 17 distinctly illustrates that the F_{max} of the transformed matrix gradually decreased with increasing drug loading. This observation aligns with the concurrent deceleration in matrix formation at elevated drug concentrations. Notably, all ISM formulations exhibited a consistently low $F_{\text{remaining}}/F_{\text{max}}$ deformation ratio (<1). This implies a higher level of plasticity compared to elasticity, suggesting that the ISMs possess favorable attributes for retention and easy adaptation to the unique contours of the gum cavity in patients [167, 168]. This characteristic is highly desirable as it ensures the ISMs conform well to the specific anatomical site, enhancing patient comfort.

Moreover, the noteworthy aspect emerges when considering the $F_{\text{remaining}}/F_{\text{max}}$ deformation ratios across ISMs with various drug concentrations. The similarity in these ratios underscores the uniform adhesive quality of the ISMs to the target site. This advantageous property significantly contributes to preventing the dosage form from undesirably slipping out of the confined space of the periodontal pocket [167].

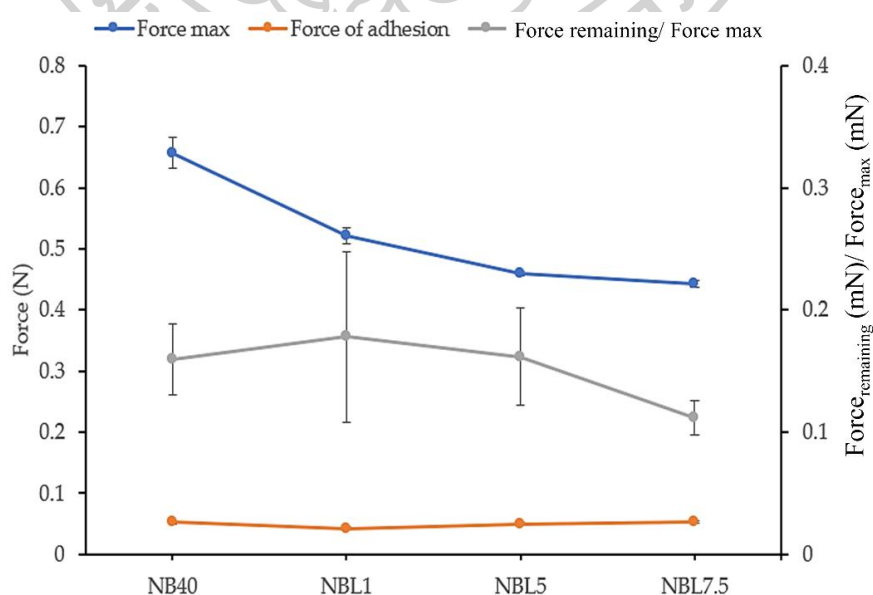


Figure 17 Mechanical properties of LH-loaded BOR-based ISMs after phase alteration for 24 h (n=3).

4.1.1.5 Water induced crystallization study through Wide Angle X-ray Scattering (WAXS) analysis

The crystallinity property of the NB40 system was investigated using WAXS analysis. The study aimed to understand the impact of gradually adding water to the ISM matrix until reaching the water tolerance endpoint. The initial WAXS pattern exhibited distinct peaks corresponding to NMP, indicating the crystalline nature of the solvent within the NB40 ISM matrix. As water was incrementally added, the WAXS pattern remained consistent with NMP peaks up to 10% of the water tolerance value (NB40W1) as demonstrated in Figure 18. However, a significant transition occurred beyond this 10% threshold, falling within the acceptable range of water levels for this system. Notably, as the water content exceeded this threshold (NB40 matrix), the WAXS pattern began to undergo transformation. It's particularly intriguing that peaks corresponding to BOR started to emerge in the pattern, albeit with reduced intensity and subtle alterations compared to the intact BOR peaks. This pivotal transition in the WAXS pattern highlights the phenomenon of crystallization within the NB40 system as it approaches its water tolerance value endpoint [170]. Moreover, this water-induced crystallization of BOR led to changes in the crystallinity morphology from the initial intact BOR material. This transformation might be influenced by the organic solvent present in the formula. It can be attributed to the interactions between BOR and water molecules within the matrix, particularly at the point where sufficient water is introduced.

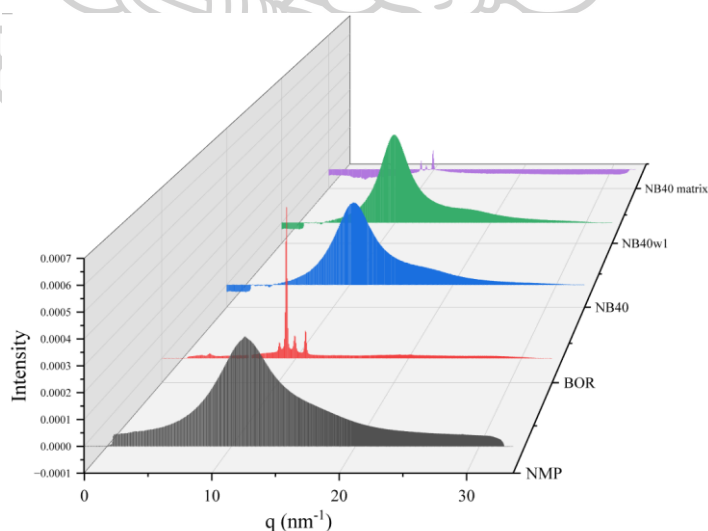


Figure 18 WAXS diffractogram of water-induced ISM system.

4.1.1.6. Microscopic Interface Interaction

The microscopic interface interaction between the ISMs and agarose gel was examined using natural light, as shown in Figure 19. This analysis aimed to understand the mechanism of phase transformation near the target site, which contains aqueous fluid [143, 158, 159]. The red line in the figure represents the initial boundary between the agarose gel (left) and the ISM formulation (right). In the case of NB40, the growth of the BOR matrix was rapid, resulting in a homogeneous, dark, and dense matrix mass that moved forward into the formulation faster compared to LH-loaded ISMs Figure 19 (A)). Additionally, a denser layer of small BOR crystals was observed separate from the matrix mass. This indicates that the increased solvent exchange, facilitated by water invading the formulation, promoted the phase inversion of the dense liquid ISM into matrices and subsequently into the matrix mass after most of the solvent was removed [17, 159]. The growth of the matrix continued to occur, involving nucleation and supersaturation-induced crystallization [143, 171]. The growth of the BOR-based *in situ* forming matrices at the interface with the agarose rim changed rapidly with an increase in BOR concentration in DMSO [17]. In the case of LH-loaded ISMs, heterogeneous thinner matrices were observed, along with some breakage of the BOR crystal layer, consistent with the slow matrix formation and low matrix hardness mentioned previously in section 4.1.1.4. *In Situ* Matrix Formation and 4.1.1.5. Mechanical Properties, respectively. The higher viscosity in ISMs with elevated drug concentrations might impede solvent exchange and hinder the matrix formation process of BOR. Intriguingly, some BOR matrices managed to penetrate the agarose gel, indicating that small BOR molecules, in conjunction with NMP, could partially infiltrate the gel and precipitate due to their insolubility when exposed to the higher polarity of the inner agarose's water phase. In Traditional Chinese Medicine (TCM), BOR is believed to facilitate the transport of other active compounds to specific tissues or organs, particularly the brain, owing to its enhanced ability to traverse the blood–brain barrier [4]. Thus, its small hydrophobic nature promotes penetration, and increased drug loading prolongs penetration into the BOR matrix. Consequently, some dissolved BOR molecules diffuse through these heterogeneous matrices into deeper agarose layers and undergo crystallization.

The diffusion of water was tracked using a fluorescent probe and sodium fluorescein from the agarose gel (left) into the ISM formulations (right) under a fluorescent microscope (Figure 19 (B)). The red line represents the interface between the agarose and the ISM formulation. Sodium fluorescein is an orange-red, odorless powder that is freely soluble in water and sparingly soluble in alcohol [171]. It absorbs blue light with peak excitation occurring at wavelengths

between 465 and 490 nm, and the resulting fluorescence occurs at yellow-green wavelengths of 520–530 nm [172]. In NMP series, sodium fluorescein does not exhibit fluorescence. However, when dissolved in a high-water medium, it manifests as a yellow-green color. The diminishing intensity of the green hue within the agarose gel (Figure 19 (B)) is linked to the diffusion of sodium fluorescein and water into the internal formulation. This water diffusion catalyzes the creation of a BOR matrix, visibly seen as an opaque band at the interface, progressively expanding within the formulation. Correspondingly, some BOR matrices infiltrate the agarose gel, consistent with previous stereoscope observations (Figure 19 (B)). The wider fluorescent coloration of NB40 and the dense opaque BOR matrix band suggest more effective matrix formation compared to the drug-loaded ISMs. The green color mapping delineates the area influenced by water influx resulting from sodium fluorescein diffusion in the water phase. As sodium fluorescein in PBS infiltrates the rosin-based ISM, the presence of PBS triggers matrix modification via phase separation, leading to rosin precipitation [173]. The gradual sodium fluorescein movement, along with sufficient water, from the agarose into the formulation accentuates a brighter green hue. Consequently, examining the interface boundary and tracing sodium fluorescein's movement offers crucial insights into water mobility and the behavior of matrix formation during the phase inversion of ISMs.



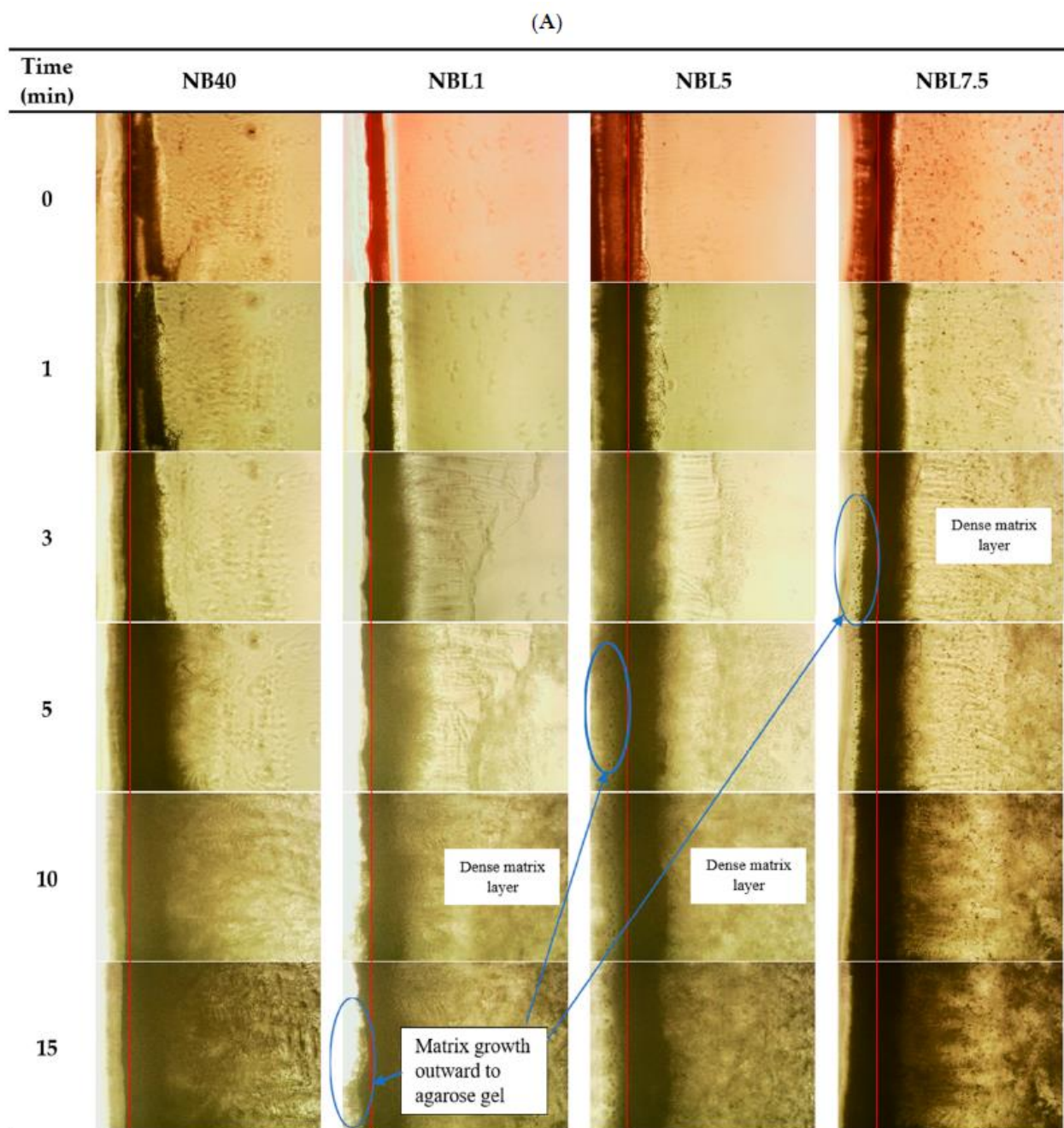
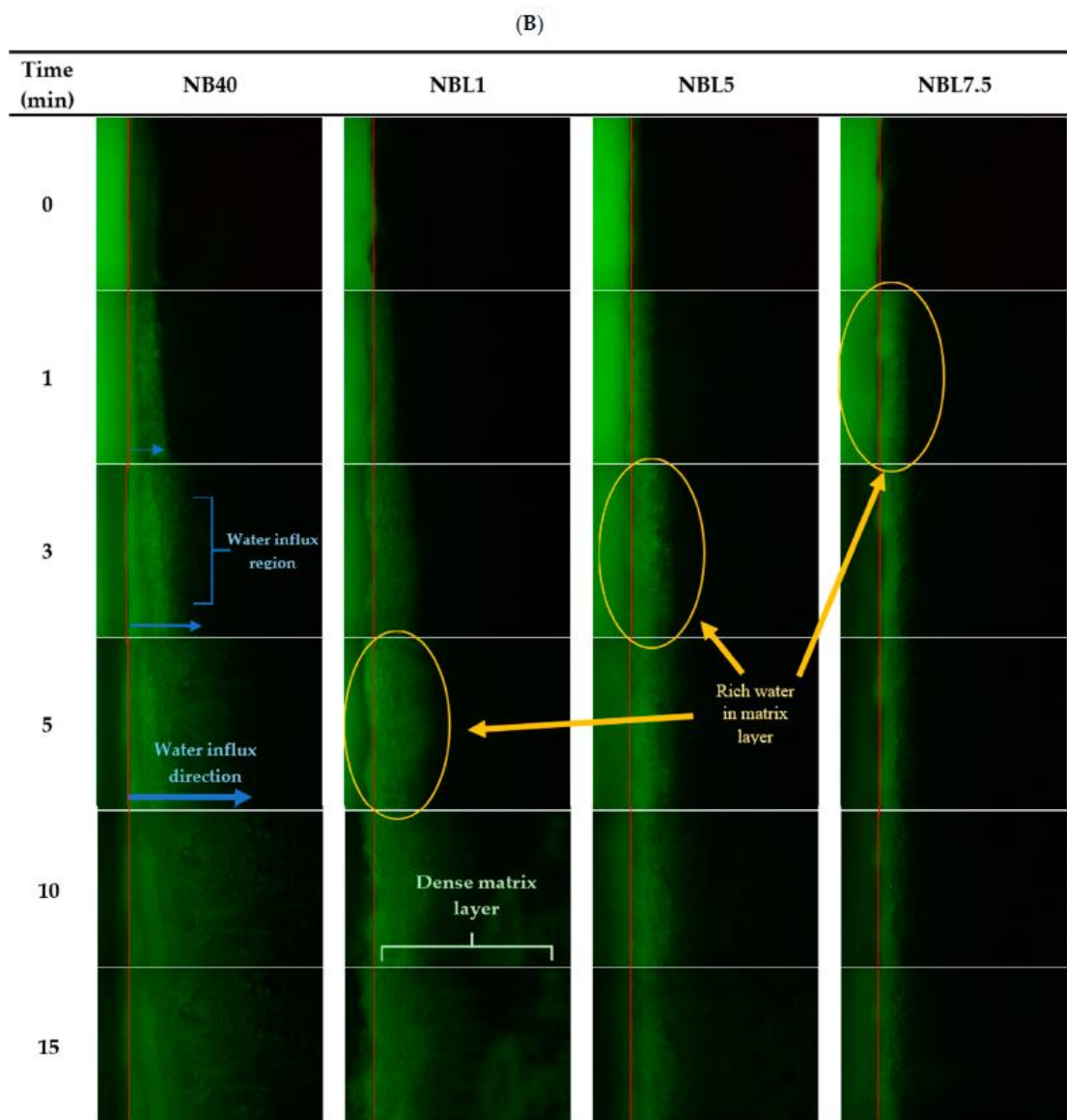


Figure 19 Matrix formation at the interface of the aqueous phase and ISMs under visible light (A) and fluorescence light (B) at magnification $\times 100$ under an inverted microscope. (The red line is the interface between agarose (left side) and formula (right side)).



4.1.1.7. Drug Content and *In Vitro* Drug Release

The LH content in the NL1, NL5, NL7.5, NBL1, NBL5, and NBL7.5 formulations was found to be 99.01 ± 0.84 , 101.77 ± 0.26 , 100.82 ± 0.16 , 100.83 ± 0.32 , 99.36 ± 0.42 , and 98.72 ± 0.72 , respectively. These formulations were prepared using a simple mixing process, resulting in LH content close to 100% of its intended addition. Figure 6 shows the drug release profiles of LH from the BOR-based ISMs in PBS pH 6.8. The BOR concentration for these ISM preparations was 40% w/w because it was the highest concentration for incorporating LH. In addition, the BOR-based *in situ* forming matrix was recently fabricated to load vancomycin HCl by varying the concentration of the matrix-forming agent. This demonstrated that 40% w/w BOR in DMSO was appropriate for sustained drug release [17]. The insufficient transformation rate into matrix was mentioned for the BOR-ISM systems at lower 40% w/w, in which they might escape by flowing as a liquid from the periodontal pocket before matrix formation. However, the impact of drug concentration on physicochemical properties of the BOR-based ISM has not been reported. NMP was selected as a solvent because of its low toxicity (oral and dermal LD₅₀ in rodents of 3600–7700 mg/kg) and its utilization in a commercial dosage form such as Atridox® for periodontitis treatment [94, 174]. The drug release behavior of these LH-loaded BOR-based ISMs was conducted by the cup method to mimic the surrounding environment of the periodontal pocket [94, 175]. Moreover, several research publications have employed this technique to follow the drug release of ISM formulations [24, 25, 176]. The LH release from the control groups, including NL1, NL5, and NL7.5, was greater than those of their ISM formulations (Figure 20). All control groups reached the plateau state of drug release within two days; meanwhile, those of the drug-loaded BOR-based ISMs were gradually liberated over 8 days. Although, the release profiles of BOR-free and BOR-based solutions containing 1% w/w LH were similar in that they had a slightly lesser amount of released drug. The commercial product Atridox®, containing DH, achieved sustained drug release for seven days [94, 174]. Thus, the LH-loaded BOR-based *in situ* forming matrices demonstrated the ability to control drug release for at least eight days. After an initial burst release from drug deposited on the surface of the *in situ* forming matrices, the drug was controlled to release more slowly (Figure 20). Usually, the hydrophobic matrix, such as solid BOR mass, following complete phase alteration, should impede drug liberation more efficiently than its initial liquid and matrix states [177]. These characteristics make the BOR-based *in situ* forming matrices promising for controlled release applications, offering enhanced patient compliance by reducing the frequency of drug administration [176, 178]. If more patient-specific factors can be identified, such as the state of periodontitis, disease severity, shape of the patient's periodontal pockets, or hereditary traits that affect how drugs respond or the growth of pathogens, these

parameters might be used to determine the properties and components of the formula to suit each patient. Therefore, the designed ISM containing different drug loading, rate of matrix formation and drug release characteristics could be personalized to specific patients' needs to insure the best therapeutic response with the highest efficacy and safety margin [179].

The release data were fitted to different mathematical models, and the degree of goodness-of-fit was evaluated using the coefficient of determination (R^2), Akaike information criterion (AIC), and model selection criterion (MSC) [180]. The high values of R^2 and MSC together with the low value of AIC are signified to indicate the best fit or high degree of goodness of curve fitting [180]. The estimated parameters obtained after fitting release data with zero-order, first-order, Higuchi's, Korsmeyer–Peppas's, and Peppas–Sahlin's equations are also presented in Table 8. The release profiles of most formulations showed a high degree of goodness-of-fit with the Peppas–Sahlin's equation, indicated by high R^2 values close to 1.0. The estimated parameters obtained from the curve fitting provided insights into the release mechanisms. Higher k_1 values suggested that diffusion was the main mechanism of drug release, while higher k_2 values indicated dominant polymer relaxation or heterogeneous erosion [180, 181]. Negative k values could typically be observed and indicated as one term in the model for compensation of producing the best fit of data; nevertheless, this should not be included when interpreting the release mechanism comparing the k value [180]. By comparison, most of the drug release from control groups without BOR addition followed the first-order release kinetic (Table 8) in which the drug was freely liberated from the solution with concentration dependence. Fickian diffusion was the predominant release mechanism observed in the NBL1 and NBL5 formulations, as evidenced by their higher k_1 values and good fit with Higuchi's equation (Table 8). The release mechanism of imatinib mesylate from the rosin-based ISM [182] and DH from the lime peel oil-loaded rosin-based formulations [183] mainly obeyed Fickian diffusion. Generally, a Fickian diffusion-driven matrix release mechanism is related to a concentration gradient, diffusion distance, and swelling degree [184]. The release mechanism of NBL7.5 appeared to be closer to first-order release kinetics but also showed a good fit with Higuchi's equation and a higher k_1 value in the Peppas–Sahlin's equation, suggesting a Fickian diffusion mechanism similar to NBL1 and NBL5. The initial burst release from the *in situ* forming matrices was attributed to drug deposited near the gel surface, while the subsequent transition from the matrix state to the solid-like BOR matrix effectively retarded drug diffusion, leading to prolonged release.

The sustained release of LH from the BOR-based ISM is particularly relevant in inhibiting microbial growth, as LH has been reported to be effective

against anaerobic bacteria, including *P. gingivalis*, commonly found in the oral cavity [[89]. The attempts to characterize the topography of BOR matrices after a dissolution test were also undertaken using scanning electron microscopy, though its sublimable manner made this mission impossible. This unique sublimable property of BOR has been applied to fabricate highly porous, low-density, and non-effervescent floating tablets [70]. Besides, BOR was reported as an enhancer for drug absorption in the gastrointestinal tract and drug distribution to the brain [61]. It is effective and safe to use as an ocular penetration enhancer by increasing transcorneal penetration of both hydrophilic and lipophilic compounds [63]. Therefore, the BOR-based ISM might promote some antimicrobial drug penetration through periodontal tissue to eradicate pathogens that have invaded in the case of aggressive periodontitis. Moreover, the delivery system of this developed ISM should cause less drug resistance and diminish the drug's side effects [185]. Meanwhile, it could deliver the drug at the infectious site locally with apparent high drug loading to completely eliminate periodontal pathogens from the chronic periodontal pockets [186]. The minimum inhibitory concentration (MIC) value of LH against *P. gingivalis* was 16–32 $\mu\text{g/ml}$ as reported by Heung-Sik and Hyung-keun [187]; therefore, the drug release from NBL5 and NBL7.5 could achieve the MIC drug level on the first day and sustain drug release above the MIC over 8 days and, thus, inhibit microbial growth.

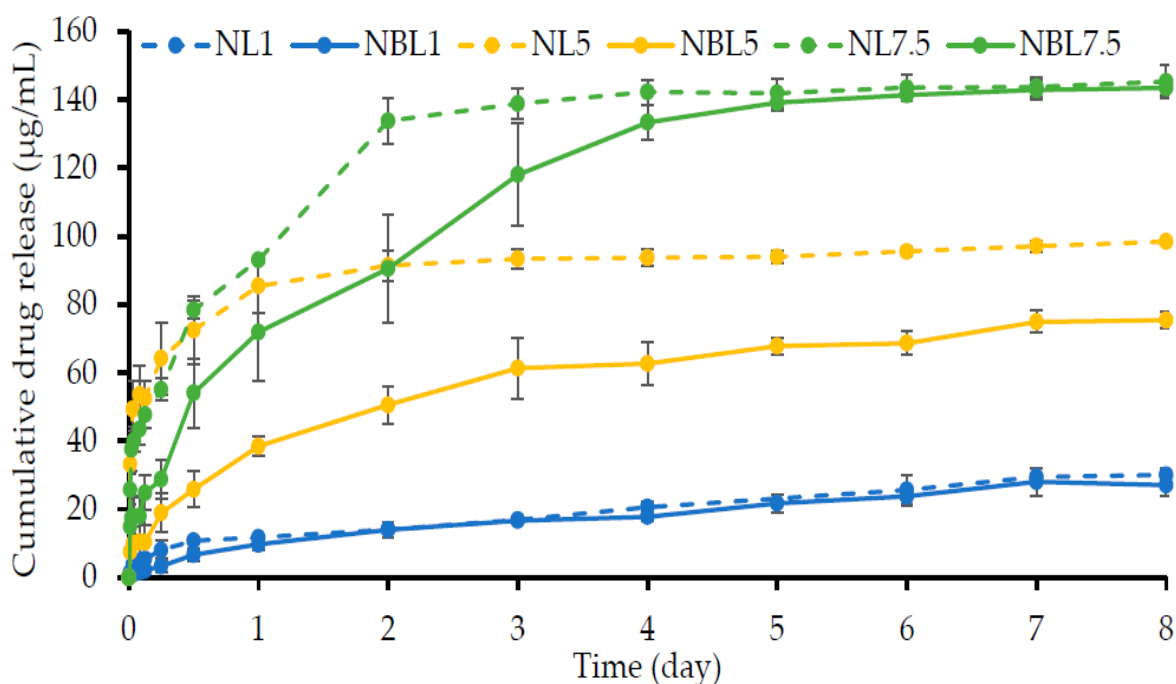


Figure 20 LH release from BOR-based ISMs in PBS pH 6.8 using the cup method (n = 6).

Table 8 Degrees of goodness-of-fit and estimated parameters from drug release profile fittings of LH-loaded BOR-based ISMs in PBS pH 6.8 using the cup method.

Formulation	Modeling	Criteria for Model Selection			Kinetic Parameters		
		R^2	AIC	MSC			
NL1	Zero order	0.7505	116.6260	1.1271	$k_0 = 14.408$		
	First order	0.8812	106.1227	1.8774	$k_1 = 0.363$		
	Higuchi's	0.9615	90.1917	3.0153	$k_H = 35.490$		
	Korsmeyer–Peppas's	0.9708	87.6678	3.1956	$k_{KP} = 39.953$	$n = 0.423$	
	Peppas–Sahlin's	0.9393	49.8984	2.4443	$k_1 = 33.959$	$k_2 = 6.109$	$m = 0.311$
NL5	Zero order	1.3967	155.4462	-1.5498	$k_0 = 16.864$		
	First order	0.7183	123.0316	0.6112	$k_1 = 6.485$		
	Higuchi's	0.0977	143.5405	-0.7561	$k_H = 44.503$		
	Korsmeyer–Peppas's	0.9380	49.9655	2.6167	$k_{KP} = 79.212$	$n = 0.173$	
	Peppas–Sahlin's	0.9488	49.4285	2.6764	$k_1 = 97.941$	$k_2 = -17.041$	$m = 0.216$
NL7.5	Zero order	0.1328	155.7117	-0.1901	$k_0 = 16.238$		
	First order	0.8631	126.0518	1.6636	$k_1 = 1.397$		
	Higuchi's	0.7421	136.2893	1.0237	$k_H = 41.861$		
	Korsmeyer–Peppas's	0.9515	48.4314	2.8900	$k_{KP} = 64.463$	$n = 0.328$	
	Peppas–Sahlin's	0.9534	48.5188	2.8803	$k_1 = 38.209$	$k_2 = 26.017$	$m = 0.247$
NBL1	Zero order	0.8892	113.1104	1.9081	$k_0 = 12.892$		
	First order	0.9700	90.2398	3.4328	$k_1 = 0.261$		
	Higuchi's	0.9834	84.4966	3.8157	$k_H = 30.920$		
	Korsmeyer–Peppas's	0.9791	36.1348	3.6937	$k_{KP} = 29.361$	$n = 0.527$	
	Peppas–Sahlin's	0.9777	36.8636	3.6026	$k_1 = 29.806$	$k_2 = -0.508$	$m = 0.585$
NBL5	Zero order	0.6336	125.7564	0.7413	$k_0 = 11.854$		
	First order	0.8453	111.2304	1.7097	$k_1 = 0.249$		
	Higuchi's	0.9395	98.3918	2.5656	$k_H = 29.375$		
	Korsmeyer–Peppas's	0.9512	55.5895	2.8777	$k_{KP} = 35.368$	$n = 0.389$	
	Peppas–Sahlin's	0.9819	47.7392	3.6627	$k_1 = 37.226$	$k_2 = -1.288$	$m = 0.547$
NBL7.5	Zero order	0.6748	141.8234	0.9285	$k_0 = 15.467$		
	First order	0.9573	109.5144	2.9478	$k_1 = 0.570$		
	Higuchi's	0.9477	111.4238	2.8284	$k_H = 38.303$		
	Korsmeyer–Peppas's	0.9697	36.5037	3.2068	$k_{KP} = 44.108$	$n = 0.495$	
	Peppas–Sahlin's	0.9730	35.8043	3.3067	$k_1 = 47.833$	$k_2 = -2.585$	$m = 0.549$

4.1.2. Antimicrobial Activities

The antimicrobial activities of the developed ISM formula, compared with its solvent and drug solutions, were evaluated against *S. aureus* ATCC 25923, *E. coli* ATCC 8739, *C. albicans* ATCC 10231, and *P. gingivalis* ATCC 33277, which are commonly associated with periodontitis [108, 188, 189]. All LH-loaded BOR-based ISMs inhibited the growth of all test microbes (Table 9 and Figure 21) in a dose-dependent manner. However, their inhibition zone diameters were smaller than those of their drug solutions without BOR. This result clearly demonstrated that the BOR matrix formed due to the solvent exchange induced by water from the agar media, which retarded the diffusion of the drug.

Significantly larger inhibition zones were observed for all samples against *S. aureus* ATCC 25923, *P. gingivalis* ATCC 33277, and *E. coli* ATCC 8739, indicating the efficient inhibition of their growth by the developed ISMs. However, NBL1 showed a significantly smaller inhibition zone diameter than NL1 ($p < 0.05$) (Table 9). Practically, LH is a reserve drug for infections caused by strains of *Staphylococci* and other *Gram*-positive microorganisms that are resistant to penicillin or for patients allergic to penicillin [190]. In dentistry, LH is effective in treating acute and chronic bone infections and soft-tissue facial infections [86, 88], and it is susceptible to an anaerobe bacterium including *P. gingivalis* [89].

For *C. albicans* ATCC 10231, NMP exhibited dominant antifungal activity, while LH also showed some activity. NMP exhibited a large inhibition zone, although smaller than those of NL1, NL5, and NL7.5 solutions (Table 9). Previous studies have reported the good inhibition of intact NMP and NMP-loaded thermosensitive matrices against *C. albicans* and bacteria [176, 191, 192]. Thus, these ISMs and their drug solutions also inhibited these fungus and bacteria. The drug-loaded ISMs presented a significantly ($p < 0.05$) smaller inhibition zone of antifungal activity than their drug solutions because the BOR matrix formation retarded drug diffusion. NMP showed significantly smaller inhibition zone diameter against *S. aureus* ATCC 25923 and *P. gingivalis* ATCC 33277 than drug-loaded ISMs and drug solution. This smaller inhibition zone diameter of some BOR-based ISMs can be attributed to the retardation of NMP diffusion by the BOR matrix, consistent with the drug release mechanism described previously. The ISM matrices obtained from fatty acids [158, 159] [36], natural resins [25, 173, 182, 183], oligosaccharide [176], and other polymers [24, 156] have been reported to impede drug diffusion and decrease the diameter of the microbial inhibition zone while enabling sustained drug release.

BOR has been used in traditional Chinese medicine for over 1600 years [193, 194]. Chinese medicine preparations such as Angong Niuhuang pills and Xingnaojing injection, which contain BOR, are widely used in the clinical treatment of stroke [195, 196]. BOR has also demonstrated anti-fibrotic activity by possibly inhibiting fibroblast mitosis, collagen production, and TIMP-1 production, making it safe for the treatment of oral submucous fibrosis [197]. BOR has low toxicity and has been tested for skin irritation in human volunteers and mice [3]. In immune cells, BOR at

concentrations of 0.5 and 5 $\mu\text{g/ml}$ did not induce significant toxicity and even increased cell viability [71]. Therefore, the use of BOR as a matrix-forming agent in ISMs is of interest due to its safety, efficient antimicrobial activities, and ability to modulate drug release. The oral absorption bioavailability of LH is 20 to 30% in the fast state and decreases in the fed state [198]. The high local drug concentration with decreasing undesirable side effects was achieved by delivery to the periodontal pocket. The release of LH from chitosan films for periodontitis treatment showed the sustained release without any burst effect for 5 consecutive days [199].

The LH-loaded BOR-based ISMs NBL5 and NBL7.5 released the drug in amounts reaching the MIC value against *P. gingivalis* on the first day and sustained drug release above the MIC value for over 8 days, considering the previously reported MIC value of LH against *P. gingivalis* as 16–32 $\mu\text{g/ml}$ [187]. While there are safety data available for BOR's medical applications, further clinical experiments are required to investigate the efficacy and safety of these LH-loaded BOR-based ISM formulations. The treatment of osteomyelitis and soft tissue infections using LH has been reported for its implications in the field of dentistry because of its effective ability to penetrate bone and severely infected tissue [200]. Moreover, LH incorporated in a tricalcium phosphate carrier could accelerate wound healing and reduce complications, such as alveolar periostitis, pain, trismus, and atrophy of the alveolar process, after surgical extraction of impacted third molars [201]. LH-loaded chitosan and chitosan–gelatin matrices have also been reported for inhibiting local infections of the oral cavity [202]. In addition to intravenous or intramuscular delivery, the use of ISMs could be an alternative treatment for site-specific microbial infections, providing high local drug concentrations while minimizing undesirable side effects. Typically, the ISM for periodontal pocket drug delivery is a freshly prepared dosage form. The constituted product of Atridox[®] for periodontitis treatment is a viscous liquid after freshly prepared with a concentration of 10% of DH [94]. Upon contact with the crevicular fluid, the liquid product solidifies and then allows for controlled release of a drug for a period of 7 days. Therefore, the instability of developed ISM should not be noticeable. The BOR-based matrix of an *in situ* forming system showed self-degradation *via* evaporation from our group investigation [203]. The BOR-based *in situ* forming system with PBS-induced phase inversion was checked for its *in vitro* degradation of BOR matrix over time. The evaporation behavior data of the BOR-based *in situ* forming system is valuable for developing the self-degradable ISM for periodontitis. Whilst there are safety data on BOR's medical applications as mentioned above, these LH-loaded BOR-based ISM formulations need to be further investigated through clinical experiments for their efficacy and safety.

Table 9 Clear zone diameter of the LH-loaded BOR-based ISM systems against *S. aureus* ATCC 25923, *E. coli* ATCC 8739, *C. albicans* ATCC 10231 and *P. gingivalis* ATCC 33277 (n = 3).

Formula	Clear Zone Diameter (Mean $\pm$ SD)			
	<i>S. aureus</i> ATCC 25923	<i>E. coli</i> ATCC 8739	<i>C. albicans</i> ATCC 10231	<i>P. gingivalis</i> ATCC 33277
NMP	17.3 $\pm$ 0.6 ^a	17.0 $\pm$ 1.0	35.7 $\pm$ 0.6	18.3 $\pm$ 1.2 ^e
NL1	30.3 $\pm$ 0.6 ^b	18.0 $\pm$ 0.0	36.3 $\pm$ 0.6	20.3 $\pm$ 0.6
NBL1	27.7 $\pm$ 1.2 ^b	12.3 $\pm$ 0.6 ^c	23.3 $\pm$ 0.6 ^d	19.3 $\pm$ 1.0
NL5	35.3 $\pm$ 1.5	18.3 $\pm$ 0.6	40.0 $\pm$ 0.0	25.0 $\pm$ 1.0
NBL5	33.7 $\pm$ 0.6	13.0 $\pm$ 1.0	26.3 $\pm$ 1.5 ^d	25.3 $\pm$ 1.2
NL7.5	35.7 $\pm$ 0.6	19.0 $\pm$ 1.0	37.7 $\pm$ 1.5	25.0 $\pm$ 1.0
NBL7.5	35.0 $\pm$ 1.0	14.3 $\pm$ 0.6	23.7 $\pm$ 2.5 ^d	26.7 $\pm$ 0.6

The superscripts ^{a-e} in the columns represent a significant difference ($p < 0.05$) within the tested formulations.

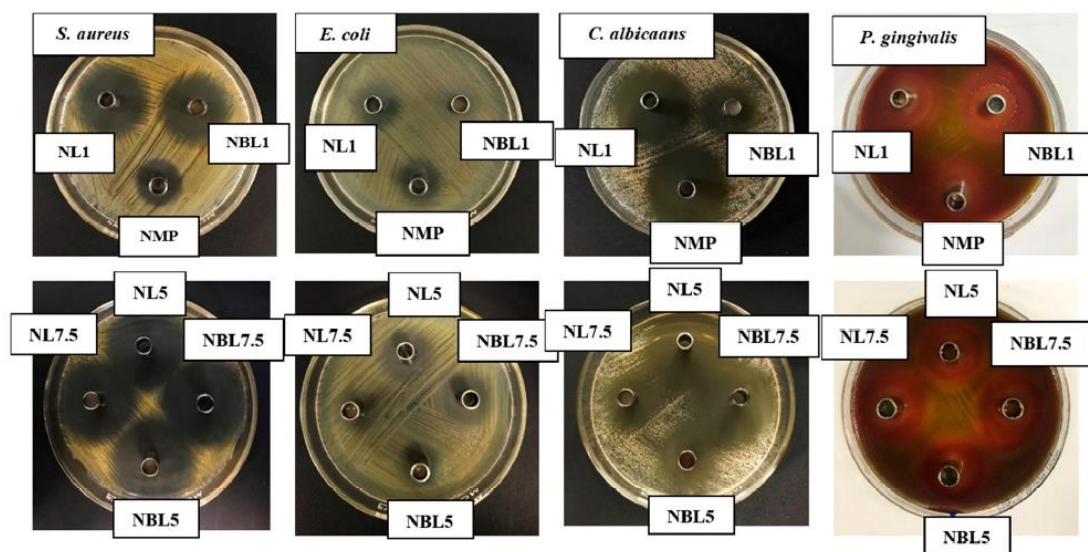


Figure 21 Inhibition zone of LH-loaded BOR-based ISM systems against *S. aureus* ATCC 25923, *E. coli* ATCC 8739, *C. albicans* ATCC 10231 and *P. gingivalis* ATCC 33277.

4.1.3. *In silico* molecular study of drug loaded ISM

The MD simulation was strategically designed to replicate the interface at the water contact region of various BOR-based ISMs. This was achieved by constructing a simulation box where the left side represented the ISM system and the right side represented the water environment. The molecular ratios were defined based on the formula content and water tolerance values obtained from previous experiments (Figure 22). In the case of low concentration BOR-based ISM formulas, as illustrated in Figure 22 (A) and (C) for DB10 and NB10 respectively, the simulation revealed the dynamic behavior of BOR molecules. These formulations demonstrated unhindered movement of BOR molecules without significant agglomeration throughout the 200 ns simulation period [204]. However, the behavior of water (WAT) molecules differed with faster inward diffusion into the DB10 system compared to the NB10 system. This discrepancy in WAT diffusion behavior contributed to the observed difference in homogeneity achievement within the respective boxes. Turning attention to high concentration BOR systems, Figure 22 (B) and (D) depicted the phase transformation of DB40 and NB40, respectively. Notably, the movement of WAT molecules exhibited distinct differences compared to the low concentration cases. WAT molecules diffused more slowly into the high concentration ISM systems. As for BOR, it exhibited continuous movement within its units over the 200 ns simulation, indicating that BOR did not crystallize into a solid structure during this initial phase. This attribute proved advantageous as it facilitated water exchange with the solvent, enhancing the system's phase transformation capability and promoting a burst release of the loaded drug (LH) at the initial stage. To delve into the dynamics of LH release during the initial phase transformation, simulations were conducted for NL and NBL, as presented in Figure 22 (E) and (F). LH molecules, represented in purple, were evenly dispersed around the BOR molecules and exhibited relatively free movement in both NL and NBL systems. However, a significant distinction was observed in the movement of WAT molecules. This divergence suggested that while BOR might not crystallize into a solid structure, it still effectively controlled LH release during the initial phase transformation. This control mechanism involved hindering the inward diffusion of WAT molecules into the ISM system, thereby preventing LH dissolution and release into the environment. In conclusion, the MD simulations provided valuable insights into the behavior of various BOR-based ISM formulations during phase transformation. The simulations highlighted the advantageous properties of BOR in facilitating controlled drug release and phase alteration by effectively managing water diffusion and enhancing homogeneity within the system.

(A)

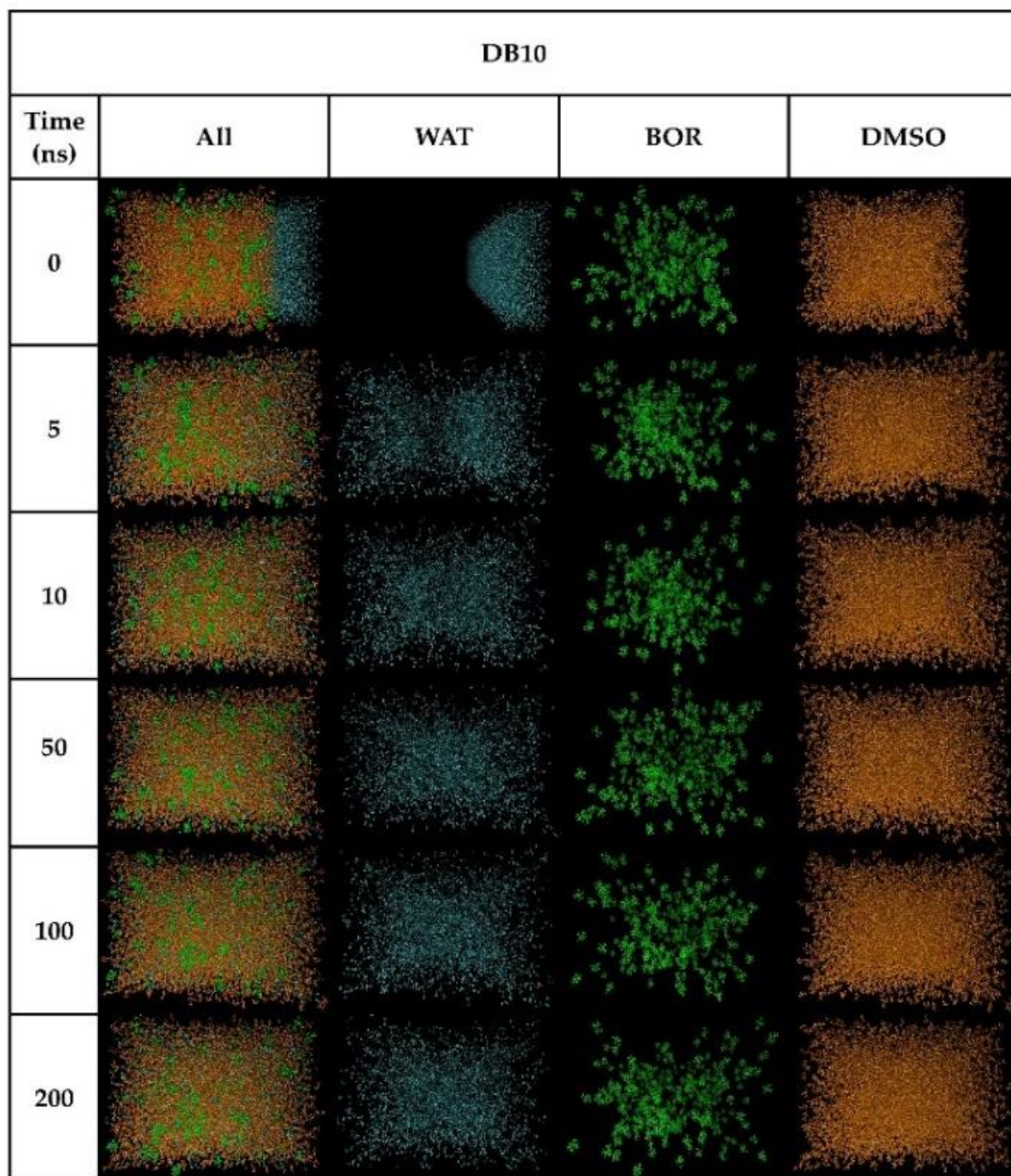


Figure 22 Molecular dynamic modeling of solvent exchange mechanism focusses at interface of each ISMs against aqueous, (DB10 (A), DB40 (B), NB10 (C), NB40 (D), NL5 (E) and NBL (F) after running in MD simulation in 200 ns.

(B)

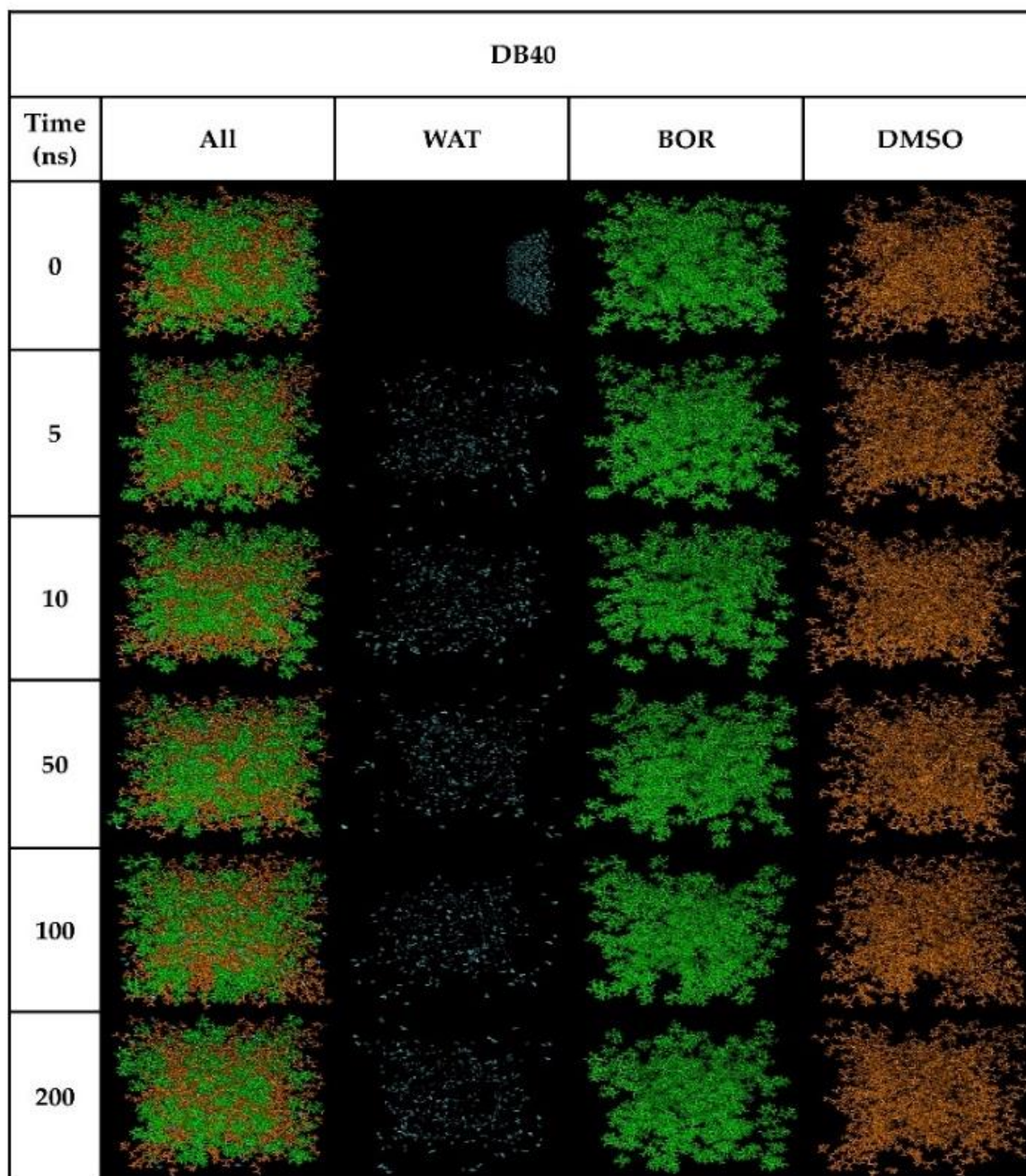


Figure 22 Molecular dynamic modeling of solvent exchange mechanism focusses at interface of each ISMs against aqueous, (DB10 (A), DB40 (B), NB10 (C), NB40 (D) NL5 (E) and NBL (F) after running in MD simulation in 200 ns (continued).

(C)

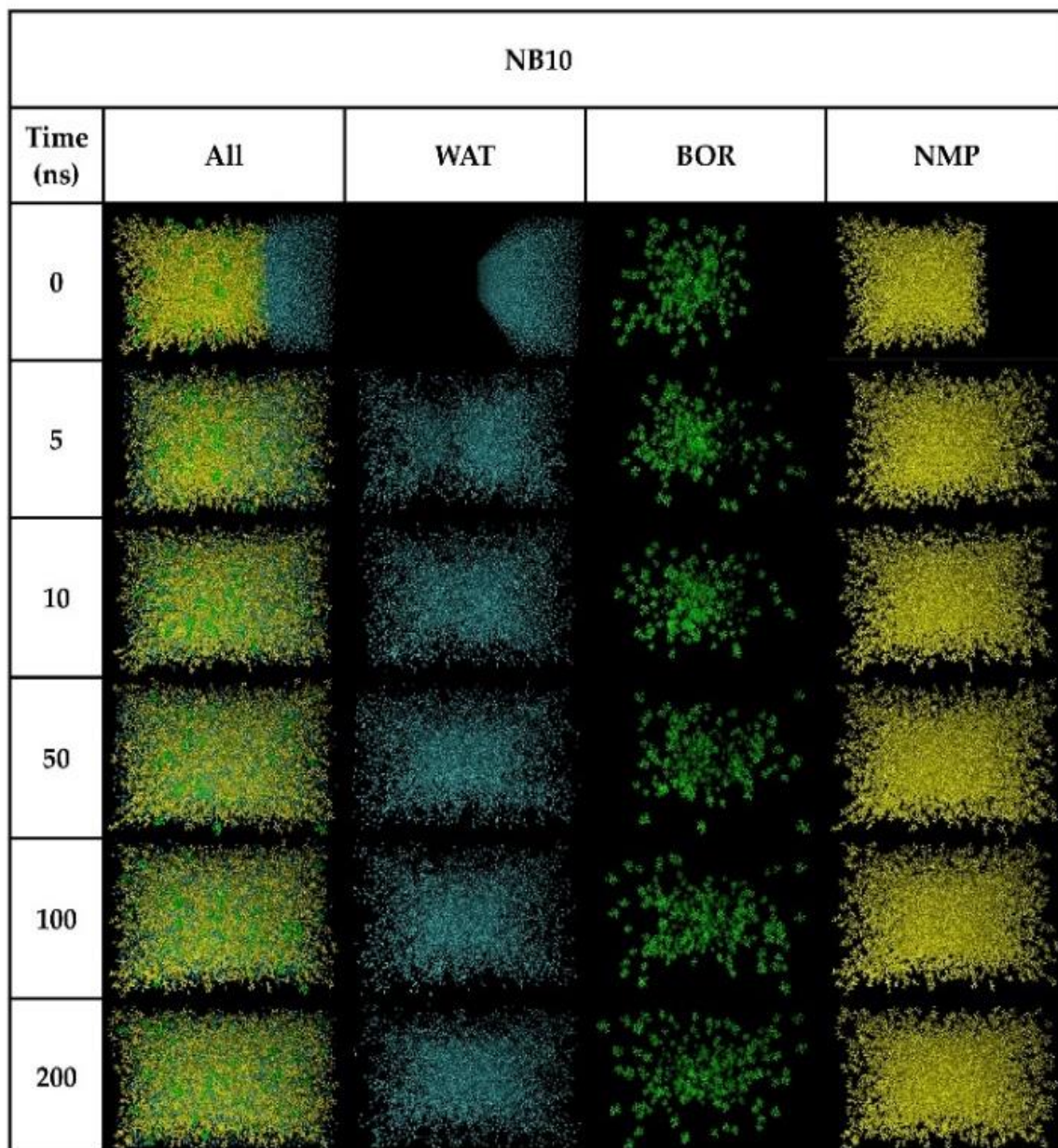


Figure 22 Molecular dynamic modeling of solvent exchange mechanism focusses at interface of each ISMs against aqueous, (DB10 (A), DB40 (B), NB10 (C), NB40 (D) NL5 (E) and NBL (F) after running in MD simulation in 200 ns (continued).

(D)

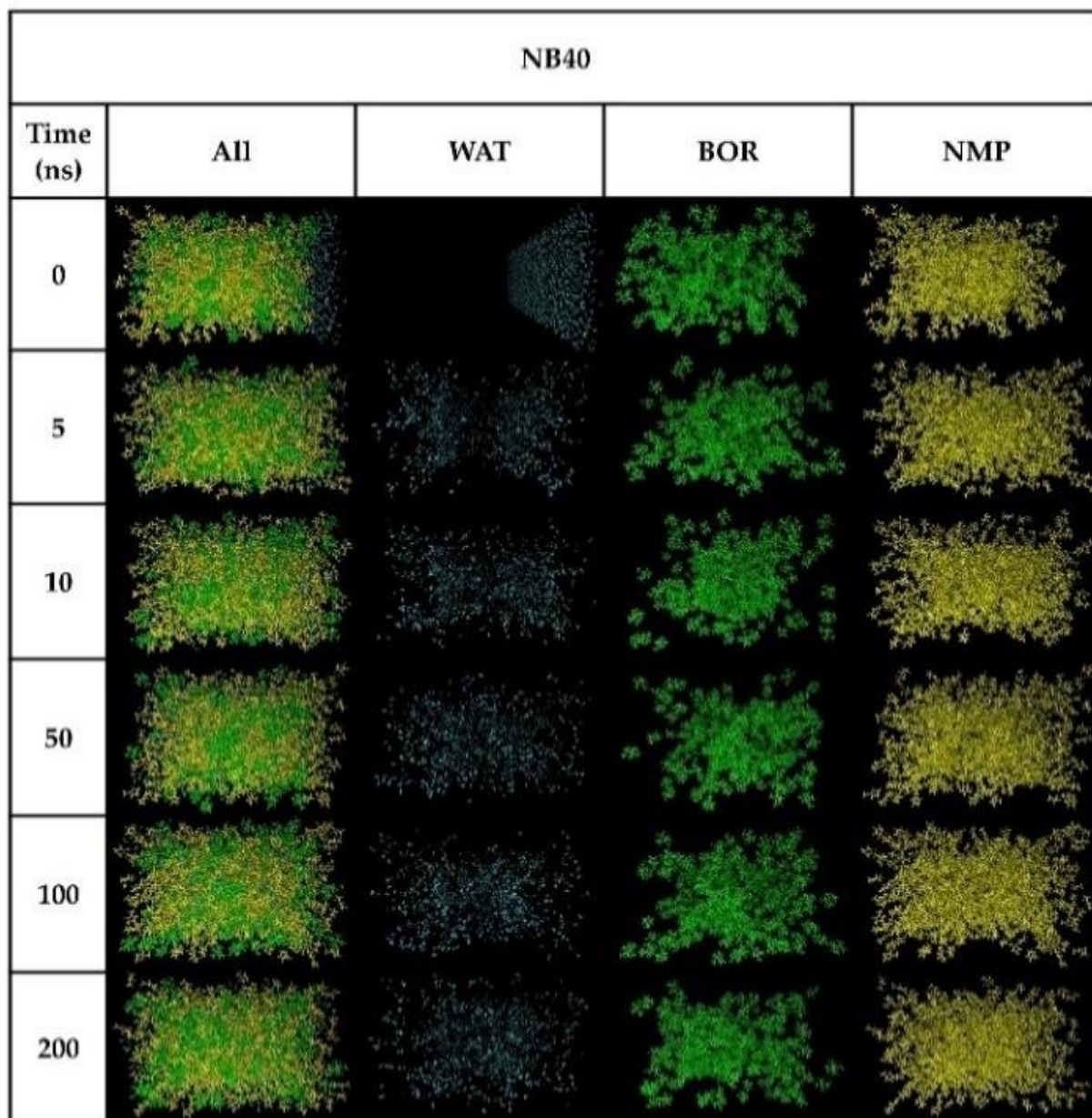


Figure 22 Molecular dynamic modeling of solvent exchange mechanism focusses at interface of each ISMs against aqueous, (DB10 (A), DB40 (B), NB10 (C), NB40 (D) NL5 (E) and NBL (F) after running in MD simulation in 200 ns (continued).

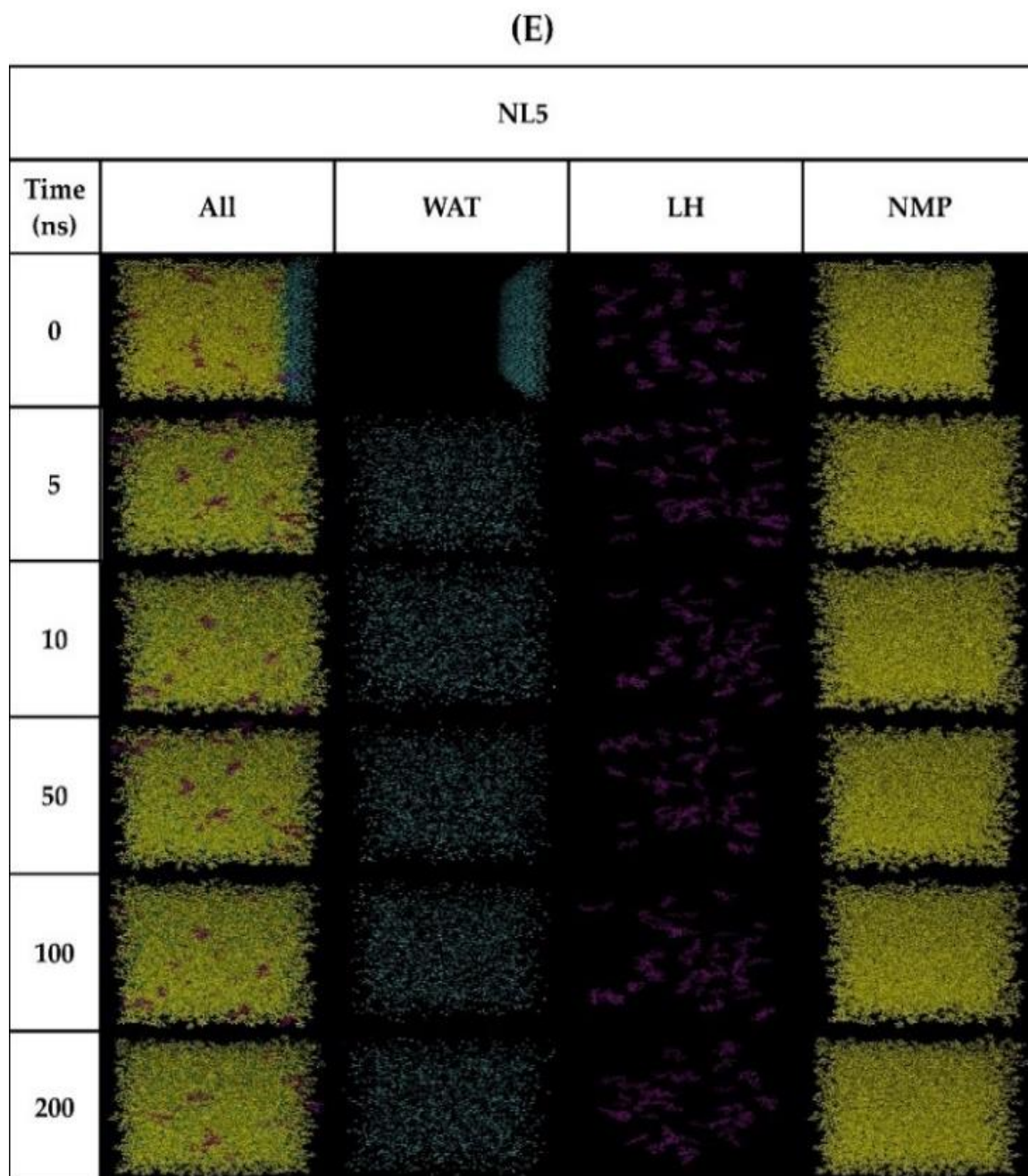


Figure 22 Molecular dynamic modeling of solvent exchange mechanism focusses at interface of each ISMs against aqueous, (DB10 (A), DB40 (B), NB10 (C), NB40 (D) NL5 (E) and NBL (F) after running in MD simulation in 200 ns (continued).

(F)

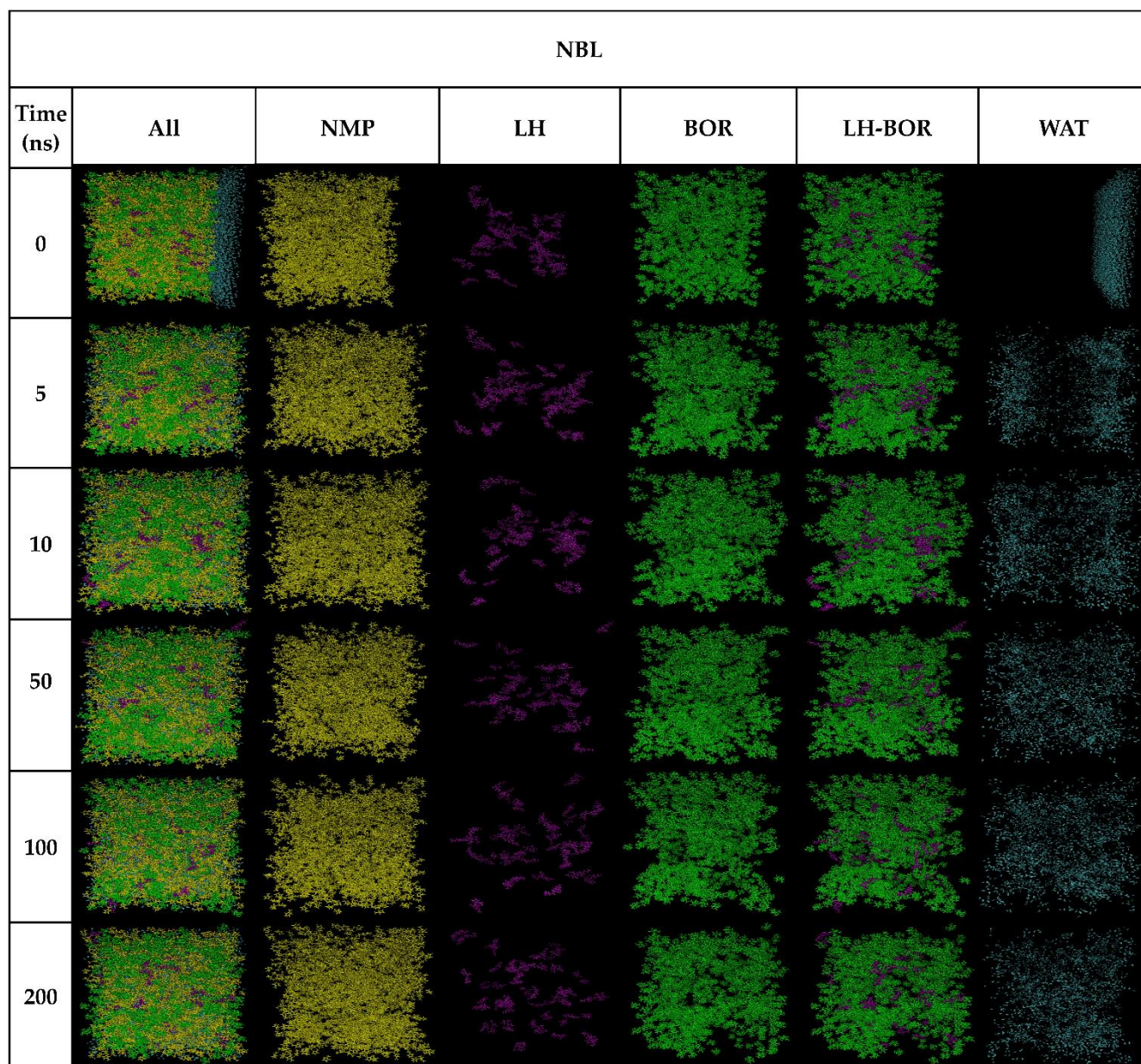


Figure 22 Molecular dynamic modeling of solvent exchange mechanism focusses at interface of each ISMs against aqueous, (DB10 (A), DB40 (B), NB10 (C), NB40 (D) NL5 (E) and NBL (F) after running in MD simulation in 200 ns (continued).

4.1.3.1. RMSD and Diffusion constant

The RMSD parameter serves as a reflection of molecular fluctuations during the simulation, shedding light on their dynamic behavior over time. RMSD values for each molecular type throughout the simulation periods are presented in Figure 23. These graphs exhibit a consistent pattern characterized by an initial phase of elevated RMSD values, followed by a plateau phase where RMSD values stabilize or experience a minor increase. Intriguingly, the presence of LH molecules displayed a distinct phenomenon. The RMSD values of LH were slightly reduced upon the addition of BOR, demonstrated by the RMSD curves of NL5 and NBL in Figure 23 (A), suggesting that BOR had the capacity to moderate LH movement during the initial phase of the simulation. This could be attributed to intermolecular interactions between BOR and LH molecules. RMSD of BOR molecules, depicted in Figure 23 (B), exhibited differing trends between DMSO and NMP series. Notably, DMSO systems showed higher RMSD values for BOR molecules compared to NMP systems, both for 10% and 40% w/w BOR content. This indicated that BOR molecules displayed higher fluctuation in DMSO environments than in NMP environments after exposure to water. Interestingly, the RMSD of BOR was progressively reduced with increasing BOR content and the addition of LH. This reduction suggested that LH, similar to BOR, might contribute to retarding the movement of BOR molecules, likely through intermolecular interactions. Exploring solvent exchange behavior, RMSD of the organic solvent and water molecules were monitored, as shown in Figure 23 (C) and (D), respectively. The fluctuation of DMSO was significantly reduced with increasing BOR content, especially in the DB10 to DB40 series. This indicated that BOR concentration influenced solvent exchange dynamics during the initial stage of phase transformation. Moreover, the introduction of LH brought forth intriguing dynamics. NBL formulations exhibited more pronounced solvent and water fluctuation than NB40, indicating that LH could potentially interfere with BOR's solvent exchange retarding ability. This was manifested through water diffusion into the ISM system due to the hydrophilic properties of LH. To delve deeper into molecular movement and diffusion throughout the phase transformation, diffusion constant values were calculated and presented in Table 10. The diffusion constants, providing complementary information to the RMSD results, were particularly informative. A comparative analysis of DMSO and NMP systems showcased a noteworthy observation. The diffusion constants of LH, organic solvents, and water molecules within the DMSO system were consistently reduced upon the addition of BOR, highlighting BOR's impact on limiting molecular movement. Interestingly, a similar trend was observed in LH-loaded systems, with diffusion constants of solvent, water, and LH all experiencing reduction upon the addition of BOR. In conclusion, the RMSD and diffusion constant analyses offer an intricate understanding of molecular dynamics during phase transformation. BOR's presence, along with LH's influence, plays a pivotal role in regulating molecular movement and solvent exchange behavior.

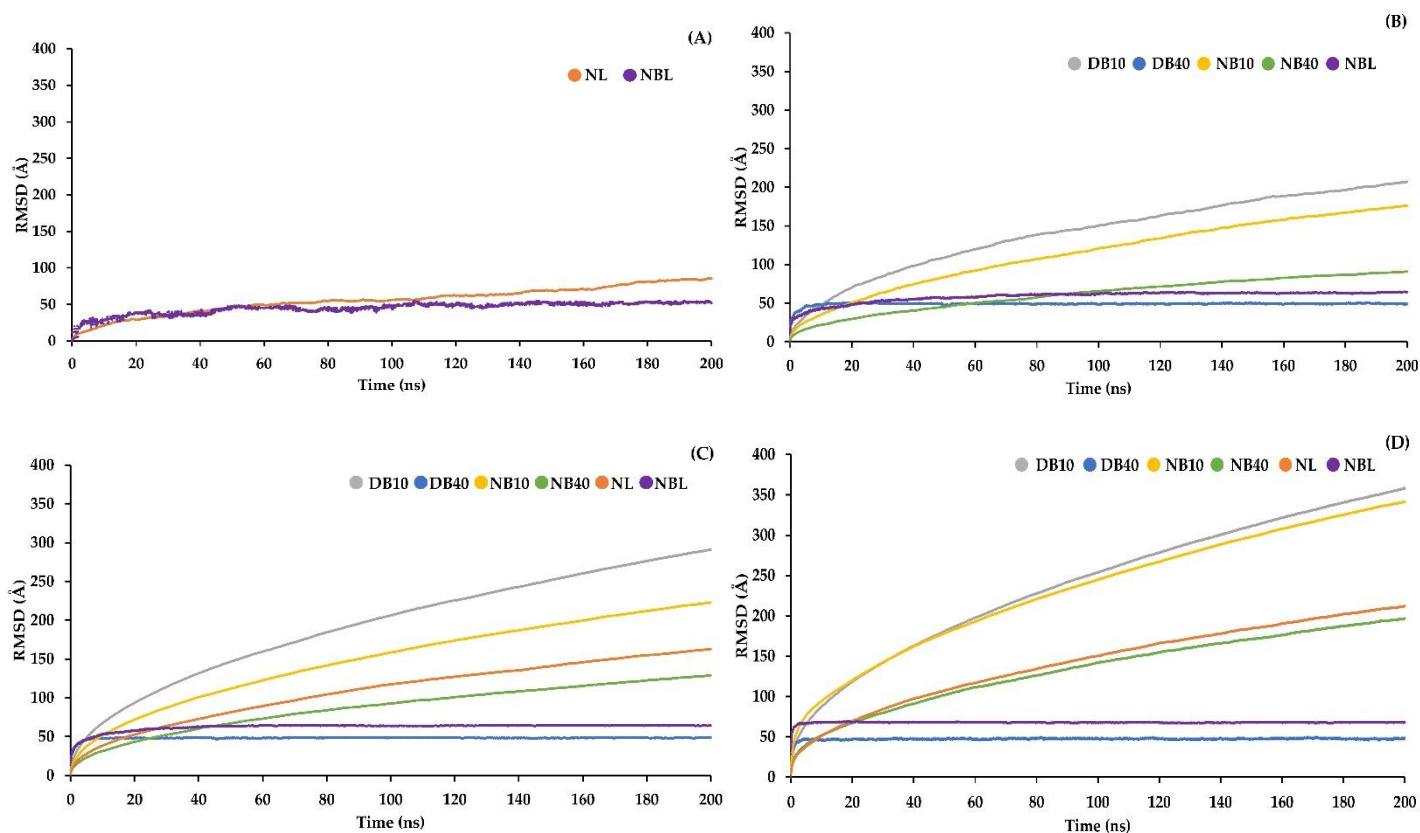


Figure 23 Root mean square deviation (RMSD) of LH (A), BOR (B), organic solvent (C) and water (D) conformation relative to the starting point of the MD simulations box contact with water.

Table 10 Diffusion constant of each substance in formulations box contact with water.

Composition	Formulation					
	DB10	DB40	NB10	NB40	NL	NBL
DMSO	7.0310	4.9494	-	-	-	-
NMP	-	-	4.1468	1.3654	2.2079	1.1929
BOR	3.5659	2.1896	2.6594	0.7022	-	0.5257
LH	-	-	-	-	0.5551	0.2071
WAT	10.6364	6.9445	9.5258	3.1901	3.7381	3.2533

4.1.3.2. Radius of gyration

The radius of gyration analysis offers valuable insights into the compactness and structural arrangement of molecular entities within the system. A smaller radius of gyration value indicates a tighter compactness of the molecules, implying a more condensed and organized configuration [205]. Remarkably, upon the addition of BOR, the radius of gyration values exhibited a significant reduction, signifying a notable enhancement in the compactness of the molecules. This phenomenon was particularly evident in the transition from the black to purple line in Figure 24, where the size of LH demonstrated a marked decrease following the inclusion of BOR. Additionally, a compelling observation emerged concerning the influence of BOR content. As the concentration of BOR increased, the radius of gyration value of BOR exhibited a prominent decrease. This trend was indicative of a heightened compactness and orderly arrangement of BOR molecules, further validating the impact of BOR on the system's structural organization. Furthermore, a noteworthy distinction was noted between the NMP and DMSO series. The NMP series showcased a more compacted configuration of BOR molecules compared to the DMSO series. This distinction suggested that the fluctuation dynamics of solvent molecules played a pivotal role in influencing the compactness of BOR molecules, particularly during the initial stages of phase transformation. In essence, the radius of gyration analysis not only sheds light on the compactness and arrangement of molecules but also highlights the intricate interplay between BOR content, solvent fluctuations, and the resulting structural changes. This comprehensive understanding of molecular dynamics and interactions enriches our insights into the mechanisms governing the initial phase of phase transformation.

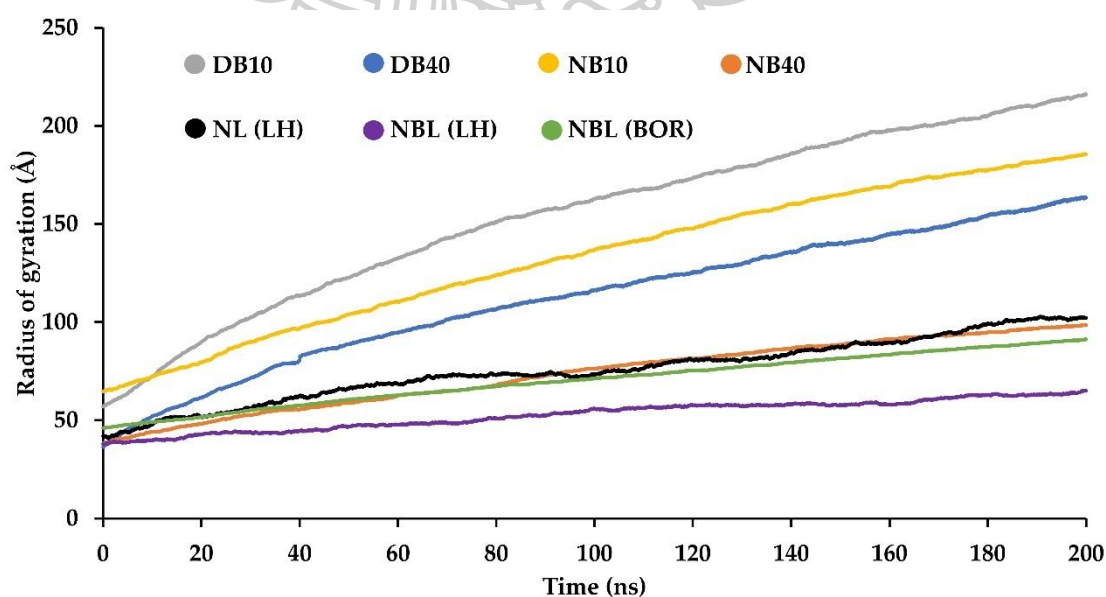


Figure 24 Radius of gyration of LH and BOR conformation of the MD simulations box contact with water.

4.1.3.3. H-bonding amount

Hydrogen bonds play a pivotal in intermolecular interactions, exert a significant influence on various physicochemical processes. This study examined the formation of hydrogen bonds within the system, focusing on interactions involving BOR and LH molecules. The investigation revealed intriguing insights into the hydrogen bonding network. Figure 25 (A) and (B) depicted the number of hydrogen bonds formed, both between BOR molecules and between BOR and LH molecules, in the DMSO and NMP series, respectively. Notably, the intermolecular hydrogen bonding interactions within BOR molecules exhibited an intriguing trend. As the concentration of BOR increased, the number of hydrogen bonds formed also displayed an upward trajectory. This behavior suggests that higher BOR concentrations contribute to the creation of a denser network of hydrogen bonds within the BOR entities. Additionally, a marginal disparity was observed between the DMSO and NMP series, with the former showcasing a slightly higher number of hydrogen bonds. An absorbing observation arose in LH-loaded ISMs. The hydrogen bonding pattern displayed a nuanced shift. The interaction between BOR and LH exhibited notably higher hydrogen bonding compared to BOR-BOR interactions. This finding implies that the presence of LH molecules does not significantly interfere with BOR's inherent hydrogen bonding interactions during the initial phases of BOR matrix formation. This observation is intriguing, suggesting that LH molecules are effectively retained within the matrix through BOR-LH hydrogen bonding interactions, which potentially contribute to retarding LH movement. This intricates interplay of hydrogen bonding interactions underscores the multifaceted dynamics at play during the initial stages of phase transformation.



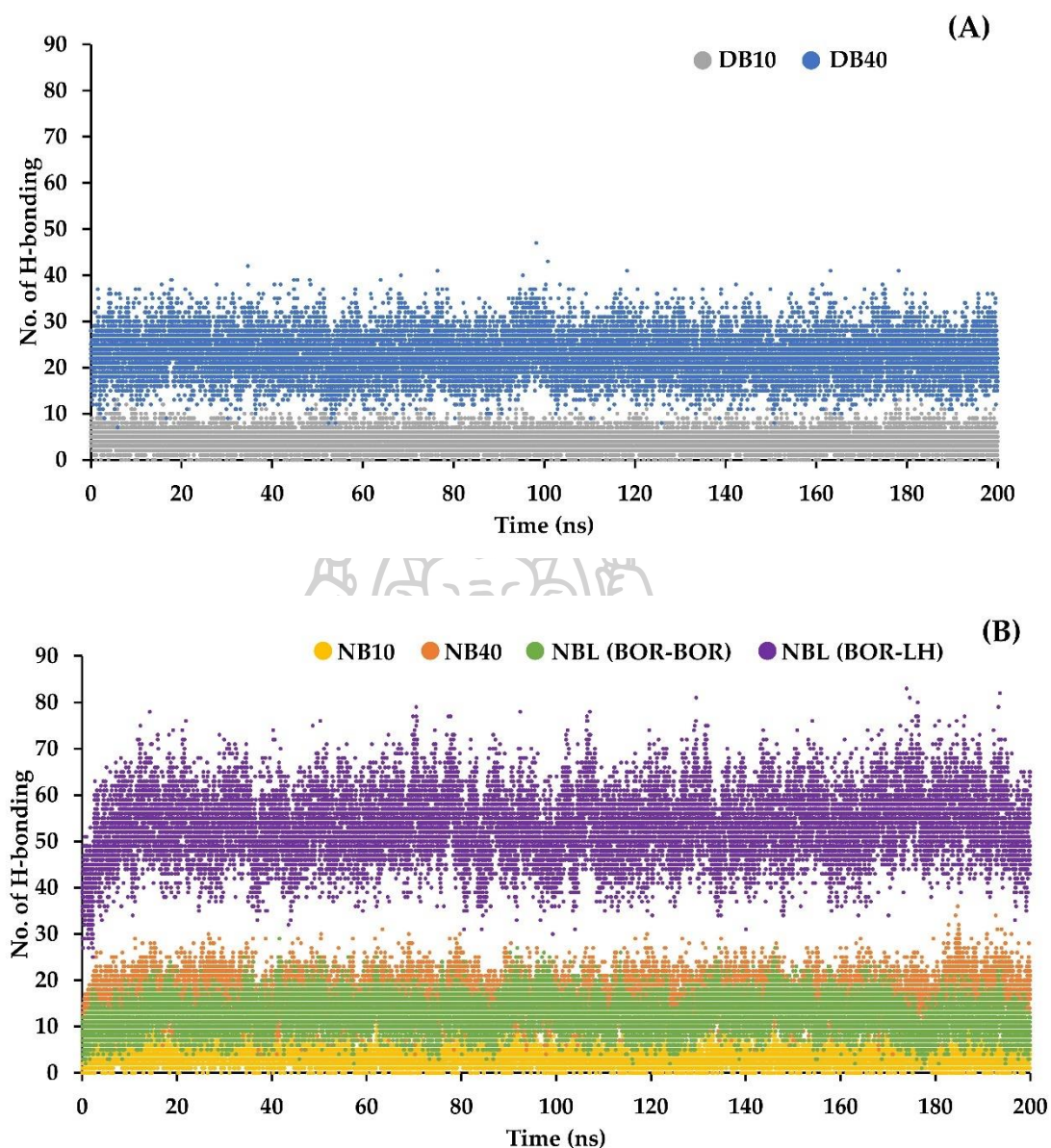


Figure 25 hydrogen bonds amount of DMSO series (A) and NMP series (B) ISM formulations box contact with water.

Summary

In summary, the present study effectively engineered LH-loaded ISMs utilizing a 40% BOR matrix-forming agent coupled with NMP as the solvent. These ISMs exhibited favorable attributes including low viscosity and surface tension, rendering them easily injectable and well-distributed upon application. The matrix formation process, driven by solvent exchange and the creation of a hydrophobic BOR matrix, intricately influenced the water tolerance and overall matrix characteristics. However, the incorporation of LH significantly impacted both solvent exchange and matrix formation dynamics, resulting in altered matrix properties. The BOR matrix displayed remarkable adaptability, showcasing plasticity that accommodates the contours of the gum cavity, thereby enhancing patient comfort. Diffusion studies involving sodium fluorescein underscored the intricate interplay between water behavior and BOR matrix formation at the interface boundary. The LH-loaded ISMs exhibited distinctive BOR matrices that were thinner and heterogeneous, aligning with the observed matrix formation and hardness outcomes. The drug release mechanism adhered to Fickian diffusion principles, as confirmed by the fitting of Peppas–Sahlin and Higuchi equations. Notably, formulations NBL5 and NBL7.5 exhibited a rapid attainment of the MIC of LH HCl on the first day, followed by sustained drug release above MIC for an impressive 8 days. The efficacy against diverse microorganisms, including *S. aureus* ATCC 25923, *E. coli* ATCC 8739, *C. albicans* ATCC 10231, and *P. gingivalis* ATCC 33277, further highlights the potential of these formulations as potent localized drug delivery systems for managing periodontitis. Delving into the molecular realm, the MD simulation outcomes emphasized the pivotal role of LH-BOR interactions in shaping matrix formation, influencing solvent exchange dynamics, and modulating the release of LH during the initial phase of phase transformation. However, a comprehensive evaluation in clinical settings is warranted to ascertain their efficacy and safety. This study underscores the multifaceted potential of LH-loaded BOR-based ISMs in periodontal treatment, while also opening avenues for future research into these promising systems.

Part 2: 4.2. DH-loaded Solvent Removal-induced IBU-based *In Situ* Forming Matrix

4.2.1. Physicochemical properties

4.2.1.1. Physical appearance

DH and IBU were dissolved in DMSO and NMP in a rapid process due to the excellent solubility of both drugs in these aprotic solvents. However, attempts to dissolve IBU at concentrations exceeding 40% w/w in these solvents proved challenging, resulting in the formation of precipitates composed of excess drug particles. The formulation success of IBU-based ISMs was validated by the presence of clear, colorless, and low-viscosity solutions. Increasing the IBU content within these ISM solutions did not alter their visual characteristics. The introduction of DH into the IBU-based ISMs caused the previously colorless solution to transition to a clear yellowish solution, attributable to DH's inherent yellowish coloration.

4.2.1.2. Density, pH, and viscosity

NMP and DMSO exhibited density values of 1.0265 ± 0.0008 and 1.0935 ± 0.0007 g/cm³, respectively. The inclusion of IBU induced a reduction in density for both the DMSO series (i.e., from 1.0845 g/cm³ to 1.0586 g/cm³) and NMP series (i.e., from 1.0264 g/cm³ to 1.0245 g/cm³), as detailed in Table 11 because the density of IBU was 1.029 less than that of DMSO and close to that of NMP [206]. IBU's inherent hydrophobicity and lower density played a pivotal role in diminishing the overall density of the formulations. Additionally, these determined density values served as crucial parameters for subsequent surface tension calculations. The introduction of DH into the ISM formulation led to an increased density, reaching 1.3809 g/cm³ [207].

The pH levels of the pharmaceutical formulations were assessed to evaluate their potential for irritation at the target site. The pH values for the organic solvents, IBU-based ISMs, and DH-loaded ISMs are summarized in Table 11. It was observed that the pH values of both DMSO and NMP were significantly higher than those of the formulations ($p < 0.01$). Upon the progressive addition of IBU, the pH levels of DMSO and NMP exhibited gradual reductions. This phenomenon could be attributed to IBU's classification as a propionic acid NSAID, wherein its COOH segment readily donated a proton to the formulation [74, 208]. Furthermore, the incorporation of DH led to a marked reduction in the pH of both formulations due to the presence of HCl from the hyclate salt [24, 25, 27]. Specifically, the pH values for DID40 and DIN40 were recorded as 4.93 ± 0.16 and 5.04 ± 0.08 , respectively. These pH levels were well within the acceptable range, closely approximating the pH of 6.8 found in periodontal pocket tissue. Moreover, considering the minimal dosage form applied during administration to the periodontal pocket, these pH values were deemed suitable.

The viscosity of IBU-loaded ISMs exhibited a modest increase in response to higher IBU content, as outlined in Table 11. Notably, ID40 and IN40 formulations recorded significantly elevated viscosity values compared to the other formulations ($p < 0.05$). This increase in viscosity can be attributed to intermolecular interactions involving the COOH segment of IBU, the S=O component of DMSO, and the C=O

group of NMP, presumably forming hydrogen bonds that limit solvent molecule mobility. Furthermore, the introduction of DH into the formulation led to a substantial rise in system viscosity, surpassing that of DH-free formulations ($p < 0.05$). This effect was primarily induced by the steric hindrance characteristics inherent to the DH structure, which impeded solvent molecule movement. [27, 28]. The incorporation of IBU and DH into the formulations reduced solvent content, thereby increasing solute concentration and environmental viscosity. Comparatively, the viscosities of the DMSO and NMP series were similar. Specifically, the viscosities of ID40, IN40, DID40, and DIN40 were measured at 12.28 ± 0.07 , 13.86 ± 0.10 , 18.61 ± 0.19 , and 20.82 ± 0.37 cP, respectively. Remarkably, DID40 and DIN40 exhibited significantly lower viscosity (<20 cP) compared to polymer-based ISMs, owing to the low density, small size, and simple structure of the loaded IBU [208]. These IBU-loaded ISMs demonstrated significantly lower viscosity than other polymer-based ISMs reported in previous studies. For instance, formulations containing 20% w/w ethyl cellulose, 30% w/w bleach shellac, and 35% w/w Eudragit RS exhibited viscosities of 2,200, 120, and 300 cPs, respectively. Additionally, ISMs with 25% w/w ethyl cellulose, 35% w/w bleach shellac, and 40% Eudragit RS were too viscous to flow [156, 209]. Natural resin-based ISMs with benzoin at 25% to 50% w/w in NMP and DMSO displayed viscosities ranging from 6.61–171.46 and 19.77–137.03 cP, respectively, while propolis-based ISMs exhibited 119–155 and 80.96–577.90 cP, respectively [155]. The advantage of low-viscosity IBU-based ISMs lies in their easy preparation through conventional mixing techniques and their suitability for administration via injection, similar to ISMs using saturated fatty acids [157, 158] or BOR [17] as the matrix-forming agents.

4.2.1.3. Surface tension and contact angle

Typically, surface tension at fluid-air interfaces arises from the strong cohesive forces among liquid molecules, promoting a natural tendency to minimize liquid surface area. Notably, surface tension values for binary mixtures of IBU-based and drug-loaded ISMs remain undocumented in prior literature. The surface tension values for the solvents, IBU-based ISM, and DH-loaded IBU-based ISM are presented in Table 11. Specifically, NMP and DMSO exhibited surface tensions of 39.31 ± 0.28 and 43.95 ± 0.13 mN/m, respectively. Interestingly, the addition of IBU at concentrations ranging from 10% to 40% w/w led to a reduction in the surface tensions of DMSO and NMP, decreasing from 40.18 ± 0.41 to 35.10 ± 0.87 and from 39.42 ± 0.19 to 36.91 ± 0.24 , respectively. Moreover, the addition of DH to the ISM formulation decreased the surface tension. This reduction of surface tension due to the attractive or cohesive force among solvent molecules around the surface region occurred through the interference of dissolved IBU and DH molecules. Both DMSO and NMP are polar aprotic solvents with strong dipole force and hydrogen bonding interactions between their molecules, whereas IBU is a monocarboxylic acid presenting a steric hindrance effect [46, 162, 210]. Thus, DMSO and NMP have a higher bonding strength between their molecules than IBU. For a binary mixture, the

component with the strongest molecular interaction typically simmers down in the bulk fluid phase instead of interacting on the droplet surface. Practically, the compound having the lowest molecular interaction is normally expelled from the bulk fluid to the droplet surface because of the apparent attractive forces between solvent molecules [31, 211, 212]. This reduction in surface tension could have advantageous implications for the formulation's administration and DH delivery to the target membrane. The decreased surface tension enhances their spreadability on the tissue surface by reducing the cohesive force of the liquid droplet.

The contact angle of ISMs is a direct indicator of the formulation's spreadability and wettability on a specific surface. Incorporating IBU tended to decrease the contact angle of formulations made with DMSO or NMP as solvents on glass slides and paraffin surfaces. This reduction indicates increased hydrophobicity due to enhanced adhesion on these surfaces, influenced by the addition of IBU. The decrease in contact angles on both surfaces followed an IBU concentration-dependent pattern, illustrating robust hydrophobic interactions between IBU molecules and the paraffin's hydrophobic surface (Log $K_{o/w}$ of 3.97) [208]. However, higher contact angles were noted at 30% and 40% IBU concentrations due to increased viscosities hindering droplet spreadability on these surfaces [213]. The agarose surface, simulating a periodontal pocket-like environment, was used for contact angle measurement due to its composition of ion species dissolved in water, creating a suitable surface for these assessments. The contact angles of IBU-loaded ISMs on the agarose surface were notably higher compared to pure organic solvents like DMSO and NMP. This was due to the solvent exchange mechanism caused by the *in-situ* transformation of the solution into a solid-like matrix, reducing the spreadability of the initial IBU-loaded ISM droplet [17]. These obtained matrices from solvent removal at the contact interface between the formulation droplet and agar decreased the spreadability of the initial IBU-loaded ISM droplet [17]. While the contact angles of DMSO and NMP were minimal and significantly lower than those of the formulations ($p < 0.01$; Table 11), the contact angles decreased with increasing IBU concentration or the addition of DH to the ISM system (Table 11). This reduction suggests the drugs' impact on weakening intermolecular interactions, similar to the impact on surface tension, leading to increased spreading and decreased contact angles, indicating challenges in matrix formation. Despite relatively high contact angle values, they remained below 90° , signifying good wettability on target surfaces [43]. The wettability of ISM is required for the adhesive quality between the formulation and the target surface to forbid the undesirable slipping out of the drug-loaded IBU matrix from the periodontal pocket [162, 214].

Table 11 Physical properties of IBU in DMSO or NMP and DH-loaded solvent removal-induced IBU-based ISM systems (n=3).

Formula	Density $\pm$ S.D. (g cm ³)	pH $\pm$ S.D.	Viscosity $\pm$ S.D. (cP)	Surface tension $\pm$ S.D. (mN/m)	Contact angle $\pm$ S.D. (Degree)		
					Glass Slide	Agarose gel	Paraffin
ID10	1.0845 $\pm$ 0.0012	7.66 $\pm$ 0.06	4.02 $\pm$ 0.23	40.18 $\pm$ 0.41	18.75 $\pm$ 0.75	18.56 $\pm$ 3.62	55.17 $\pm$ 1.63
ID20	1.0758 $\pm$ 0.0020	6.99 $\pm$ 0.04	5.73 $\pm$ 0.37	36.32 $\pm$ 1.17	16.98 $\pm$ 3.86	16.10 $\pm$ 1.45	50.71 $\pm$ 2.49
ID30	1.0678 $\pm$ 0.006	6.38 $\pm$ 0.10	7.87 $\pm$ 0.17	35.94 $\pm$ 0.26	15.16 $\pm$ 1.19	16.50 $\pm$ 0.98	46.74 $\pm$ 4.26
ID40	1.0586 $\pm$ 0.0011	5.87 $\pm$ 0.04	12.28 $\pm$ 0.07 ^b	35.10 $\pm$ 0.87	15.21 $\pm$ 0.25	15.98 $\pm$ 0.56	50.57 $\pm$ 4.76
IN10	1.0264 $\pm$ 0.0008	8.26 $\pm$ 0.19	3.80 $\pm$ 0.06	39.42 $\pm$ 0.19	12.43 $\pm$ 2.32	26.01 $\pm$ 0.64	50.04 $\pm$ 0.87
IN20	1.0260 $\pm$ 0.0003	7.50 $\pm$ 0.20	5.95 $\pm$ 0.45	38.39 $\pm$ 0.76	11.51 $\pm$ 1.04	27.95 $\pm$ 0.65	49.03 $\pm$ 1.74
IN30	1.0253 $\pm$ 0.0006	6.55 $\pm$ 0.08	8.17 $\pm$ 0.27	37.41 $\pm$ 0.69	16.63 $\pm$ 1.65	20.83 $\pm$ 0.61	47.42 $\pm$ 2.62
IN40	1.0245 $\pm$ 0.0009	5.79 $\pm$ 0.07	13.86 $\pm$ 0.10 ^b	36.91 $\pm$ 0.24	15.68 $\pm$ 0.51	18.69 $\pm$ 0.60	51.30 $\pm$ 1.40
DID40	1.0690 $\pm$ 0.0000	4.93 $\pm$ 0.16	18.61 $\pm$ 0.19 ^c	34.81 $\pm$ 1.88	15.42 $\pm$ 0.80	17.22 $\pm$ 0.82	53.60 $\pm$ 3.08
DIN40	1.0260 $\pm$ 0.0004	5.04 $\pm$ 0.08	20.82 $\pm$ 0.37 ^c	36.73 $\pm$ 0.89	15.31 $\pm$ 1.31	20.05 $\pm$ 0.60	52.74 $\pm$ 0.71
NMP	1.0265 $\pm$ 0.0008	10.08 $\pm$ 0.58 ^a	2.04 $\pm$ 0.13	39.31 $\pm$ 0.28	31.08 $\pm$ 0.40 ^d	7.12 $\pm$ 1.51 ^e	54.94 $\pm$ 1.31 ^f
DMSO	1.0935 $\pm$ 0.0007	10.02 $\pm$ 0.11 ^a	1.98 $\pm$ 0.09	43.95 $\pm$ 0.13	33.98 $\pm$ 1.25 ^d	4.30 $\pm$ 1.19 ^e	64.70 $\pm$ 0.81 ^f

The superscripts ^{a, d} and ^e in the columns represent a significant difference ($p < 0.01$) and the superscripts ^{b, c} and ^f in the columns represent a significant difference ($p < 0.05$) within the tested formulations.

4.2.1.4. Water tolerance

The water tolerance value is a pivotal parameter indicating the ability of the solution formulation to withstand phase changes induced by the aqueous phase. In this study, the water tolerance property of the prepared IBU-based ISM systems was evaluated by determining the amount of water required to induce turbidity, marking the phase separation endpoint [158, 159]. A higher water tolerance was noted when a larger volume of water was needed to reach this endpoint. The amount of water needed to achieve the phase separation point or water tolerance value of DH-loaded IBU-based ISMs is presented in Table 12. With the increase in IBU content in ISMs, the water tolerance notably showed a tendency to decrease, signifying sensitivity to the aqueous phase. At elevated IBU concentrations, the solvent was insufficient to

fully dissolve the drug once the solvent diffused out, leading to a reduction in water tolerance for the ISMs. Additionally, the introduction of DH into the formulation resulted in a decrease in water tolerance. The increase in both IBU concentration and the inclusion of DH augmented the overall solute molecules while reducing the solvent component within the system. Consequently, IBU reached its saturated dissolving point more readily due to its low hydrophilicity. Furthermore, the gradual introduction of water into the ISM system caused a reduction in the system's LogP due to water's higher dielectric constant compared to the organic solvent of ISMs. For reference, the dielectric constant values of DMSO, NMP, and water are 47, 32, and 78.54, respectively, at 20°C. [153, 163]. Practically, hydrophobic substances like IBU tend to crystallize due to the LogP reduction phenomenon. Within the ISMs, those in the DMSO series exhibited lower water tolerance compared to the NMP series. This difference can be attributed to the LogP values; the LogP of DMSO is -1.35 , which is lower than that of NMP at -0.38 . [215], which induced the crystallization of IBU to crystallize and the easier phase separation from the DMSO solution than from the NMP solution. The decreasing water tolerance of saturated fatty acid-based ISM in DMSO was evident as the carbon chain of fatty acids was longer or their concentrations were higher [157, 158]. Lauric acid-based ISM in DMSO systems required less water than the systems using NMP as a solvent to achieve the endpoint because DMSO has higher water miscibility than NMP because of its higher polarity [216], leading to the fast phase separation of lauric acid from DMSO. The increasing temperature of the system from room temperature to body temperature could increase the water tolerance of all formulas (Table 12) because the ideal solubility of a crystalline solute in a liquid solvent is increased proportionally by increasing the temperature or entropy of the system [164]. Hence, the process of matrix formation in IBU-based ISMs within the periodontal pocket at body temperature might present some level of difficulty compared to the *in vitro* matrix formation experiment. However, despite this potential challenge, the small dosage form injection could potentially be enveloped or immersed in the crevicular fluid within the periodontal pocket, triggering the induction of phase transformation.

Table 12 Water tolerance value of IBU in NMP or DMSO and DH-loaded solvent removal-induced IBU-based ISM systems after titration with deionized water at temperatures of 25°C and 37°C (n=3).

Formula	deionized water amount		% water tolerance	
	(mean $\pm$ S.D. (μ l))		(mean $\pm$ S.D.)	
	25°C	37°C	25°C	37°C
ID10	893.33 $\pm$ 9.43	1046.67 $\pm$ 24.94	26.27 $\pm$ 0.20	29.45 $\pm$ 0.49
ID20	726.67 $\pm$ 9.43	806.67 $\pm$ 24.94	22.47 $\pm$ 0.23	24.34 $\pm$ 0.57
ID30	593.33 $\pm$ 24.94	846.67 $\pm$ 24.94	19.13 $\pm$ 0.65	25.24 $\pm$ 0.55
ID40	480.00 $\pm$ 16.33	613.33 $\pm$ 24.94	16.06 $\pm$ 0.46	19.65 $\pm$ 0.64
IN10	1300.00 $\pm$ 28.28	1733.33 $\pm$ 24.94	34.14 $\pm$ 0.49	40.87 $\pm$ 0.35
IN20	1126.67 $\pm$ 9.43	1440.00 $\pm$ 28.28	31.00 $\pm$ 0.18	36.48 $\pm$ 0.46
IN30	766.67 $\pm$ 24.94	1133.33 $\pm$ 18.86	23.41 $\pm$ 0.58	31.13 $\pm$ 0.36
IN40	566.67 $\pm$ 18.86	766.67 $\pm$ 24.94	18.43 $\pm$ 0.50	23.41 $\pm$ 0.58
DID40	463.04 $\pm$ 10.27	604.38 $\pm$ 17.22	12.92 $\pm$ 1.04	17.02 $\pm$ 0.44
DIN40	550.41 $\pm$ 24.16	748.11 $\pm$ 20.16	15.03 $\pm$ 0.70	20.79 $\pm$ 0.41

4.2.1.5. *In situ* matrix transformation

The process of matrix formation in IBU-based ISMs was monitored in a simulated periodontal fluid environment. Over time, these ISMs underwent a transition from a liquid solution to a solid matrix, forming an opaque mass upon injection into the PBS pH 6.8 buffer at the base of the test tube, as depicted in Figure 26 (A). Only ID30 and ID40 formulations demonstrated complete matrix formation within a 30-minute timeframe. Initially, the injected solution retained a liquid state, gathering at the base of the test tube and showing no signs of mixing with PBS. Over time, the IBU-loaded ISMs underwent a gradual transformation into spherical, moderately soft matrices that ascended from the bottom. This transformation was aided by the diminishing density of the formulation as the organic solvent diffused outward. For the NMP series, the ISMs appeared as a cloudy emulsion scattered across the bottom of the test tube, lacking the formation of a cohesive unit, unlike what was observed in the DMSO series. The delayed transformation can be attributed to the strong interaction between IBU and these specific solvents [208, 215, 216]. The presence of DH-loaded IBU-based ISMs slowed down the transformation process compared to the DH free formulations. This alteration was evident in the color change of the matrix from its initial appearance to a yellowish hue, reflecting the color of DH. Despite reducing the water tolerance of the ISMs, the speed of matrix formation didn't seem directly correlated to water tolerance. Instead, the increase in viscosity caused by DH hindered solvent exchange and delayed the removal of solvents from the formulation, impacting the overall transformation rate. To obtain a clear understanding of phase transition, the morphological change of the formulation after filling the formulations into the agarose well was observed under a stereoscope, as shown in Figure 26 (B). The opaque ring expansion into the inner agarose well of the formulation was evident with time, particularly for high IBU loading formulas, such as ID30, IN30, and IN40. Formulations using DMSO as a solvent with high IBU loading exhibited more rapid phase transformation than that using NMP because of the higher polarity of DMSO provoking more miscibility between this solvent and the water phase, resulting in a rapid solvent exchange behavior and thereafter notable phase separation of IBU [157, 216]. This result corresponded with water tolerance, as previously mentioned.

Focusing on the microscopic aspect of matrix formation around the interface among ISMs and aqueous solutions, the experiment was performed by dropping the formulation beside the PBS pH 6.8 agarose gels under an inverted microscope and the results are presented in Figure 27. This experimental design was conducted to investigate the phenomenon at the boundary of the formulation, which faced the target site containing the biological fluid [157, 158]. Notably, during the matrix formation process of all formulations, three different layers, namely, the matrix formation layer (G layer), the droplet fluctuation layer (D layer), and the clear ISM solution layer (C layer), were generated, as shown in Figure 27. The initial phase of matrix transformation presented only a thin D layer between the interface and the C layer. Focusing on the D layer (Figure 28), the movement of droplets occurred from the

inner part or C layer outward to into the G layer; thus, this phenomenon was similar to emulsion droplet movement. As time passed, the D layer expanded to the C layer, and the droplets in D layer progressively decreased in size, resulting in increased turbidity and decreased velocity. When water-miscible solvents are present in the formulation, the diffusion of water into the ISM solution leads to a decrease in solvent concentration and the formation of unstable compositions. This triggers a phase separation process, resulting in the formation of two distinct phases: a D layer consisting of droplets of the IBU-lean phase and an IBU-rich phase with a demixing gap, while the inner layer remains a single homogeneous phase. The movement of droplets towards the aqueous phase at the interface is driven by the affinity between the water-miscible solvent and water, as the system seeks to minimize the free energy and achieve a more stable state. This leads to the rapid migration of tiny droplets from the interlayer to the aqueous phase, where the water concentration is higher. The slower movement and accumulation of larger droplets at the interlayer can be attributed to factors such as differences in droplet size, viscosity, and interfacial interactions [217]. These factors influence the dynamics of droplet movement during the self-matrix transformation process. Furthermore, the accumulation of IBU crystals at the G layer can be explained by the preferential solubility of IBU in the aqueous phase compared to the polymer-rich phase. As water penetrates the interlayer, it carries dissolved IBU and upon reaching the interface, the solubility of IBU decreases, leading to the precipitation and accumulation of IBU small crystals. By understanding the thermodynamic principles underlying phase inversion, the formation of polymer-lean droplets and polymer-rich phases, as well as the interplay between droplet characteristics and interfacial phenomena [218]. Matrix growth at the interface of BOR-based *in situ* forming matrices and agarose rim occurred over time, and its formation became more rapid with the increase in BOR concentration in DMSO [17]. By comparison, the DMSO series ISMs presented more droplets with more fluctuation in droplet size than the NMP series. At higher concentrations of IBU, the droplet amount and its movement in the D layer increased. The G and D layers were also thicker than the low-IBU concentration formulation. The DH-loaded formulation presented a slower movement of droplets than the DH-free formulation. Moreover, the change of the D and G layers was slower; therefore, the higher viscosity of the formulation because of the addition of DH interrupted solvent removal and retarded the IBU matrix formation process. Most of the phase transformations observed in previous studies on polymer or matrix-forming materials in ISGs or ISMs (e.g., PLGA, ethyl cellulose, shellac, fatty acid, cholesterol, BOR, Eudragit RS) resulted in the sudden formation of a solid matrix upon injection into the target site, making it challenging to study the phase transformation process [25, 27, 28, 98, 158, 159, 218, 219]. However, IBU undergoes transformation into a matrix form, allowing for continuous observation of the transformation process. This unique property of IBU-based *in situ* matrices offers valuable insights into the phenomenon during transformation, contributing to a better understanding of the underlying mechanisms.

(A)

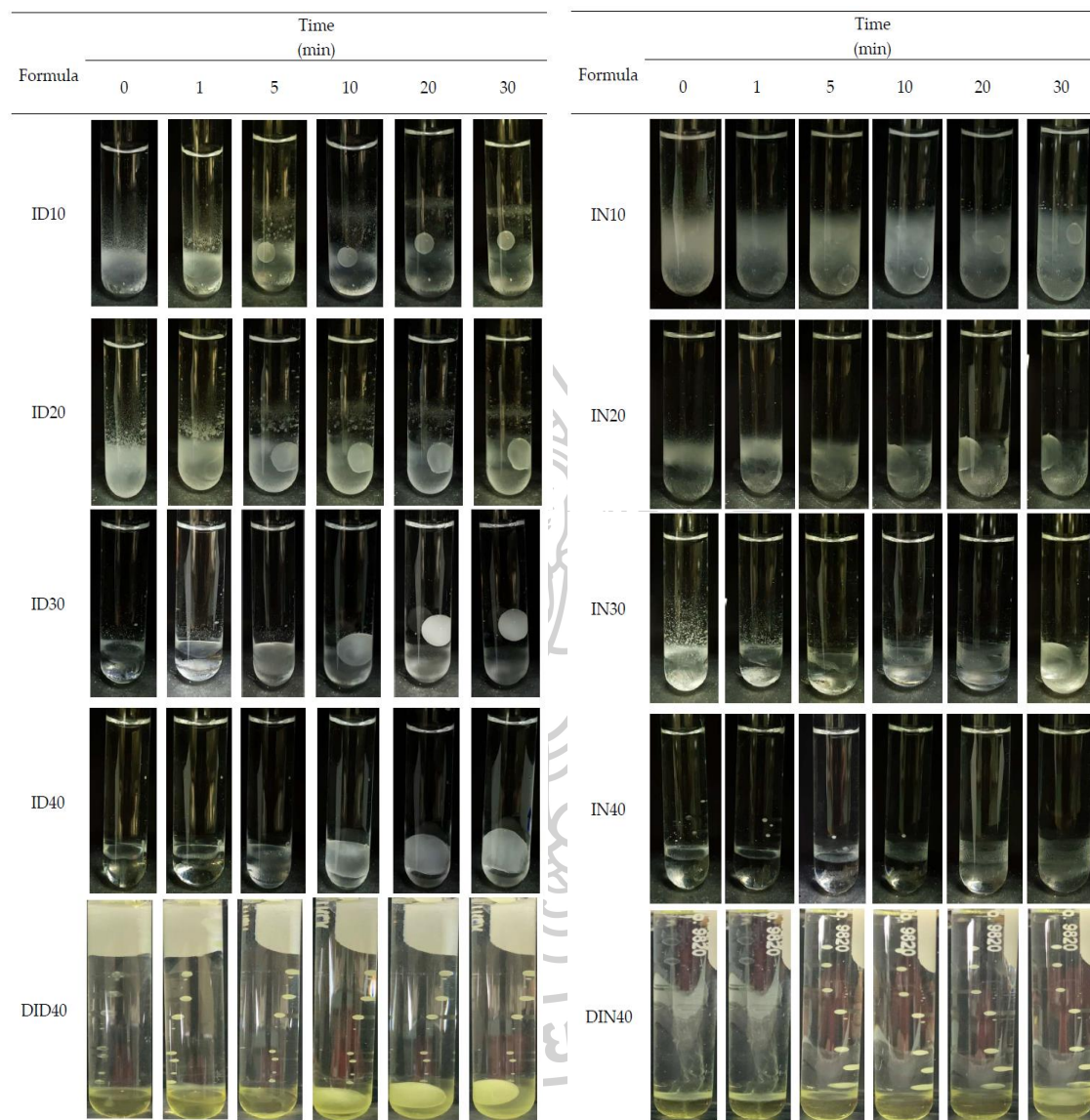


Figure 26 *In vitro* matrix formation behavior of IBU-based ISM and DH-loaded IBU-based ISM in PBS (A) and cross-sectional view of matrix formation of IBU-loaded ISM systems using DMSO and NMP as the solvents in the agarose well under a stereo microscope at a magnification of $\times 12$ (B).

(B)

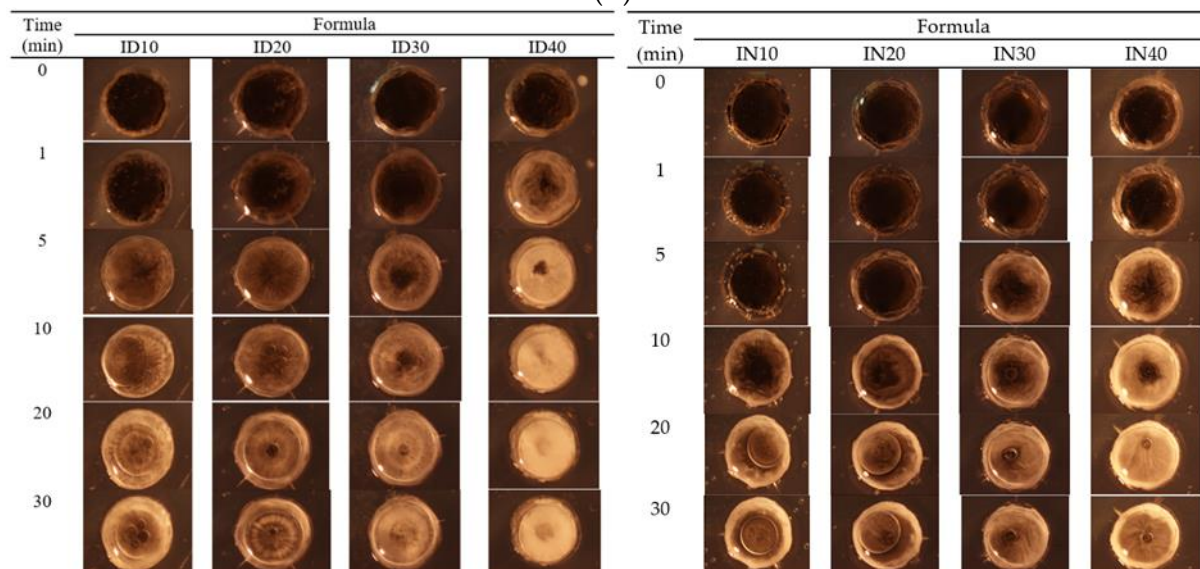


Figure 26 *In vitro* matrix formation behavior of IBU-based ISM and DH-loaded IBU-based ISM in PBS (A) and cross-sectional view of matrix formation of IBU-loaded ISM systems using DMSO and NMP as the solvents in the agarose well under a stereo microscope at a magnification of $\times 12$ (B) (continued).

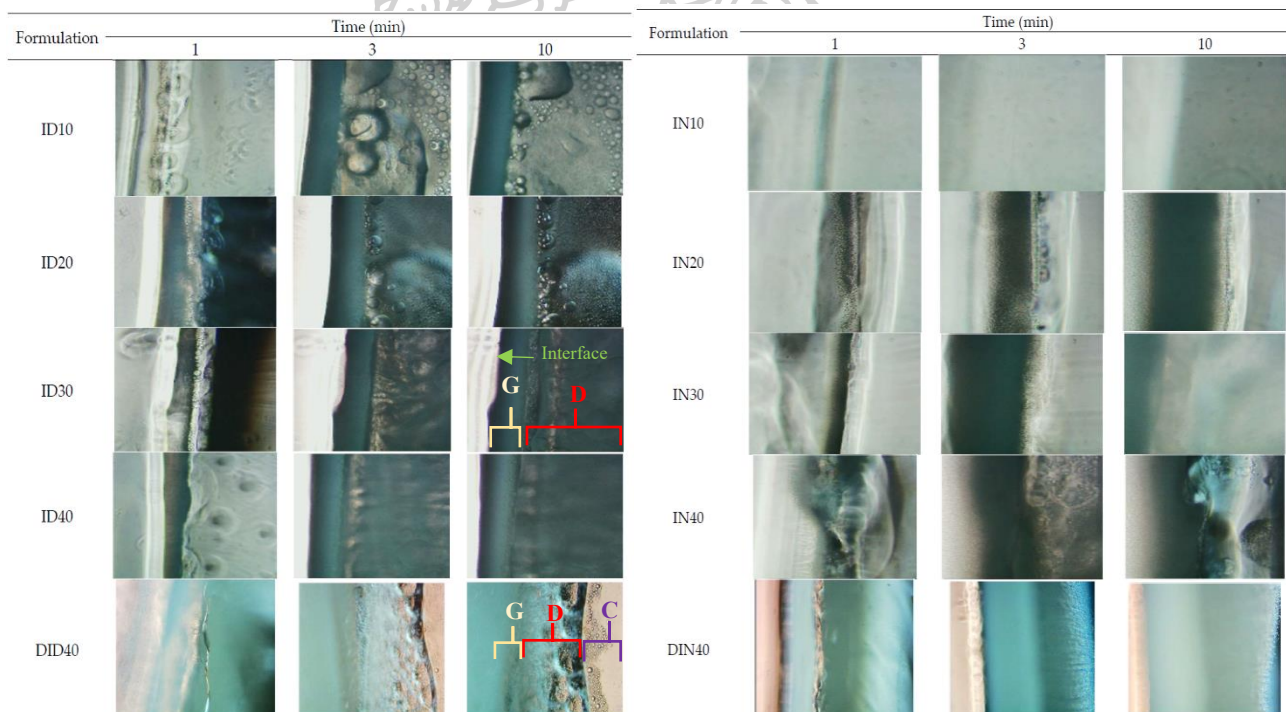


Figure 27 Matrix formation at the interface of the aqueous phase and ISM systems during the initial period (i.e.; 1, 3, and 10 min) at a magnification of $\times 60$ under an inverted microscope. Abbreviations: G = matrix layers, D = droplet fluctuation layers, C = clear ISM layers.

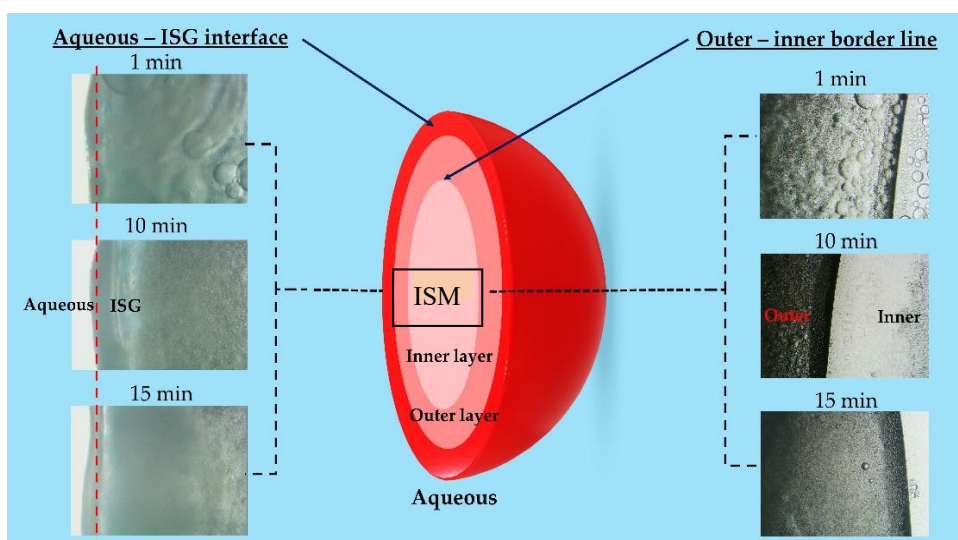


Figure 28 D-layer of IBU-based ISM for monitoring droplet movement behavior at a magnification of $\times 100$ under an inverted microscope.

4.2.1.6. Injectability properties

The injectable formulation requires precise control of both injection force and dosage quantity to effectively eliminate pathogenic microbes. Additionally, since these formulations can transition from a liquid solution to a solid matrix state, it is crucial to administer ISM formulations swiftly to ensure uniform matrix formation. [46, 155]. The effort and force required for injection appeared to rise with higher IBU content in the formulation, notably more so in the NMP series compared to the DMSO series (as indicated in Table 13), aligning with the viscosity data presented in Table 11. DH-loaded IBU-based ISMs exhibited the same trend, in which DIN40 exhibited slightly higher force and work of injection than DID40. The force and work of injection of DIN40 were 2.543 ± 0.022 N and 48.357 ± 0.560 N/mm, respectively, and those of DID40 were 2.365 ± 0.013 N and 43.242 ± 0.200 N/mm, respectively. The force and work of injection of these two solvents were quite significantly less than the formulations ($p < 0.05$). The force of injection of DH-loaded IBU-based ISMs was significantly lower than the acceptable level of force for injectable formulation (< 50 N), indicating that it was conveniently applied to the administration site [220]. In previous research on polymer-based ISMs involving materials like ethyl cellulose, Eudragit RS, and bleach shellac [27, 156], these materials showcased notably higher viscosity and required more force for injection compared to the IBU-based ISMs. This lower viscosity and enhanced injectability could potentially reduce discomfort during the injection of the formulation at the specific site of interest.

4.2.1.7. Mechanical properties

The mechanical characteristics of the transformed matrices within simulated periodontal pockets were evaluated using a texture analyzer. These matrices were expected to adhere to the pocket's surfaces and withstand the forces exerted by jaw movement, maintaining their shape to effectively fit within the periodontal pocket [166]. The mechanical properties were assessed by measuring both the maximum and remaining forces, providing insights into the matrices' elasticity and plasticity. The adhesion force and mechanical properties of the transformed matrices, specifically IBU-based and DH-loaded IBU-based ISMs, are summarized in Table 13. The addition of DH led to increased matrix hardness, evident in the maximum compression forces observed for ID40 and DID40, measuring 0.736 ± 0.001 and 1.678 ± 0.161 N, respectively. Additionally, DIN40 exhibited higher hardness compared to DID40, as indicated by the maximum compression force. DIN40 also displayed greater remaining force and mechanical properties while maintaining a similar adhesion force to DID40. This result corresponded with the previous report of bleached shellac-based ISM, with the mechanical hardness of its transformed matrix prepared with three solvents ordered as 2-pyrrolidone > NMP > DMSO, which was influenced by the phase separation rate [214]. The obtained transformed bleached shellac systems prepared with NMP and DMSO were more likely plastic or able to adapt their geometry to dynamic changes, whereas those prepared with 2-pyrrolidone were elastic [214]. During the texture analysis, the probe successfully pulled back all fabricated formulations, indicating low adhesive strength against the texture analyzer probe. All obtained matrices remained attached to the agarose basements, albeit with varying degrees of adhesion, as detailed in Table 13. This adhesive quality between the transformed ISMs and the target site was intentionally designed to prevent undesired dislodgement of the dosage form from the periodontal pocket [162, 214].

Table 13 Summary of the injectability and mechanical parameters of DH, IBU, and remnants from DH-loaded IBU-based ISM formulations (n = 3; mean $\pm$ SD).

Formulation	Injectability Properties			Mechanical Properties		
	Injection Force (N)	AUC of injection (N·mm.)	Maximum Force (N)	Remaining Force (N)	Adhesion Force (N)	Mechanical Properties
DMSO	0.734 ± 0.047^a	12.339 ± 0.375^c	-	-	-	-
ID40	1.837 ± 0.010	33.882 ± 0.663	0.736 ± 0.001	0.275 ± 0.009	0.083 ± 0.002	0.374 ± 0.013
DID40	2.365 ± 0.013	43.242 ± 0.200	1.678 ± 0.161	0.792 ± 0.093	0.091 ± 0.001	0.471 ± 0.019
NMP	0.904 ± 0.171^b	12.195 ± 0.369^d	-	-	-	-
IN40*	1.930 ± 0.053	36.291 ± 0.081	-	-	0.130 ± 0.003	-
DIN40	2.543 ± 0.022	48.357 ± 0.560	2.623 ± 0.079	1.477 ± 0.057	0.087 ± 0.009	0.563 ± 0.005

*IN40 did not transform into matrices and (-) not determined, the superscripts ^{a-c} in the column represent a significant difference within the tested formulations (p < 0.05).

4.2.1.8. *In vitro* drug release

DH-loaded IBU-based ISMs were designed as a drug delivery system for the treatment of periodontitis under the concept of “using the active chemical as excipient with dual drug release” [175, 221, 222]. This approach leverages the physical and chemical properties of active ingredients to aid in preparation or improve the effectiveness of the primary pharmaceutical ingredient. The use of IBU-based ISMs with these solvents has been recognized as effective drug delivery systems due to their low viscosity, which makes them easier to inject. Furthermore, their safety in human application is supported by the low toxicity of DMSO (LD₅₀ = 2.5–8.9 g/kg) [223] and NMP (oral and dermal LD₅₀ in rodents of 3600–7700 mg/kg) [174]. The 40% w/w concentration of IBU was chosen for the ISM drug delivery system as it enabled the loading of 5% w/w DH. Lower concentrations of IBU showed slower transformation into matrices, which might result in leakage from the periodontal pocket before complete matrix formation. Hence, the 40% w/w IBU concentration was selected for loading DH in formulations like DID40 and DIN40. IBU served as the matrix-forming material to regulate DH release, reducing administration frequency, while leveraging its anti-inflammatory activity to alleviate gum inflammation. *In vitro* drug release studies of DH-loaded IBU-based ISMs were conducted using the cup method to mimic the periodontal pocket environment [17, 183]. Moreover, many research works have applied the cup method to investigate drug release. DH was liberated from various ISMs comprising 40% Eudragit RS, 30% bleached shellac, 40% beta-cyclodextrin, and 20% Ethocel in 16 h, 2 days, 7 days, and 2.5 days, respectively, using this technique [24, 25, 176, 213]. The control groups, DD and DN, exhibited immediate release upon exposure to PBS pH 6.8 solution (Figure 29). DID40 and DIN40 solutions in the cup underwent complete transformation into turbid matrices within 15 minutes and 2 hours, respectively. The final residue of DID40 and DIN40 ISMs appeared as a solid matrix, achieving full transformation within 6 hours and 1 day, respectively. Atridox®, utilizing 33.03% poly(d,l-lactide) as a polymer, regulates drug release for 7 days following the cleaning of the periodontal pocket with an irrigating solution [96, 224]. The flushing of an irrigating solution, such as chlorhexidine solution, reduced the plaque bacteria before administration with the prolonged DH delivery system [225]. Hence, the DH-loaded IBU-based ISM formulations in this study are intended to regulate DH release for at least 7 days. Examining the release profiles in Figure 29, DH dissolved in organic solvent without IBU matrix former (as DD and DN) showed rapid and complete release within the first day. In contrast, DID40 and DIN40 exhibited an initial burst release within 3 and 18 hours, respectively. Subsequently, the DH release rate decreased, transitioning into a controlled release pattern over time. This behavior of DH release is closely associated with the state of the matrices, as the solid nature of the hydrophobic IBU matrix obtained might restrict DH release more than the liquid and matrix states [177]. Moreover, the efficiency of controlling DH release from the system corresponded well with the topography of the solid matrix of IBU from the SEM result (Figure 30) because DID40 had multiple layers of tortuously organized sheet structure with a small porous surface to impede drug liberation similar to drug

diffusion through a matrix of saturated fatty acid-based ISM as previously reported [143, 157, 158]. Meanwhile, the microstructure of DIN40 exhibited needle crystals with spherical holes and greater porosity, resembling multichannels (as shown in Figure 30), facilitating drug diffusion outward into the medium. Consequently, these IBU matrix structures improved the effectiveness of DID40 in trapping DH within the system, thereby extending DH release more effectively than DIN40. On the seventh day, the percentage of DH release for DID40 and DIN40 was $38.72 \pm 0.19\%$ and $84.49 \pm 2.08\%$, respectively. Nonetheless, both formulations demonstrated efficient and controlled DH release, providing sustained local drug delivery within the periodontal pocket. This approach aids in enhancing patient compliance due to reduced medication frequency [178].

For fitting different mathematical models to obtained drug release profiles, DDSolver[®] software applies the nonlinear least square curve-fitting technique, which determines the parameter values by minimizing the sum of squares (SS) or the weighted SS [226]. The conventional or modified forms of the utilized mathematical models were chosen from the model library collected based on the established models in the previous studies [227]. The estimated parameters from the DH release profile were fitted to the obtained release profiles of DH with zero-order, first-order, Higuchi's, Korsmeyer–Peppas's, Hixon's, Hofenberg's, and Peppas–Sahlin's equations, as presented in Table 14. The DH release profile of DID40 fits quite well with Korsmeyer–Peppas's model ($R^2 = 0.9345$, AIC = 57.4259, MSC = 1.2889) and Peppas–Sahlin's model ($R^2 = 0.9871$, AIC = 13.5315, MSC = 43.4335). Therefore, DH was released from the system proportionally to the exponent of time and the release mechanism followed the Fickian diffusion mechanism dominantly that occurred through the usual molecular diffusion of the drug because of a chemical potential gradient. The dominant drug release mechanism was determined to be the Fickian diffusion mechanism because the diffusion exponent (n value in Korsmeyer–Peppas's model ($n = 0.0861$) and m value in Peppas–Sahlin's model ($m = 0.2636$)) was less than 0.45, and when comparing the k_1 and k_2 values in Peppas–Sahlin's model, k_1 was superior to k_2 ($k_1 = 53.2199$ and $k_2 = -22.6506$); thus, the ratio of the Fickian diffusion mechanism was superior to the Case II relaxation mechanism [180, 228]. DIN40 also fits Korsmeyer–Peppas's model ($R^2 = 0.9796$, AIC = 40.7243, MSC = 2.8788, and $n = 0.1984$) and Peppas–Sahlin's model ($R^2 = 0.9857$, AIC = 60.2321, MSC = 3.4269, $m = 0.2402$, $k_1 = 75.0866$, and $k^2 = -11.5256$) with the dominant drug release mechanism being the Fickian diffusion mechanism similar to the DID40 formulation.

Typically, the pharmacological treatment of periodontitis involves addressing both infection and inflammation. Therefore, monitoring the IBU release behavior from ISMs alongside DH is essential. The IBU release profiles, as depicted in Fig. 3, revealed a burst release at the initial phase, which subsided within 1 hour. Subsequently, the release pattern transitioned into a sustained release. By the seventh day, IBU release reached $35.17 \pm 2.23\%$ for DID40 and $53.19 \pm 2.66\%$ for DIN40. In the case of in situ forming implants employing poly (D, L-lactic-co-glycolic acid) as a

polymer, both IBU and chlorhexidine dihydrochloride were released in a controlled manner. Specifically, the release rate of chlorhexidine dihydrochloride was higher during the first 5 days before slowing down [222]. DID40 notably delayed IBU release more effectively than DIN40, aligning with the DH release behavior. This was due to the extended duration of transformation from the liquid state to the matrix state in DIN40 compared to DID40. During the initial phase of ISM transformation, DIN40 exhibited both liquid and matrix states, allowing IBU to move more freely and readily diffuse outward from the system, resulting in a burst release behavior [177]. Thus, DIN40 demonstrated significant burst release behavior. During the later phase of drug release from the solid state of the IBU matrix, DIN40 also displayed accelerated IBU release owing to its rougher and more porous topographical surface, providing a larger water contact area compared to DID40 [229]. Comparatively, hydrophilic drugs like DH displayed a high initial burst release and less sustained release (as observed in Figure 29) due to their compatibility with the aqueous phase. On the other hand, hydrophobic drugs such as IBU exhibited a lower initial burst release and a more sustained release owing to their strong affinity with the solvent. The substantial loading of these two active compounds in the developed ISM dosage forms ensures efficient therapeutic treatment for periodontitis [24, 25, 156]. The release profile of IBU also fits Korsmeyer–Peppas's model (DID40: $R^2 = 0.9433$, AIC = 53.1028, MSC = 2.7078, and $n = 0.3875$; DIN40: $R^2 = 0.8885$, AIC = 60.2584, MSC = 3.0058 and $n = 0.364$) and Peppas–Sahlin's model (DID40: $R^2 = 0.9474$, AIC = 52.4558, MSC = 2.7576, $m = 0.282$, $k_1 = 10.363$, and $k_2 = 5.713$; DIN40: $R^2 = 0.9665$, AIC = 60.0905, MSC = 3.0187, $m = 0.3400$, $k_1 = 20.2566$, and $k_2 = 5.3191$) (Table 15) similar to those of DH from IBU matrices. Moreover, the Fickian release mechanism was presented as the major mechanism in both DID40 and DIN40 similar to the DH release behavior. Thus, DH and IBU release from both DID40 and DIN40 were influenced by their concentration gradients through the IBU matrices.

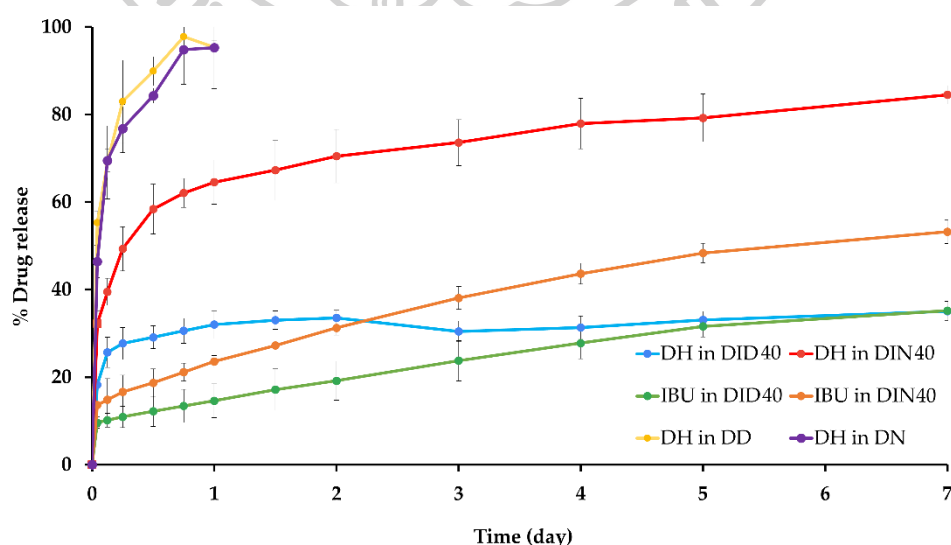


Figure 29 Comparison of the release profiles of DH and IBU from DH-loaded 40%IBU ISM systems (n=3).

Table 14 Degree of goodness-of-fit and estimated parameters from the curve fittings of the DH release profiles of different ISMs in PBS pH 6.8 using the cup method (n=3).

Formulation	Modelling	Criteria for model selection			Kinetic parameters		
		R^2	AIC	MSC			
DD	Zero order	0.4415	32.6951	-1.0092	$k_0 = 396.66$		
	First order	0.8925	39.1268	1.1125	$k_1 = 11.8593$		
	Higuchi's	0.7001	47.2998	-0.2496	$k_H = 135.2473$		
	Korsmeyer-Peppas's	0.9845	23.8737	2.6192	$k_{KP} = 105.0155$	$n = 0.1965$	
	Hixson's	0.7484	46.1530	-0.0585	$k_{HC} = 1.9307$		
	Hopfenberg's	0.8656	41.1295	0.7787	$k_{HB} = 0.0038$	$n = 4411.0392$	
	Peppas-Sahlin's	0.9905	13.9208	4.6098	$k_1 = 180.6271$	$k_2 = 96.0516$	$m = 0.3160$
DID40	Zero order	0.4050	24.4736	-1.0740	$k_0 = 136.33$		
	First order	0.0682	33.3778	-1.5290	$k_1 = 1.0087$		
	Higuchi's	0.2194	61.0878	-1.2085	$k_H = 31.3751$		
	Korsmeyer-Peppas's	0.9345	57.4259	1.2889	$k_{KP} = 29.8363$	$n = 0.0861$	
	Hixson's	0.0042	33.7183	-1.5971	$k_{HC} = 1.9307$		
	Hopfenberg's	0.2707	25.6048	-1.3568	$k_{HB} = 0.0026$	$n = 734.1810$	
	Peppas-Sahlin's	0.9871	13.5315	3.4335	$k_1 = 53.9199$	$k_2 = -22.6506$	$m = 0.2636$
DN	Zero order	0.0736	43.8395	-1.4003	$k_0 = 218.1449$		
	First order	0.8795	40.8805	0.8518	$k_1 = 9.4428$		
	Higuchi's	0.7863	36.4714	0.0734	$k_H = 144.0345$		
	Korsmeyer-Peppas's	0.9789	24.8168	2.4043	$k_{KP} = 101.2309$ $n = 0.2166$	$n = 0.2166$	
	Hixson's	0.7086	55.0420	-0.0523	$k_{HC} = 1.4516$		
	Hopfenberg's	0.8563	50.0978	0.6541	$k_{HB} = 0.0030$	$n = 5010.6982$	
	Peppas-Sahlin's	0.9940	17.4275	3.8821	$k_1 = 224.8520$	$k_2 = -157.0385$	$m = 0.4227$
DIN40	Zero order	0.2189	38.5772	-1.1486	$k_0 = 144.8828$		
	First order	0.2704	110.9836	-0.4770	$k_1 = 1.2346$		
	Higuchi's	0.1722	113.6096	-0.6790	$k_H = 42.5461$		
	Korsmeyer-Peppas's	0.9796	40.7243	2.8788	$k_{KP} = 63.3992$	$n = 0.1984$	
	Hixson's	0.3735	46.0956	-0.8243	$k_{HC} = 0.5602$		
	Hopfenberg's	0.2039	112.9868	-0.6311	$k_{HB} = 0.0004$	$n = 3235.7009$	
	Peppas-Sahlin's	0.9857	60.2321	3.4269	$k_1 = 75.0866$	$k_2 = -11.5256$	$m = 0.2402$

Remarks: R^2 = coefficient of determination; k = rate constant; AIC = Akaike information criterion; MSC = Model selection criterion.

Table 15 Degree of goodness-of-fit and estimated parameters from the curve fittings of the IBU release profiles of different ISMs in PBS pH 6.8 using the cup method (n=3).

Formulation	Modelling	Criteria for model selection			Kinetic parameters		
		R^2	AIC	MSC			
DID40	Zero order	0.3656	86.5737	0.1331	$k_0 = 6.4001$		
	First order	0.5031	83.3354	0.3822	$k_1 = 0.0818$		
	Higuchi's	0.8787	64.9331	1.7978	$k_H = 14.0786$		
	Korsmeyer-Peppas's	0.9433	53.1028	2.7078	$k_{KP} = 16.2576$	$n = 0.3875$	
	Hixson's	0.4610	84.4003	0.3003	$k_{HC} = 0.0251$		
	Hopfenberg's	0.4576	85.3405	0.2280	$k_{HB} = 0.0001$	$n = 750.6823$	
	Peppas-Sahlin's	0.9474	52.4558	2.7576	$k_1 = 10.363$	$k_2 = 5.713$	$m = 0.282$
DIN40	Zero order	0.3184	99.2511	0.0064	$k_0 = 9.907$		
	First order	0.5583	92.6213	0.5164	$k_1 = 0.1514$		
	Higuchi's	0.8885	70.2564	2.2367	$k_H = 21.8944$		
	Korsmeyer-Peppas's	0.9637	60.2584	3.0058	$k_{KP} = 25.729$	$n = 0.364$	
	Hixson's	0.4912	94.8484	0.3450	$k_{HC} = 0.0440$		
	Hopfenberg's	0.5178	94.6278	0.3620	$k_{HB} = 0.000$	$n = 1493.131$	
	Peppas-Sahlin's	0.9665	60.0905	3.0187	$k_1 = 20.2566$	$k_2 = 5.3191$	$m = 0.3400$

Remarks: R^2 = coefficient of determination; k = rate constant; AIC = Akaike information criterion; MSC = model selection criterion.

4.2.1.9. Surface topography

Surface topography analysis was performed on both intact drug powders and the water-attached surfaces of dried matrix remnants using SEM, as illustrated in Figure 30. The intact DH micrograph (Figure 30 (A, left)) indicated the different sizes of crystalline rod-shaped powders with more aggregation than that of IBU (Figure 30 (A, right)). The topography of intact IBU was a crystalline rhombic prism with various sizes and smooth surfaces. The remnant of the DH-loaded ISM (DID40) revealed a distinct surface. It displayed a rough and homogeneous sheet structure with multiple layers arranged in a tortuous fashion, and numerous small pores were observed on the structure, particularly at a high magnification of $\times 2500$. The surface topology of ID40 dried remnant is composed of a network of large IBU crystals connected, as shown in Figure 30 (B), indicating IBU recrystallization with a rather slow crystallization rate. Therefore, the deconstruction force of DID40 from the mechanical study was quite stronger than that of ID40 against compression force. This result corresponded decently with the continuous/denser topography and the more compact component of the obtained mass of DID40 than ID40. Thus, DID40 tolerated the compression force more than ID40. However, the dried remnant of DIN40 exhibited compaction of needle crystals with many small circular holes around the surface. This apparent high porosity of DIN40 indicated the high rate of solvent

exchange during its phase transformation. These topographical results from SEM explicated the DH and IBU release behavior as previously mentioned, in which the rates of liquid and drug diffusion were the limiting step of drug release through the different porous insoluble IBU matrices [230, 231]. SEM of the dried matrix of vancomycin hydrochloride-loaded lauric acid-based ISM presented a needle-shaped crystal agglomerate with a continuously intricate matrix, whereas palmitic acid dried matrix of this dosage form showed an enclosed sheath crystal with a large and simple aperture, confirmed the low tortuosity that resulted in a burst drug release and low sustainable drug liberation [143, 158]. Thus, the morphology of the transformed matrix of ISM notably influenced the drug release behavior. The aforementioned morphologically transformed matrices from saturated fatty acids and IBU were notably different from polymeric material-based ISM. The fibrous and scaffold characteristics were typically found for polymer-based ISM after solvent removal because of solvent leaching out from the matrix structure promoting various pore inner remnant structures [14, 25, 27, 31, 98]. The SEM micrograph of the remnant from DH-loaded Eudragit RS ISM contained many spherical particles connected and formed into the continuous phase in which the size of the void inside the remnant structure decreased with the increase in polymer concentration [24, 232]. This characteristic indicated the involvement of both the diffusion of the drug and the dissolution of the Eudragit RS network in controlling drug release. Therefore, the type of matrix-forming agent prominently influences the topography of the transformed structure of ISM systems after solvent removal and thereafter also affected the drug release characteristics.

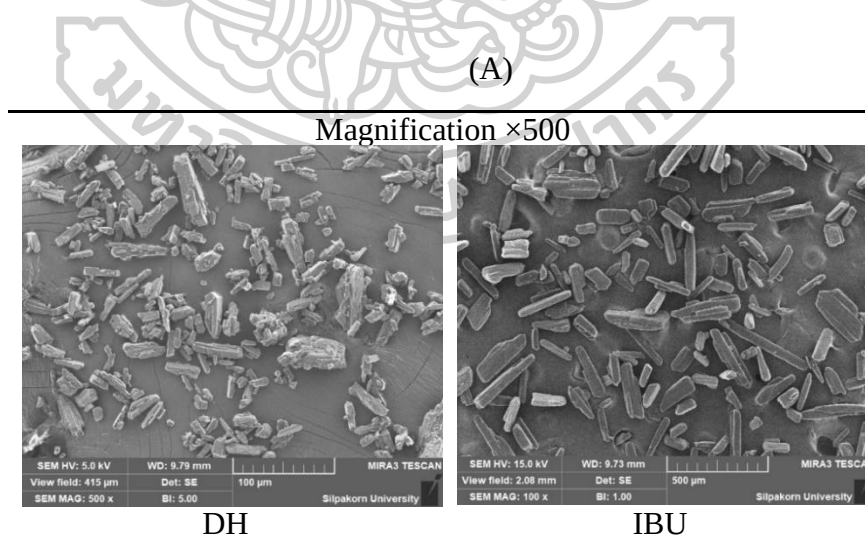


Figure 30 SEM photographs of DH and IBU powders and remnants from DH-loaded IBU ISM systems using DMSO and NMP as the solvents and IBU in DMSO (ID40) after drug release test in PBS pH 6.8 for 7 days.

(B)

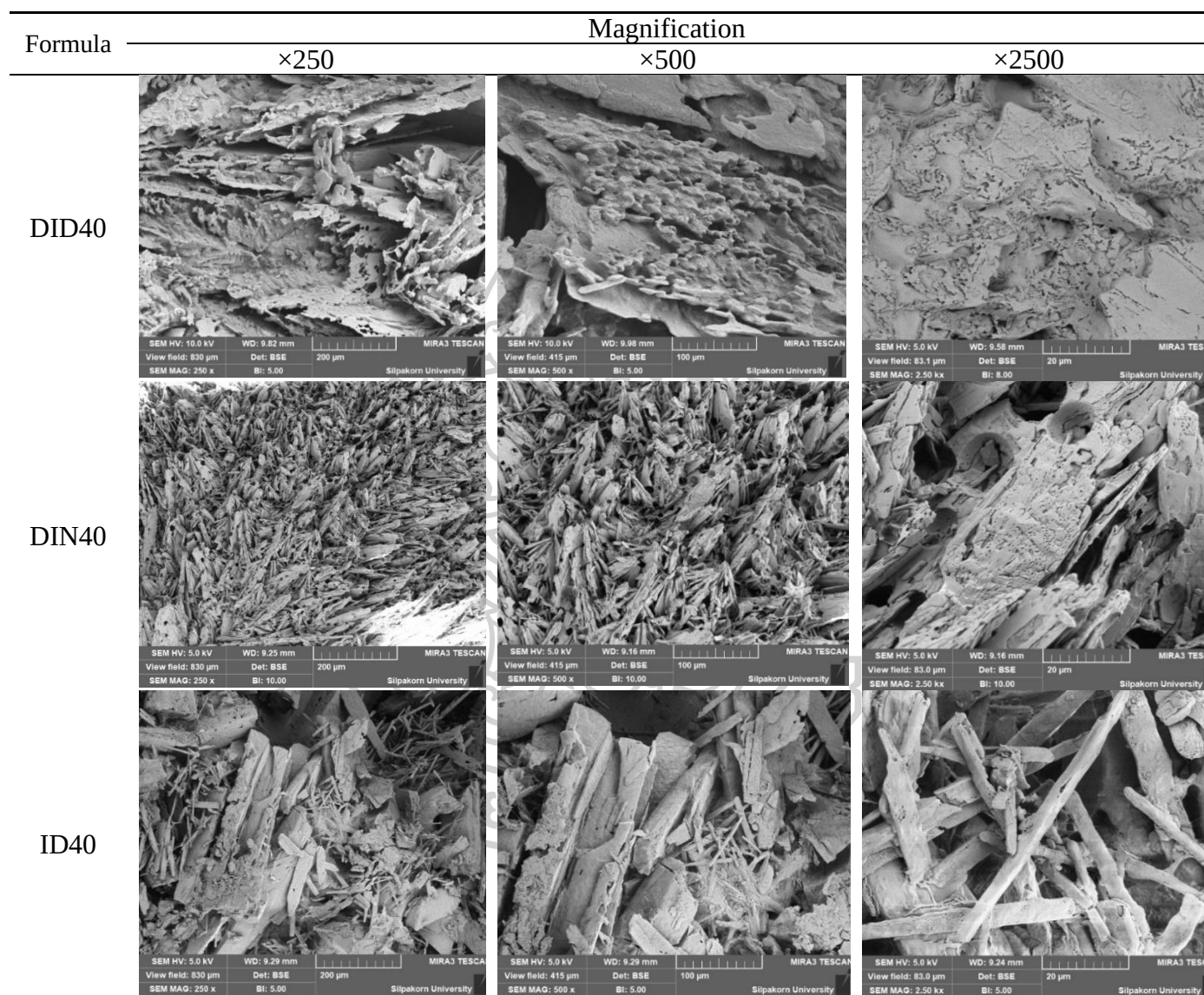


Figure 30 SEM photographs of DH and IBU powders and remnants from DH-loaded IBU ISM systems using DMSO and NMP as the solvents and IBU in DMSO (ID40) after drug release test in PBS pH 6.8 for 7 days (continued).

4.2.1.10. Differential scanning calorimeter (DSC), thermogravimetric analysis (TGA), powder X-ray diffraction (PXRD), Wide Angle X-ray Scattering (WAXS) and Fourier transform infrared spectroscopy (FTIR)

The decomposition patterns of intact components in ISMs and the residual materials after drying are illustrated in the TGA and dTGA thermograms, as shown in Figure 31 (A) and (B), respectively, with the peak positions for these patterns are listed in Table 16. In the case of intact DH, the degradation temperature exhibited two peaks at 160.72°C and 222.07 °C. On the other hand, intact IBU showed a decomposition temperature at 232.25 °C. Upon analyzing the dried remnants of both DID40 and DIN40, it became evident that they displayed similar degradation patterns, although with slightly lower temperatures when compared to intact IBU. This observation implies that these remnants predominantly consisted of IBU within the matrix debris and exhibited a lower degree of crystallinity compared to intact IBU. This evidence indicated that these remnants had only IBU left in matrix debris and presented less crystallinity than intact IBU. From the DSC thermogram (Figure 30 (C)), the endothermic peaks of intact IBU appeared at 78°C and 247°C. The peak at 78°C was the melting point of IBU [233] and the second peak indicated the decomposition of IBU, whereas DH showed two endothermic peaks at 163°C and an endothermic peak for its melting point at 202°C. The DID40 and DIN40 remnants after the dissolution test showed quite similar peak patterns as intact IBU. The first peak of DID40 and DIN40 was the melting point of IBU that presented a similar pattern of endothermic temperature, whereas the second peak of DID and DIN was related to the decomposition temperature of IBU, as presented in the TGA data (Table 16). Their endothermic peak patterns confirmed that only IBU remained in the dried remnants because the thermal properties of the DID and DIN formulations were similar to that of intact IBU.

To confirm the crystallinity of intact materials and dried remnants of ISMs, PXRD analysis was performed in an angular range of 3° to 40° 2 θ , as shown in Figure 31 (D). The PXRD diffractogram of intact IBU and DH presented sharp peaks with different patterns, indicating that IBU and DH were crystalline materials. The characteristic peaks of intact IBU were observed at 5.9°, 11.9°, 16.4°, and 19.9° 2 θ and those of DH were observed at 11° and 24.6° 2 θ . The diffractogram of DID40 and DIN40 remnants also exhibited sharp peaks with similar peak positions to that of intact IBU. Meanwhile, dried matrix remnants of DID40 and DIN40 had only characteristic IBU peaks. Furthermore, the intensity of their peaks presented a slight alteration compared with intact IBU. This change might correlate to the different recrystallization rates of IBU during its phase transformation when DMSO and NMP diffused outward whereupon these solvents exhibited a different degree of affinity with the aqueous phase, as previously mentioned. The FTIR spectra of intact IBU showed intense and well-defined bands at 1,720 cm⁻¹ (carbonyl stretching of the isopropanoic acid group) and 3,000 cm⁻¹ (hydroxyl stretching), whereas DH showed a carbonyl band between 1,714.717 cm⁻¹ and 1,649.138 cm⁻¹ (Figure 32). Both of DID40 and DIN40 debris showed similar patterns to that of intact IBU with no change

in their peaks; thus, this result confirmed that no interaction between IBU with solvent and DH occurred [235]. The characterization studies of the dried remnants of DH-loaded IBU-based ISMs indicated that the crystallized IBU mass remained with no significant modification of its chemical structure and crystalline state.

The crystallinity attributes of the IN40 system, evolving through the gradual introduction of water, were meticulously investigated using WAXS analysis. This facet of the study aimed to delve into the intricate transformations that occur when water is incrementally incorporated into the ISM matrix until reaching the prescribed water tolerance threshold. The initial WAXS profile, depicting the unperturbed IBU, NMP, and IN40 solution, is illustrated in Figure 33. Within this portrayal, the discernible peaks of IN40 align with the corresponding NMP peaks, signaling the crystalline nature of the solvent nestled within the IN40 ISM matrix. As the water content progressively increased, the WAXS pattern persisted in synchrony with the NMP peaks, manifesting through the range of 10% to 80% of the water tolerance value (IN40W1 to IN40W8). Remarkably, at the culmination of the 100% water tolerance endpoint (IN40 matrix), the WAXS pattern exclusively mirrored that of NMP. This observation underscores a pivotal aspect - the IBU molecules within the IN40 matrix seemingly did not transmute into a solid crystal structure at this juncture [170]. This microscopic insight is harmonious with the visual depiction of a liquid droplet at the inception of the phase transformation, with the absence of discernible IBU crystals following the interaction with water as Figure 28. This outcome offers a compelling perspective on the initial stages of matrix alteration, shedding light on the fluidic nature of the transformation process and the latent non-crystalline state of IBU at this point.

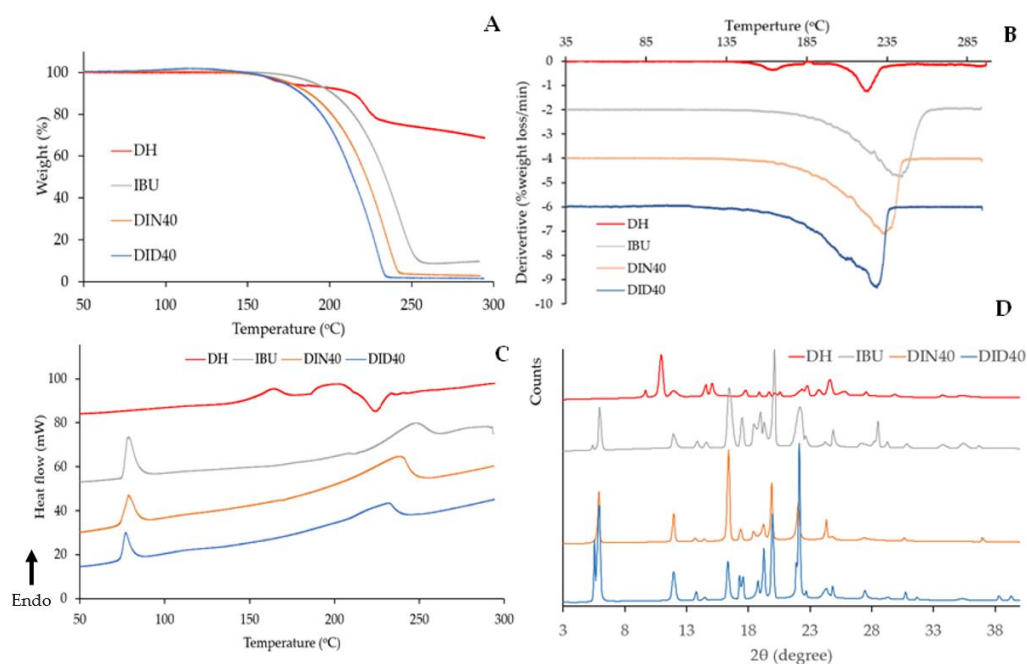


Figure 31 TGA (A), dTGA (B) and DSC (C) thermograms, and XRD diffractograms (D) of DH, IBU, and remnants from DH-loaded IBU-based ISM formulations after drug release.

Table 16 Summary of the thermal parameters of DH, IBU, and remnants from DH-loaded IBU-based ISM formulations after drug release from DSC and TGA.

Formulation	TGA parameter						DSC parameter					
	Onset temp (°C)		Degradation temp (°C)		End temp (°C)		Onset temp(°C)		T peak (°C)		Enthalpy (ΔH)(J/g)	
	Peak 1	Peak 2	Peak 1	Peak 2	Peak 1	Peak 2	Peak 1	Peak 2	Peak 1	Peak 2	Peak 1	Peak 2
DH	157.11	168.66	160.72	222.07	168.30	225.07	151.71	186.66	163.66	202.32	59.63	71.64
IBU	217.6	-	232.25	-	250.84	-	75.48	225.55	78.67	246.81	92.99	101.74
DIN40	208.5	-	229.58	-	238.80	-	74.56	198.42	78.68	237.30	79.59	300.70
DID40	199.15	-	228.69	-	232.80	-	74.15	200.9	77.01	231.29	72.10	207.95

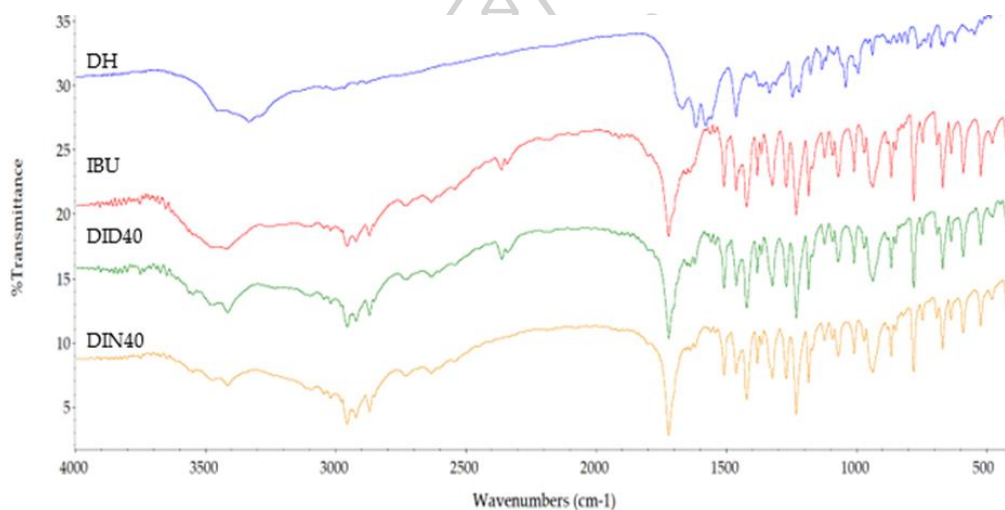


Figure 32 FTIR spectra of DH, IBU, and remnants from DH-loaded IBU-based ISM formulations after drug release.

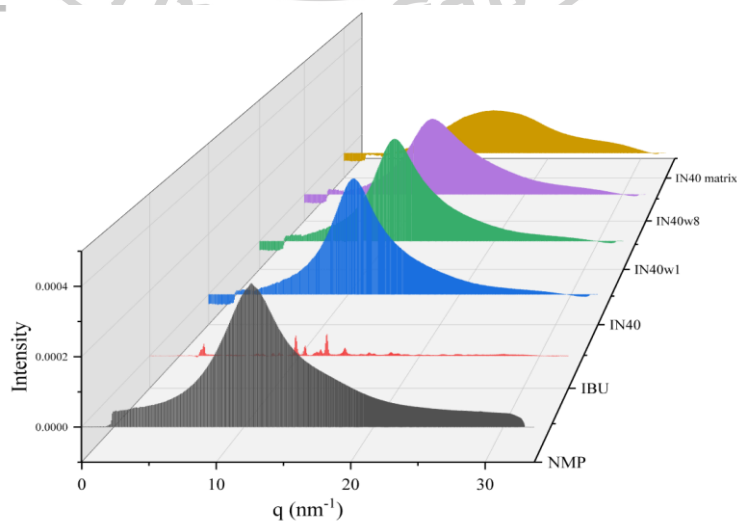


Figure 33 WAXS diffractogram of water-induced ISM system.

4.2.2. Bioactivities studies

4.2.2.1. Antimicrobial activities

The inhibition zone of the developed formula against *S. aureus* (ATCC 6538, 6532, and 25923), methicillin-resistant *S. aureus* (MRSA; *S. aureus* ATCC 4430), *E. coli* (ATCC 8739), *C. albicans* (ATCC 10231), *P. gingivalis* (ATCC 33277), and *A. actinomycetemcomitans* (ATCC 29522) from the antimicrobial activity tests via the cup agar diffusion method is illustrated in Figure 34, and their measured inhibition zone diameters are shown in Table 17. These tested microbe species were selected because all of them are associated with periodontitis, particularly pathogens, such as *P. gingivalis* and *A. actinomycetemcomitans*. DMSO and NMP are the organic solvents used as components in some pharmaceutical dosage forms because of their safety with low toxicity (LD₅₀ of DMSO and NMP are 2.5–8.9 and 4.15 g/kg, respectively) [236]. In commercial products, 33.3% w/w NMP was used as a solvent of Atridox[®] to dissolve the drug and poly(lactide) [237]. Furthermore, the US FDA has approved 50% w/w DMSO (RIMSO-50[®]) for the treatment of human interstitial or chronic cystitis, as well as various topical dosage forms containing DMSO for flap ischemia (60% DMSO), herpes zoster (5% idoxuridine in DMSO), and injection site extravasation (99% DMSO) treatment [158, 238]. However, the safety data of various medical applications of DMSO, NMP, and the developed formulations need to be determined through clinical experiments. Both DH in organic solvent solutions (DD and DN) that served as positive control groups exhibited significantly larger clear zones against all of the tested bacteria strains than the organic solvents ($p < 0.05$), particularly DD, because DMSO showed less antibacterial activity. NMP exhibited notable antimicrobial activity against *C. albicans* and *A. actinomycetemcomitans*. This interesting antimicrobial activity of NMP has been reported previously [191]. Therefore, the use of NMP as a solvent could enhance the antimicrobial activities of ISM. By comparison, NMP and DN presented no significant difference in inhibition zone against *C. albicans* because DH is not designed to inhibit this yeast and NMP efficiently inhibits fungus [24, 191].

Notably, DH-loaded IBU-based ISMs exhibited a tendency toward more inhibition zones against nearly all microbial strains when compared with DH dissolved in organic solvents (Table 17). Therefore, IBU enhanced the antimicrobial activity of DH; nevertheless, the dominant antimicrobial activities were from broad-spectrum antibiotics, such as DH. The antimicrobial activities of IBU have been reported in some literature, including against *Gram*-positive bacteria, such as *Bacillus subtilis* and *S. aureus* with a minimum inhibitory concentration (MIC) of 1.25–2.5 mg/ml [76]. Its equipotent antibacterial activity against three *Gram*-negative human pathogens, namely, *E. coli*, *Salmonella typhi*, and *Enterobacter* sp., was reported with a MIC range of 2.5–5 mg/ml [76]. The IBU matrices that occurred after phase transformation did not markedly interfere with the inhibition efficacy, whereas the other matrices transformed from ISM prepared with natural resins [155, 173, 183], cholesterol [28], polymers [156], and saturated fatty acids [158] typically diminished the inhibition zone diameter. Therefore, IBU is a promising material providing a

beneficial antimicrobial activity without lowering the inhibition efficacy when it was applied as the matrix-forming agent of ISM in addition to its capability to modulate drug release. The main pathogens of adult periodontitis are *P. gingivalis* and *A. actinomycetemcomitans*. However, the precise roles that such species play, singularly or in combination, in the pathogenesis of periodontal breakdown remain to be determined. In contrast to the majority of general infections, all of the suspected periodontal pathogens are indigenous to the oral flora, of which *C. albicans* causes refractory periodontitis [189]. *S. aureus* could be isolated from the periodontal pockets of patients with aggressive periodontitis and *E. coli* was considered a microorganism usually detected in patients with periodontitis [108]. From DH burst release on the first day of DH-loaded IBU-based ISMs, the drug amount at the initial stage was sufficient for inhibiting the growth of microbes. In patients with periodontitis, the volume of gingival crevicular fluid in the crevicular pocket ranges from 0.5 μ l to 1.0 μ l [239]. Once 0.3 g of DH-loaded IBU-based ISMs were injected into the crevicular pocket, the cumulative DH released from DID40 ISMs on the first and seventh days were 16,000 and 19,000 μ g/ml, respectively, and those from DIN40 were 32,200 and 42,500 μ g/mL, respectively, which are above the MIC of DH against *P. gingivalis* and *A. actinomycetemcomitans* (0.125 and 0.21 μ g/ml, respectively) [240-242]. Thus, the localized DH-loaded IBU-based ISM should be able to effectively eradicate the pathogens and related microbes during periodontitis treatment.



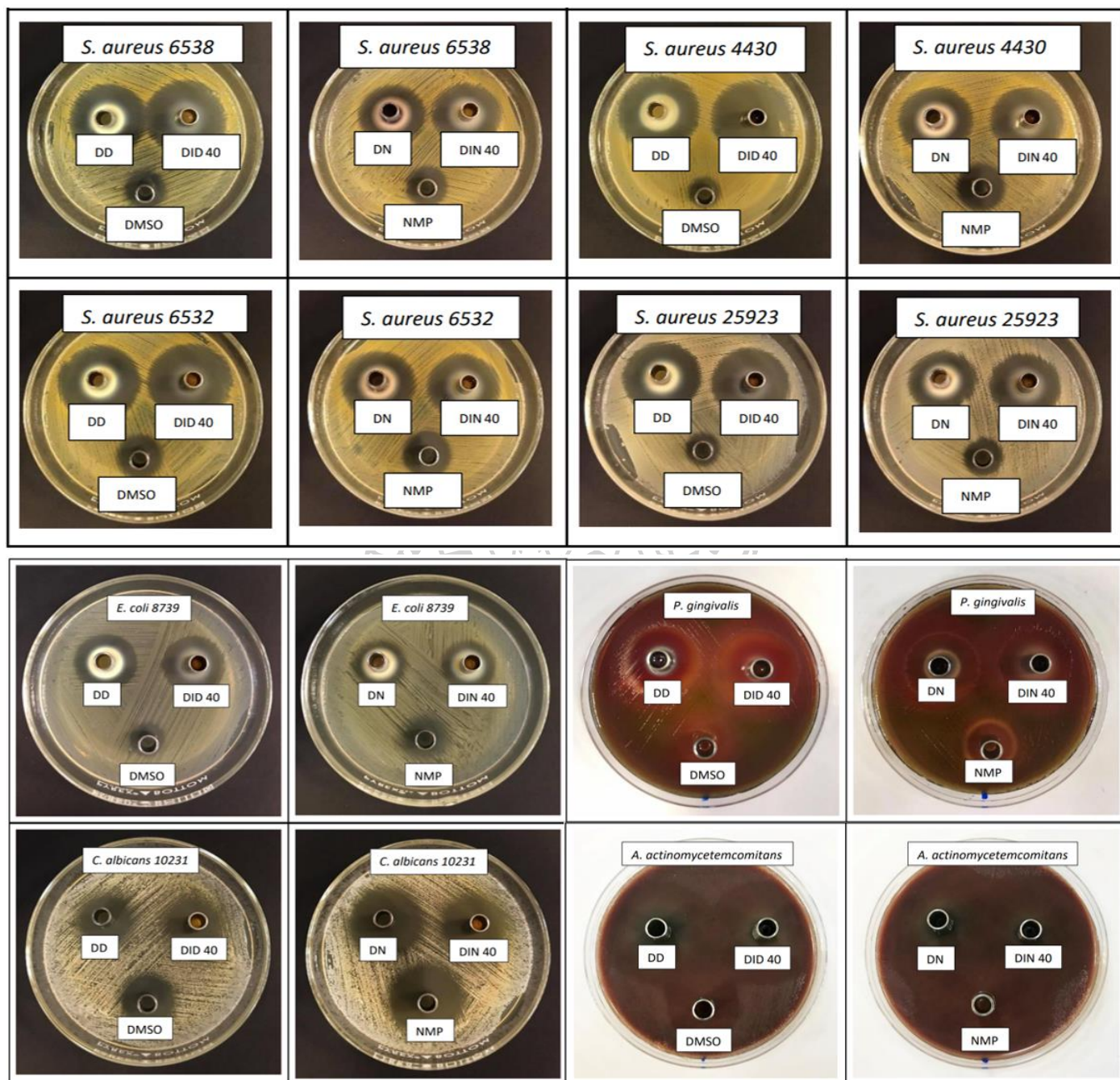


Figure 34 Inhibition zones of DH-loaded IBU-based ISM systems against *S. aureus* (ATCC 6538, 6532, and 25923), methicillin-resistant *S. aureus* (MRSA) (*S. aureus* ATCC 4430), *E. coli* ATCC 8739, *C. albicans* ATCC 10231, *P. gingivalis* ATCC 33277, and *A. actinomycetemcomitans* ATCC 29522 (n = 3).

Table 17 Clear zone diameters of DH-loaded IBU-based ISM systems against *S. aureus* (ATCC 6538, 6532, and 25923), MRSA (*S. aureus* ATCC 4430), *E. coli* ATCC 8739, *C. albicans* ATCC 10231, *P. gingivalis* ATCC 33277, and *A. actinomycetemcomitans* ATCC 29522 (n = 3).

Formula	Clear zone diameter (mm) (mean $\pm$ S.D)							
	<i>S. aureus</i> ATCC 6538	<i>S. aureus</i> ATCC 4430	<i>S. aureus</i> ATCC 6532	<i>S. aureus</i> ATCC 25923	<i>E. coli</i> ATCC 8739	<i>C. albicans</i> ATCC 10231	<i>P. gingivalis</i> ATCC 33277	<i>A. actinomycetemcomitans</i> ATCC 29522
NMP	15.7 $\pm$ 1.5	16.7 $\pm$ 0.6	16.0 $\pm$ 1.0	14.3 $\pm$ 0.6	15.3 $\pm$ 0.6	29.7 $\pm$ 0.6 ^j	17.0 $\pm$ 1.0	47.7 $\pm$ 2.1 ^a
DN	23.0 $\pm$ 1.0 ^a	25.7 $\pm$ 0.6 ^c	25.7 $\pm$ 0.6 ^d	25.7 $\pm$ 0.6 ^f	20.7 $\pm$ 1.2 ^h	29.7 $\pm$ 1.2 ^j	27.3 $\pm$ 1.5 ^l	48.0 $\pm$ 2.0 ^a
DIN40	26.0 $\pm$ 1.0 ^a	27.3 $\pm$ 0.6 ^c	27.3 $\pm$ 0.6 ^d	27.0 $\pm$ 1.0 ^f	21.3 $\pm$ 1.5 ^h	27.7 $\pm$ 1.5 ^j	27.0 $\pm$ 1.0 ^l	47.0 $\pm$ 1.0 ^a
DMSO	13.0 $\pm$ 1.0	12.7 $\pm$ 1.2	14.0 $\pm$ 1.0	12.3 $\pm$ 0.6	12.7 $\pm$ 0.6	21.0 $\pm$ 1.0 ^k	14.3 $\pm$ 0.6	24.0 $\pm$ 1.0
DD	27.7 $\pm$ 0.6 ^b	30.7 $\pm$ 0.6 ^d	32.0 $\pm$ 1.0 ^e	31.0 $\pm$ 1.0 ^g	22.7 $\pm$ 0.6 ⁱ	22.3 $\pm$ 1.2 ^k	28.7 $\pm$ 0.6 ^m	41.7 $\pm$ 1.5 ^o
DID40	29.7 $\pm$ 0.6 ^b	31.0 $\pm$ 1.0 ^d	32.3 $\pm$ 0.6 ^e	31.7 $\pm$ 0.6 ^g	23.3 $\pm$ 0.6 ⁱ	22.7 $\pm$ 0.6 ^k	30.7 $\pm$ 0.6 ^m	40.0 $\pm$ 1.0 ^o

The superscripts ^{a-o} indicates no significant difference ($p < 0.05$) using one-way ANOVA followed by the LSD posthoc test.

4.2.2.2. Anti-inflammatory activities

Aside from the antimicrobial activities, the anti-inflammatory effect on periodontitis treatment has been the focus of considerable interest because inflammation is a complicated process that typically results in pain and involves changes to membranes and increases protein denaturation and vascular permeability [243]. The protein denaturation process is influenced by external factors, such as heat, organic solvents, and extreme changes in environmental conditions, such as pH or inorganic salt concentration changes [244]. The thermal inhibition of protein denaturation of egg albumin is one of the models used to screen the *in vitro* anti-inflammatory activity of a novel substance or formulation [243, 244]. The percentage of inhibition of the protein denaturation effect on the release medium from the fabricated IBU-based ISMs and DH-loaded IBU-based ISMs on the first day is shown in Figure 35 using diclofenac sodium and 0.9% sodium chloride as the positive and negative controls, respectively. Only DIN40 exhibited higher anti-inflammatory activity than the positive control. The anti-inflammatory activity of DIN40 was superior to that of DID40 because IBU released from DIN40 was superior to that from DID40 (DIN40 was 23.58 ± 1.32 % and DID40 was 14.58 ± 3.85 %). The percentage of inhibition of ID40 and DID40 was significantly less than that of DIN40 and the positive control ($p < 0.05$). Moreover, this experiment revealed the potential of DH to synergize the anti-inflammatory activity of ISMs by increasing the IBU activity in both the DMSO and NMP series. Doxycycline suppressed the inflammatory activity *via* the reduction of the production of the cytokines IL-1 β , IL-6, TNF- α , and IFN- γ , as well as the chemokines MCP-1, MIP-1 α , and MIP-1 β , in a dose-dependent manner [245]. By comparison, the activity of NMP series ISMs was superior to that of DMSO series ISMs. A previous study reported that intact NMP exhibited more anti-inflammatory activity than DMSO [149]. NMP, 2-pyrrolidone, and diclofenac sodium expressed concentration-dependent inhibition of heat-induced protein denaturation of egg albumin with 50% inhibitory concentration (IC₅₀) values of 4.82% v/v (49.35

mg/mL), 4.83% v/v (52.29 mg/mL), and 736.50 μ g/ml, respectively [149]. NMP has also been reported to decrease inflammation by activating Krüppel-like factor 2 [246] and suppressing nuclear factor kappa B signaling [247]. The potentially diminished inflammatory activity of the developed DH-loaded IBU-based ISMs is beneficial to periodontitis treatment.

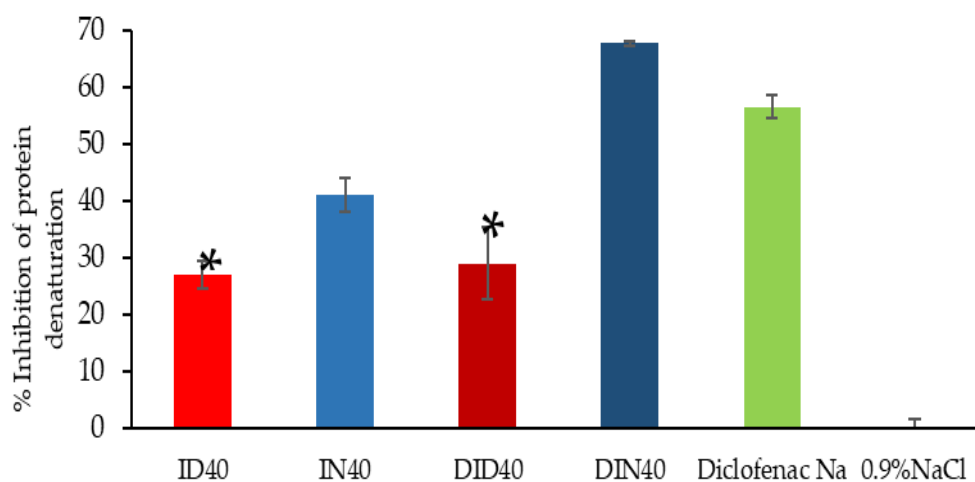


Figure 35 Percentage of inhibition of protein denaturation of DH-loaded IBU-based ISM (n=3). The asterisk symbol indicates no significant difference ($p \geq 0.05$) using one-way ANOVA followed by the LSD posthoc test.



4.2.3. *In silico* simulation results

4.2.3.1 Binding energy (effect of organic solvent)

To delve further into the intricacies of water tolerance and phase separation kinetics of IBU across different solvents, The DFT calculation was undertaken. This computational approach provided insights into the nature and strength of intermolecular interactions between IBU and solvents. The structural optimization of IBU and solvents was conducted using the Gaussian09 software at the B3LYP/6-311G* level. Subsequently, H-bonding interactions between IBU and solvents were predicted at the same theoretical level, followed by the computation of their respective binding energies. The summarized outcomes of these calculations were presented in Figure 36. Notably, the binding energy values for IBU-solvent interactions were consistently lower than those of solvent-solvent interactions. This implies that the principal interaction mechanism governing dissolved IBU molecules within the solvents is H-bonding between IBU and the respective solvent [248]. In comparison, the hierarchy of binding energy among solvent-solvent interactions was observed to be DMSO (D) > NMP (N) > Ethanol (E), respectively. This insight is pivotal in comprehending the phenomenon of phase separation within a solution. During this process, the interaction between IBU-solvent and solvent-solvent gets disrupted by the antisolvent, effectively facilitating the segregation of hydrophilic and hydrophobic phases. The calculated binding energy information is crucial in elucidating the water tolerance observed in various IBU solutions. The propensity of these solutions to tolerate water is intricately linked with the strength of solvent-solvent interaction. Within the IBU solution, the solvent assumes prominence across all IBU concentrations, exhibiting uniformity across the three types of solvents, as reflected in the IBU mole fraction. The binding energy of solvent-solvent interaction signifies a more stable and resilient factor against the disruptive forces at play.

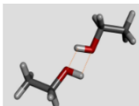
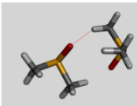
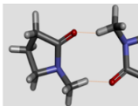
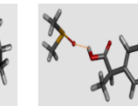
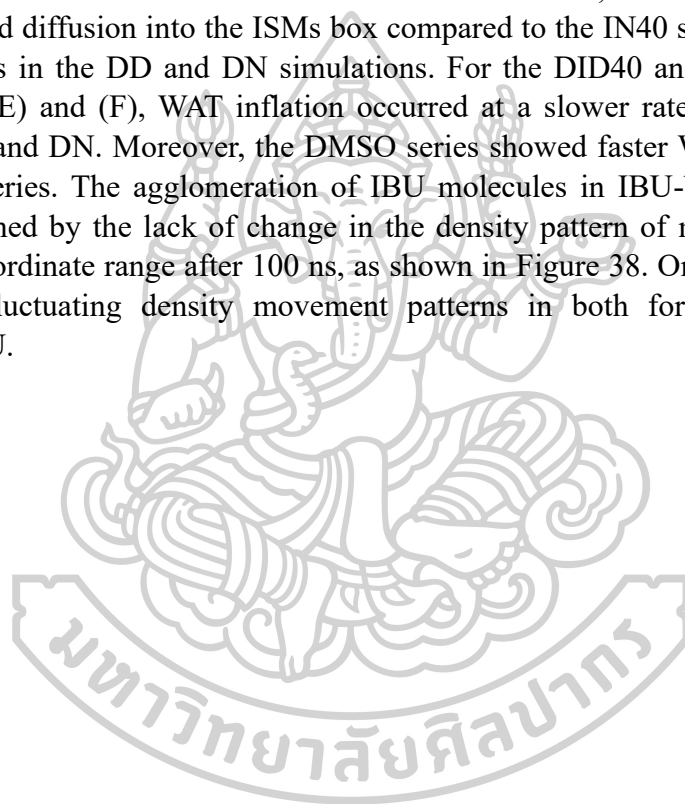
Formula	E	D	N	IE	ID	IN
Binding energy (kcal/mol)	-6.33	-2.00	-4.14	-10.97	-11.99	-9.35
Optimized molecular						

Figure 36 Binding energies and binding location of optimized molecules.

4.2.3.2. MD simulation of phase transformation

During the MD simulation experiment, the simulation boxes were setup to replicate the interface region between different phases of IBU-based ISMs (on the left side) and the aqueous phase (on the right side). The molecular ratio in each the simulation boxes were based on the formula's content and water tolerance value to ascertain the minimum required water content around the interface for inducing phase transformation, as illustrated in Figure 37. The simulation result of DD and DN

formulations (Figure 37 (A) and (B)) exhibited freely moving DH molecules without significant agglomeration. Nonetheless, there was a distinct variation in the movement of WAT molecules, with a notably quicker inward diffusion into the DD system when contrasted with the DN system. This was evident from the time it took for WAT diffusion to reach homogeneity within the box. Additionally, simulation animations of the ID40 and IN40 formulations were shown in Figure 37 (C) and (D), respectively. The simulations revealed no difference between the two IBU formulations. Over time, IBU gradually formed bulk structures while still exhibiting continuous movement within their units for up to 200 ns. This indicated that IBU did not undergo solid precipitation during the initial stage of phase transformation but rather formed clusters of IBU molecules. In terms of WAT molecule diffusion, the ID40 system showed faster inward diffusion into the ISMs box compared to the IN40 system, similar to the observations in the DD and DN simulations. For the DID40 and DIN40 systems as Figure 37 (E) and (F), WAT inflation occurred at a slower rate compared to ID40, IN40, DD, and DN. Moreover, the DMSO series showed faster WAT movement than the NMP series. The agglomeration of IBU molecules in IBU-based ISMs systems was confirmed by the lack of change in the density pattern of molecules within the 40-80 Å coordinate range after 100 ns, as shown in Figure 38. On the other hand, DH exhibited fluctuating density movement patterns in both formulations with and without IBU.



(A)

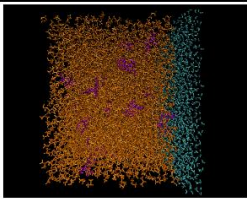
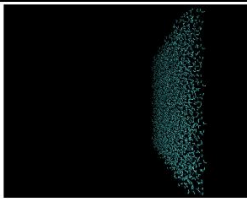
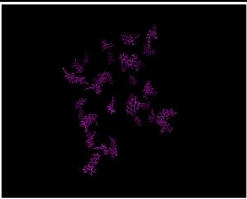
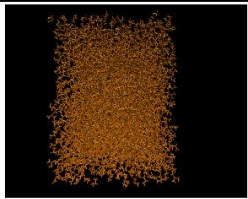
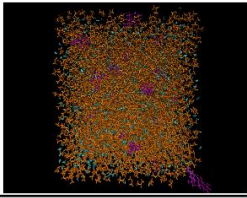
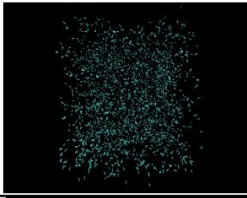
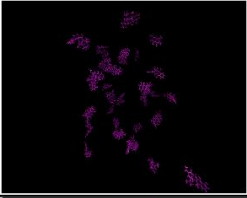
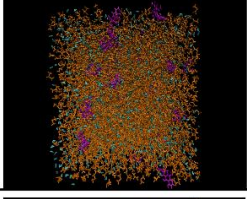
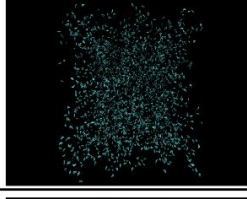
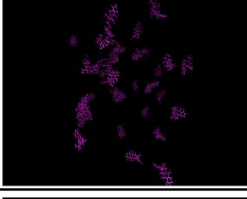
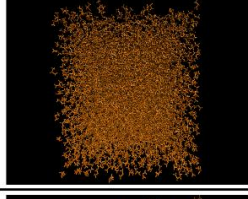
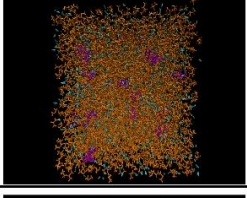
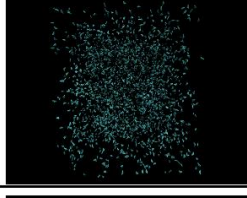
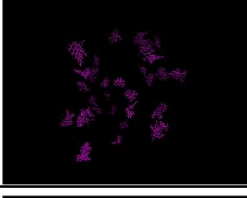
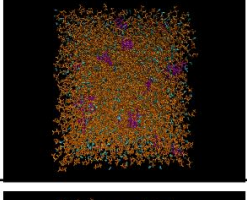
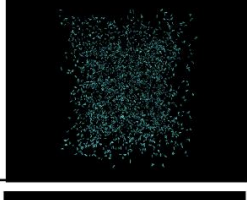
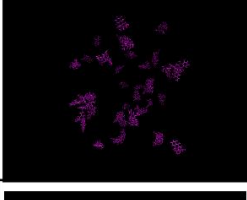
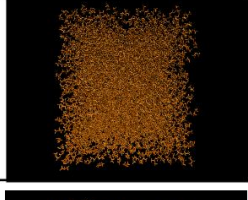
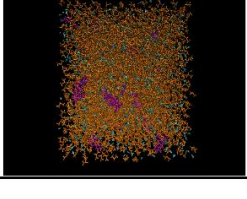
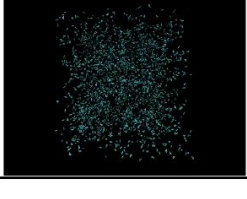
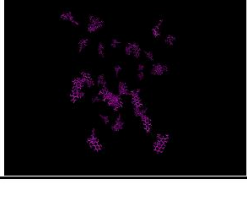
DD				
Time (ns)	All	WAT	DH	DMSO
0				
5				
10				
50				
100				
200				

Figure 37 Molecular dynamic modeling of solvent exchange mechanism focusses at interface of each ISMs against aqueous, (DD (A), DN (B), ID40 (C), IN40 (D), DID40 (E) and DIN40 (F) after running in MD simulation in 200 ns.

(B)

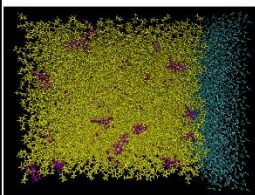
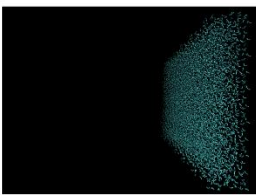
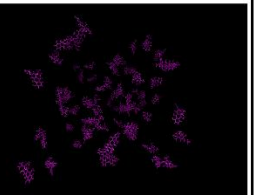
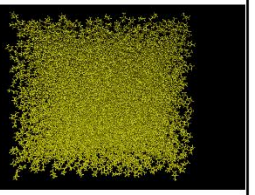
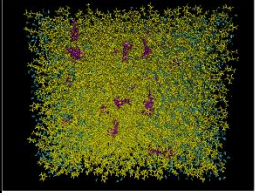
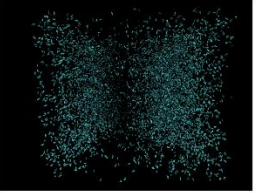
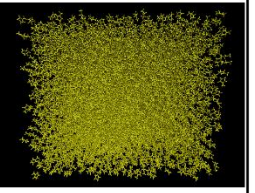
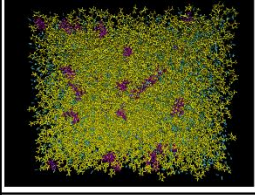
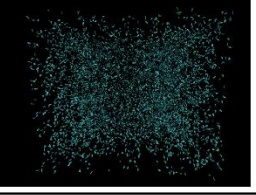
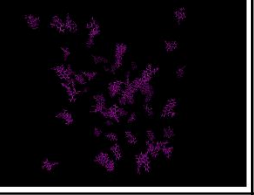
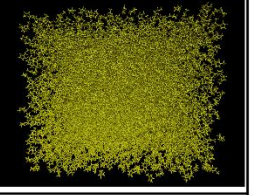
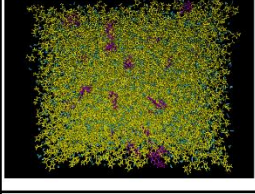
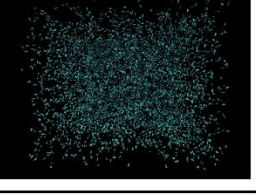
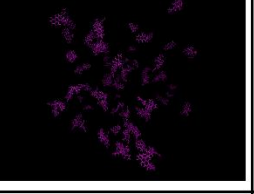
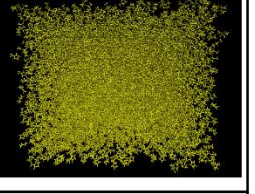
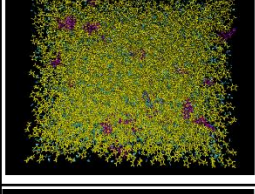
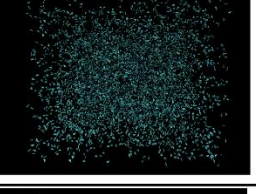
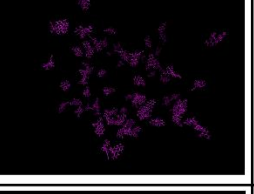
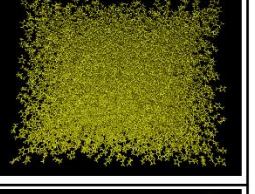
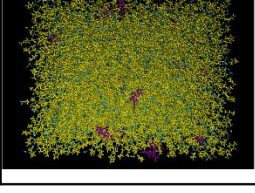
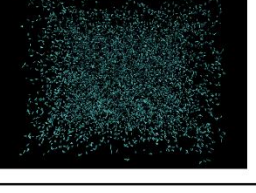
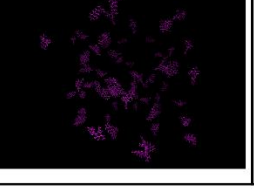
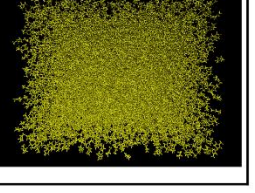
DN				
Time (ns)	All	WAT	DH	NMP
0				
5				
10				
50				
100				
200				

Figure 37 Molecular dynamic modeling of solvent exchange mechanism focusses at interface of each ISMs against aqueous, (DD (A), DN (B), ID40 (C), IN40(D), DID40 (E) and DIN40 (F) after running in MD simulation in 200 ns (continued).

(C)

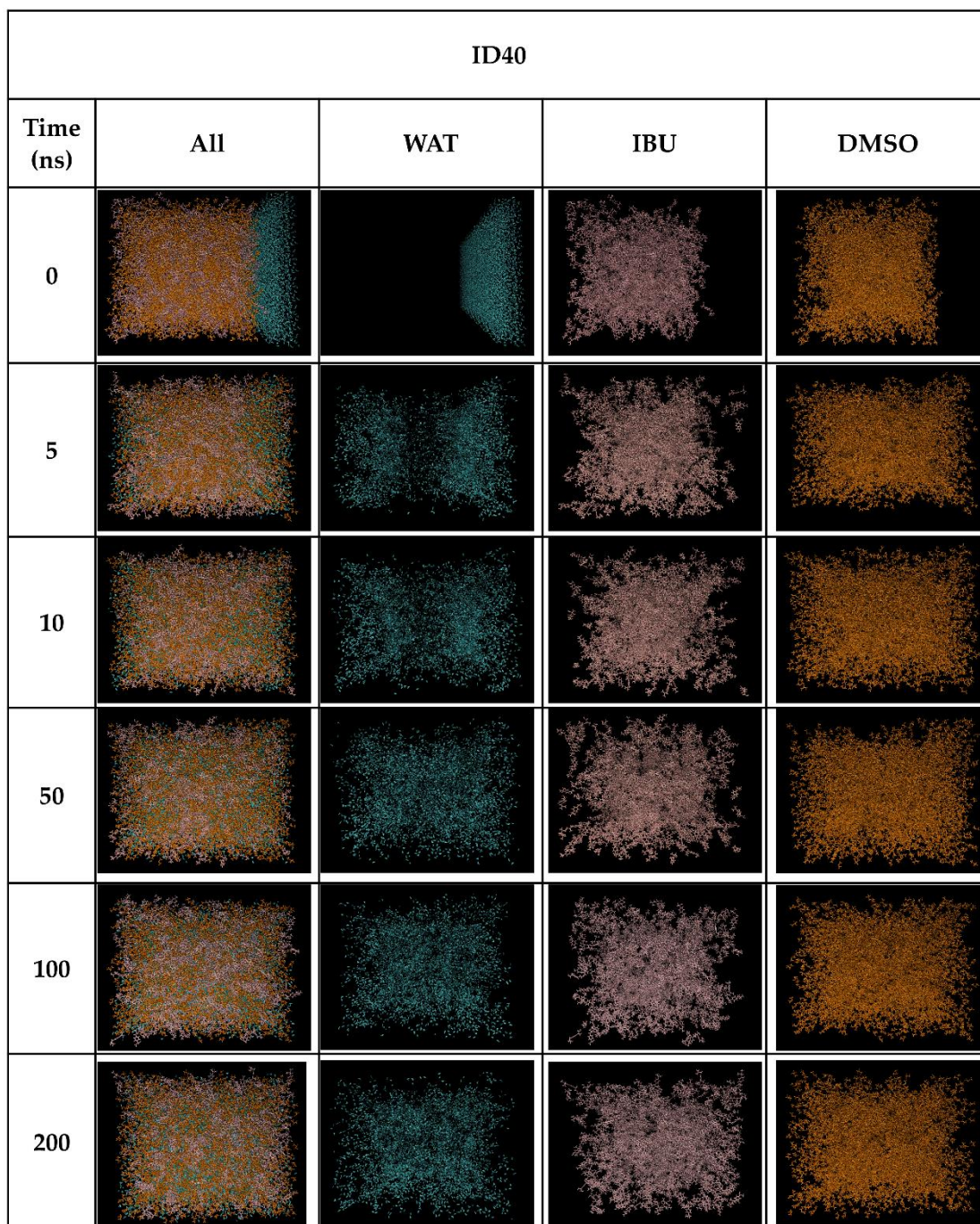


Figure 37 Molecular dynamic modeling of solvent exchange mechanism focusses at interface of each ISMs against aqueous, (DD (A), DN (B), ID40 (C), IN40(D), DID40 (E) and DIN40 (F) after running in MD simulation in 200 ns (continued).

(D)

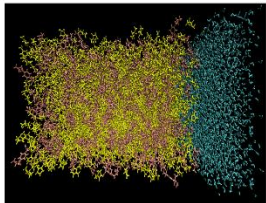
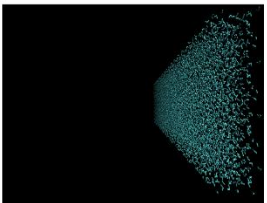
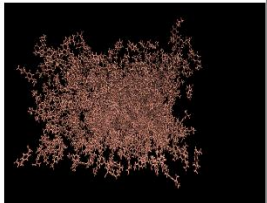
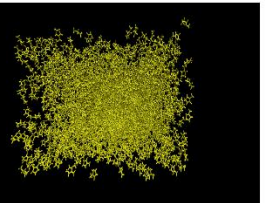
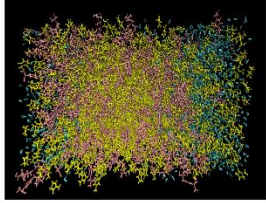
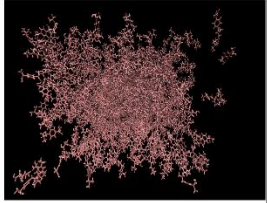
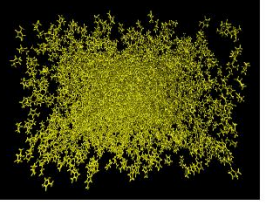
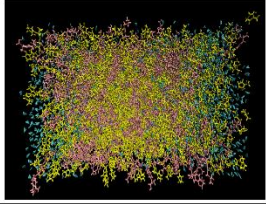
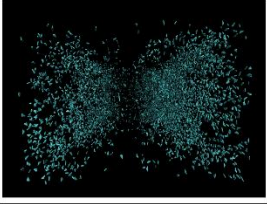
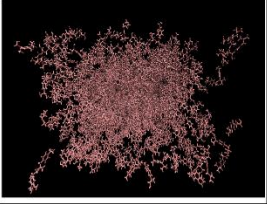
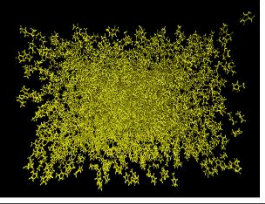
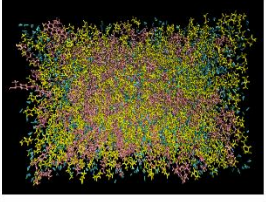
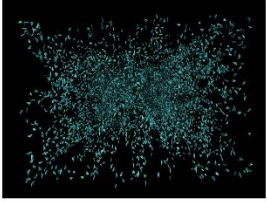
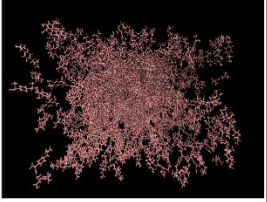
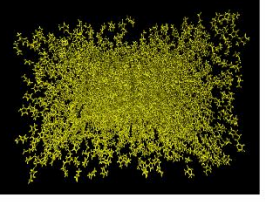
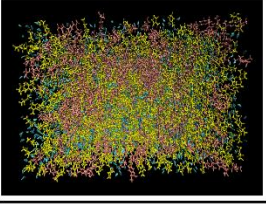
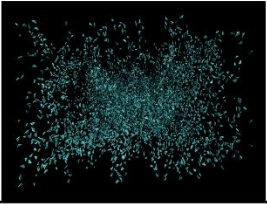
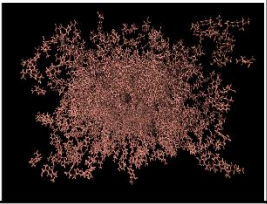
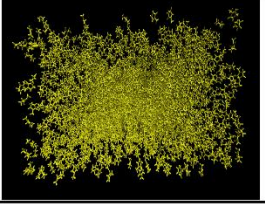
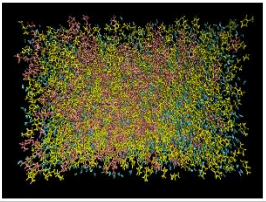
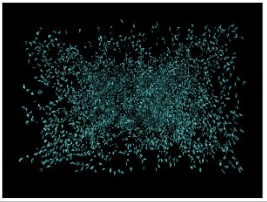
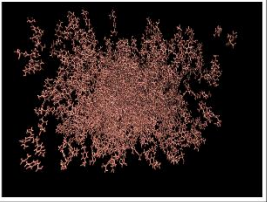
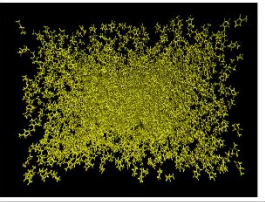
IN40				
Time (ns)	All	WAT	IBU	NMP
0				
5				
10				
50				
100				
200				

Figure 37 Molecular dynamic modeling of solvent exchange mechanism focusses at interface of each ISMs against aqueous, (DD (A), DN (B), ID40 (C), IN40(D), DID40 (E) and DIN40 (F) after running in MD simulation in 200 ns (continued).

(E)

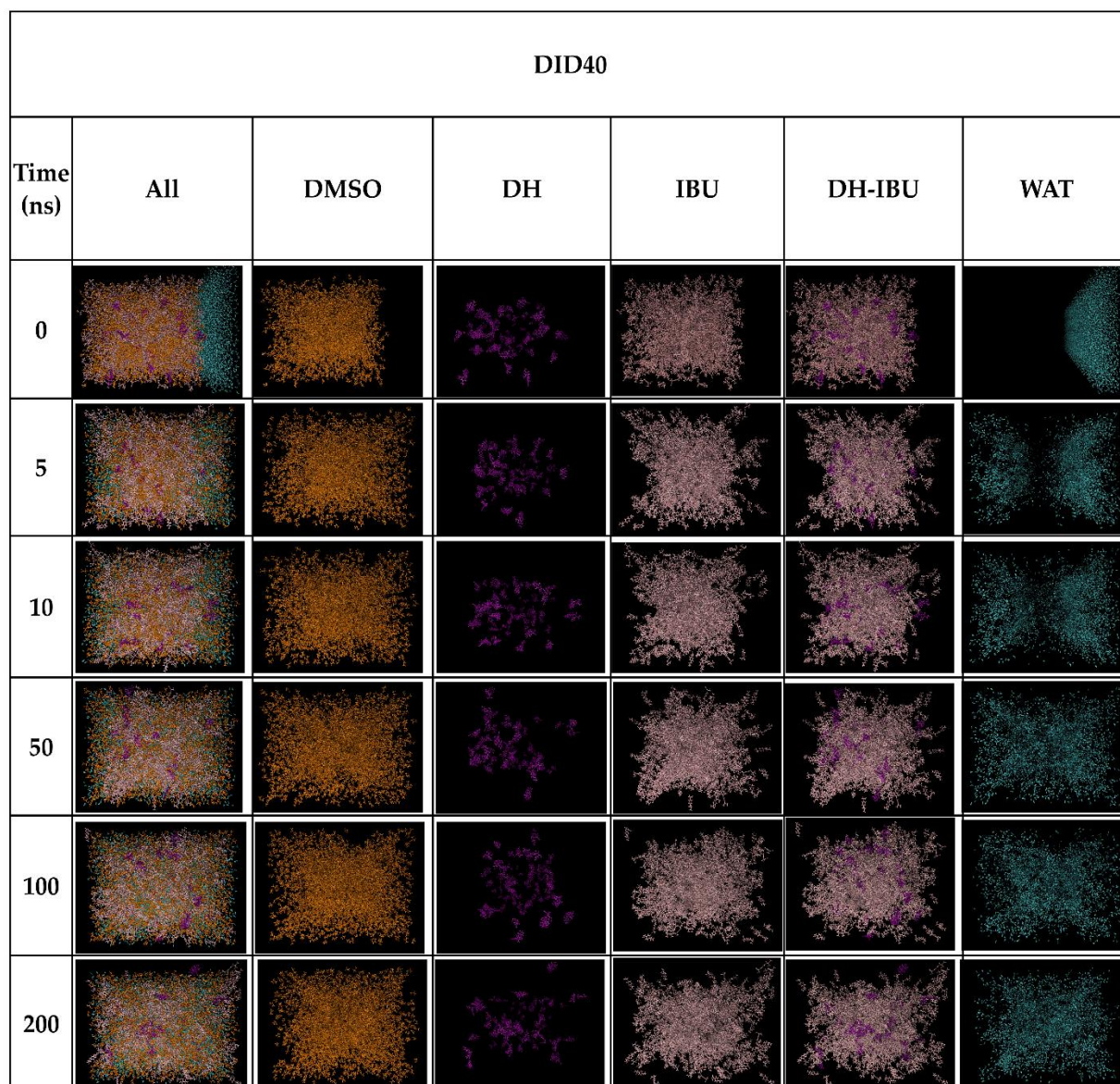


Figure 37 Molecular dynamic modeling of solvent exchange mechanism focusses at interface of each ISMs against aqueous, (DD (A), DN (B), ID40 (C), IN40(D), DID40 (E) and DIN40 (F) after running in MD simulation in 200 ns (continued).

(F)

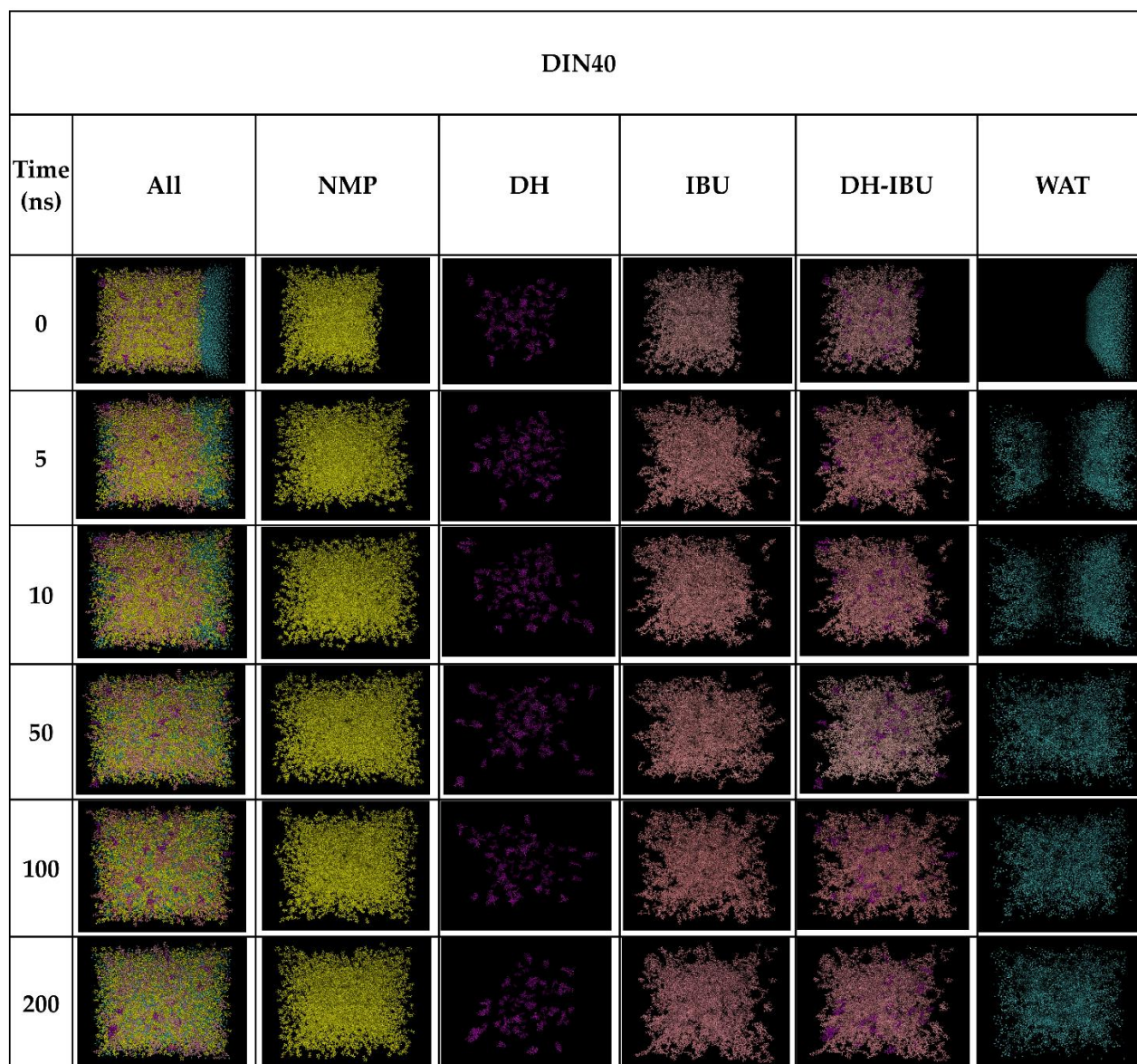


Figure 37 Molecular dynamic modeling of solvent exchange mechanism focusses at interface of each ISMs against aqueous, (DD (A), DN (B), ID40 (C), IN40(D), DID40 (E) and DIN40 (F) after running in MD simulation in 200 ns (continued).

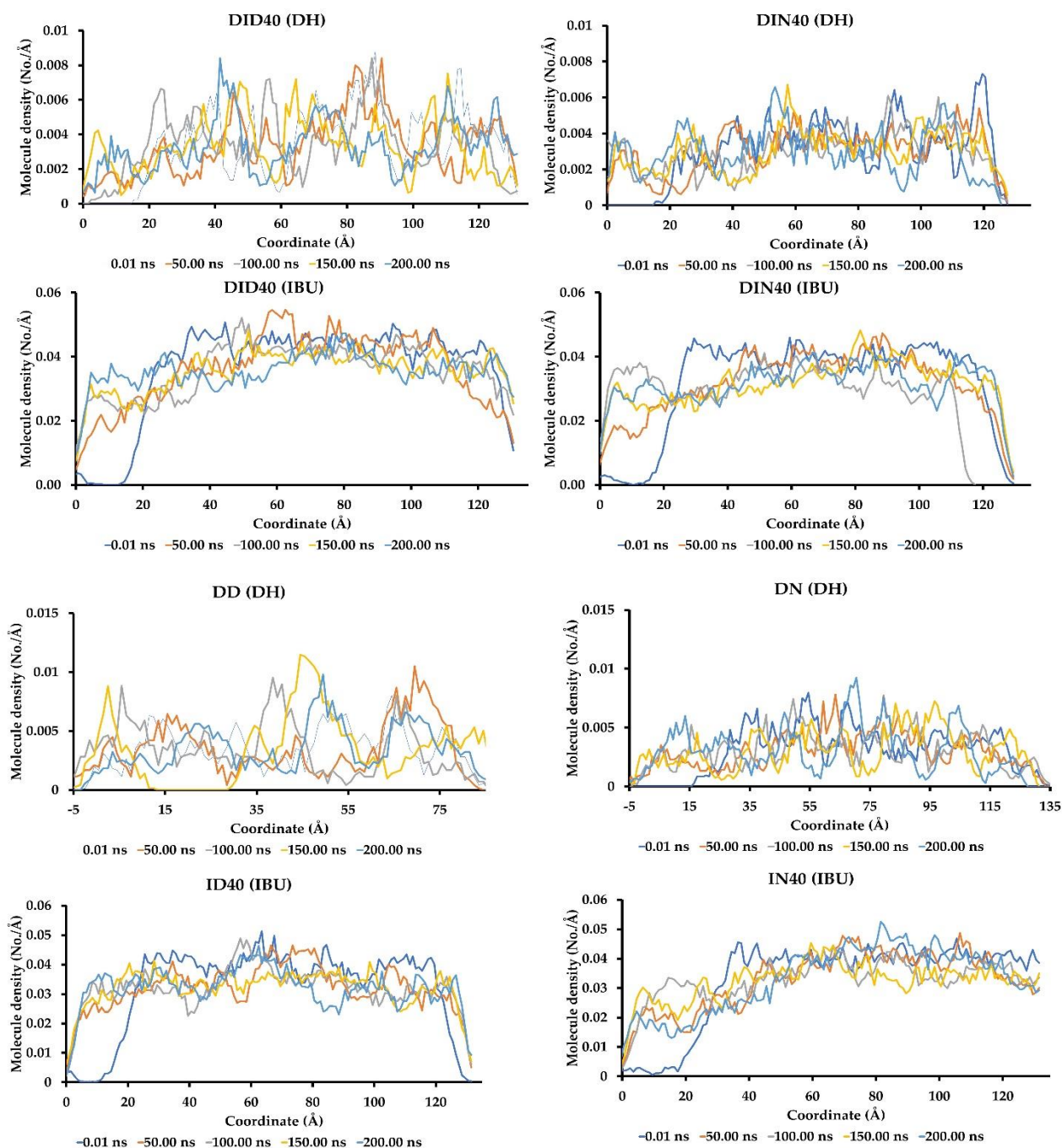


Figure 38 The density profile of IBU and DH molecules in coordinate of different formulation box after contact with water box.

4.2.3.2.1. RMSD and Diffusion constant

The RMSD parameter serves as a crucial insight into molecular fluctuations during the simulation. Throughout the simulation, the RMSD values for each molecular type, as shown in Figure 39, display a consistent pattern. This pattern typically initiates with an initial phase characterized by elevated RMSD values, subsequently transitioning into a plateau phase where the RMSD values either stabilize or exhibit minor fluctuations. Although DH molecules showed unrestricted movement without being fully obstructed by other molecules in the system, the RMSD values of DH in the DMSO system were higher than those in the NMP system. Specifically, the addition of IBU dramatically reduced the RMSD values of DH in both the first and second phases in the DMSO system. In contrast, the NMP series only showed a decrease in the RMSD values of DH during the first phase but no significant difference during the second phase. This indicated that DH movement was effectively restrained by IBU, especially in the DID40 system. The effect of IBU on DH retardation was further supported by analyzing the RMSD values of other molecules. The RMSD values of IBU (Figure 39 (B)) were slightly lower in the NMP series compared to the DMSO series, but both systems showed similar values during the second phase. IBU in the DMSO system exhibited lower mobility compared to the NMP system or showed a higher tendency to pack together more efficiently. The RMSD patterns of organic solvents and WAT molecules (Figure 39 (C) and (D)) were similar in all the formulations. In the DMSO series, the RMSD values demonstrated significantly lower values in the IBU-based ISMs system compared to the system without IBU. In the NMP series, there was a slight decrease in RMSD values after the addition of IBU. The RMSD analysis of aqueous and organic solvent results provides insights into the packing of IBU through aqueous and organic solvent exchanges. Specifically, the DID40 DMSO system and the water molecules showed higher mobility, indicating lower mobility or stronger packing of IBU compared to the DIN40 system, and further contributing to the retardation of DH movement compared to IBU in the DIN40 system. To further investigate the molecular movement and diffusion throughout the phase transformation, diffusion constant values were calculated and are presented in Table 18. The diffusion constants provide information related to the RMSD results. A comparison between the DMSO and NMP systems revealed that the diffusion constants of DH, organic solvents, and WAT in the DMSO system were significantly reduced upon the addition of IBU compared to the NMP system.

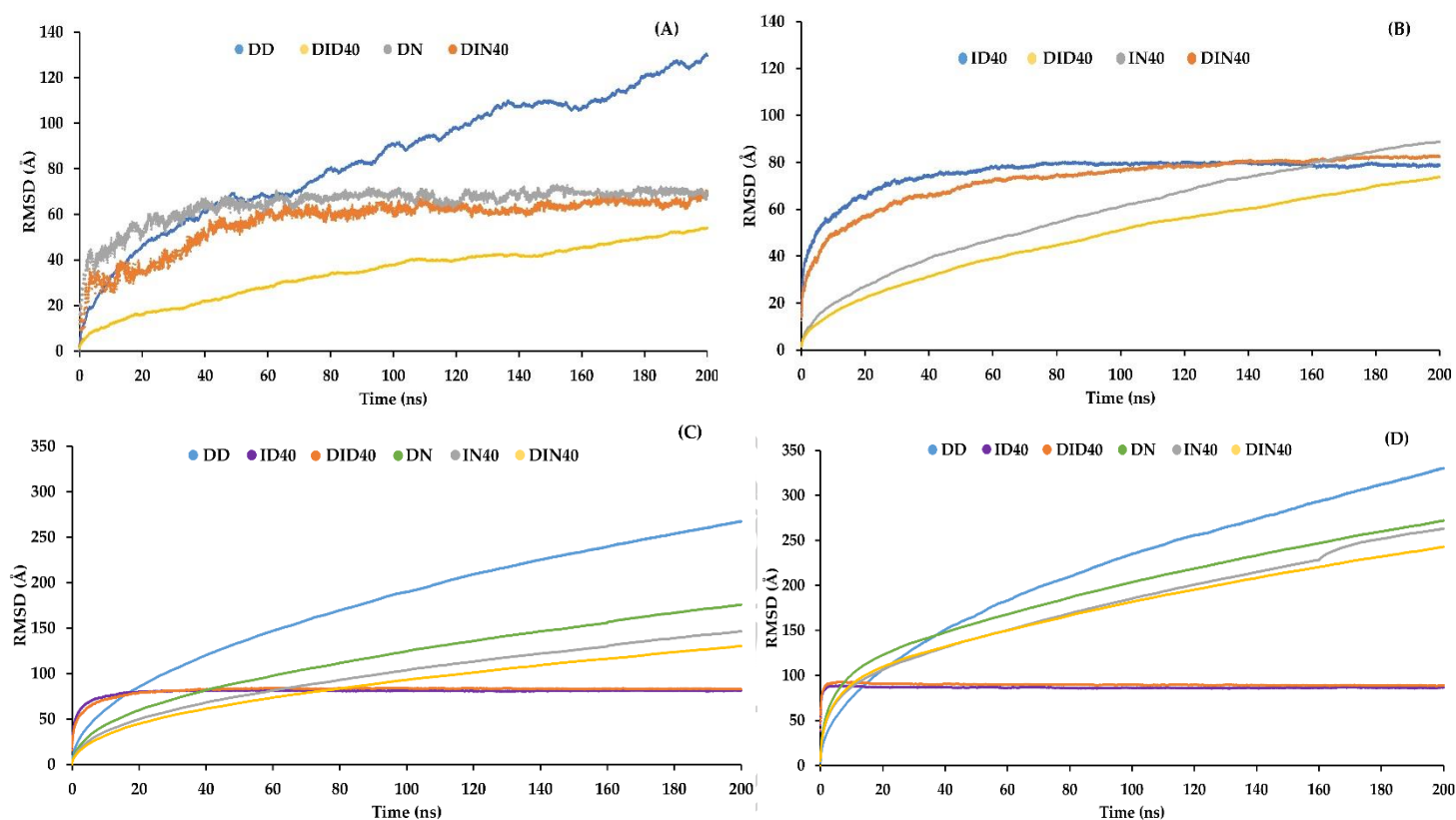


Figure 39 Root mean square deviation (RMSD) of DH (A), IBU (B), organic solvent (C), and (D) water conformation relative to the starting point of the MD simulations box contact with water.

Table 18 Diffusion constant of each substance in formulations box contact with water

Composition	Formulation					
	DD	DN	ID40	IN40	DID40	DIN40
DH	1.2452	0.3703	-	-	0.3372	0.2245
IBU	-	-	1.4820	0.6618	0.7130	0.4529
DMSO	5.8256	-	4.4566	-	2.6423	-
NMP	-	2.1479	-	2.0939	-	1.2914
WAT	9.2859	4.2385	8.1378	4.5864	5.6385	3.7678

4.2.3.2.2. Radius of gyration

The radius of gyration measures the size and structural compactness of molecules. It quantitatively measures the extent to which atoms or residues are distributed from a molecule's center of mass. A reduced radius of gyration signifies more condensed or extended molecular structure, while a larger radius suggests the opposite state [249]. In our study, the Amber program was used to calculate the radius of gyration for atoms or residues in the molecules to providing structural and dynamic information [205]. Our analysis unveiled a noteworthy observation: the introduction of IBU into the system led to a reduction in the size of DH, a phenomenon documented in Figure 40. Moreover, the DMSO series showcased a denser compaction pattern for both DH and IBU compared to the NMP series. This observation underscores the pivotal influence of IBU on retarding DH distribution within the simulation box. Furthermore, it emphasizes how the choice of organic solvent significantly impacts the rate of IBU-induced structural modifications.

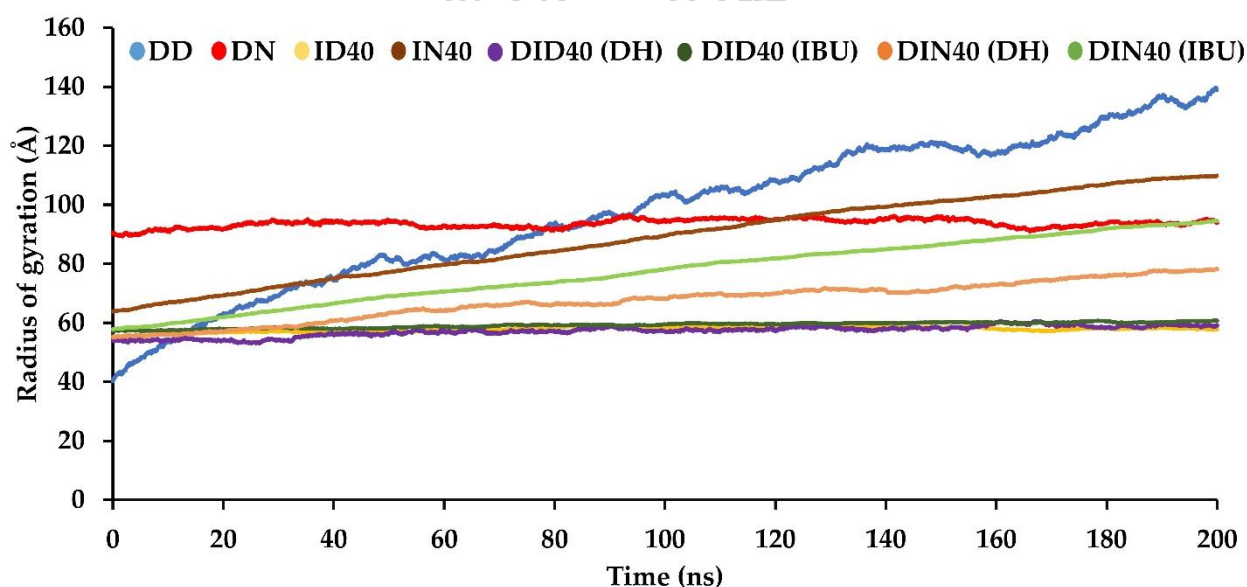


Figure 40 Radius of gyration of DH and IBU conformation of the MD simulations box contact with water.

4.2.3.2.3. Matrix-drug interaction (No. of H-bonding interaction and DH-IBU complex configuration)

Hydrogen bonds play a crucial role in intermolecular chemical bonding and can significantly influence physicochemical processes. The amount of hydrogen bonds was determined using the VMD software based on geometric criteria, including a distance criterion of within 3.5 Å between chosen donor-acceptor pairs and an angle criterion of less than 60° [250, 251]. The number of hydrogen bonds formed between IBU-IBU and DH-IBU was investigated, as shown in Figure 41 (A) and (B), respectively. The IBU-IBU interaction was found to be highest in the DID40 system, followed by the DIN40 system, ID40 system, and IN40 system. Regarding the DH-

IBU interaction, the DID40 system exhibited a higher number of hydrogen bonds compared to the DIN40 system, which was consistent with the RMSD and radius of gyration results, confirming the significant interaction between the IBU structure and DH. The presence of hydrogen bonding was crucial for DH movement facilitated by IBU, as supported by the aforementioned evidence.

The final configuration of the DH-IBU complex in the DID40 system was presented in Figure 42. To facilitate visualization, IBU and DH were depicted as a singular matrix and stick structure, respectively, with different colors representing each element. The configuration of the complex exhibited various formation types, with the majority of them showing interesting intermolecular interactions such as hydrogen bonding (indicated by yellow dotted lines) between the carboxyl group of IBU (red surface of the matrix) and the hydroxyl groups at C3, C10, and C12 of DH. This configuration represented one of the most stable forms of the DH-IBU complex. That might clarify the H-bonding location between DH and IBU that was influenced to the retardation of DH penetration and release out IBU-based ISMs system to the environment.

In summary, the MD simulation provided valuable insights into the behavior of IBU-based ISMs at the interface with the aqueous phase. The simulations demonstrated phase transformation behavior, diffusion patterns of molecules, changes in RMSD values, compactness of molecules, hydrogen bonding interactions, and the configuration of the DH-IBU complex. These findings contributed to a better understanding of the molecular dynamics and interactions within IBU-based ISMs, aiding in the development of controlled drug release systems.

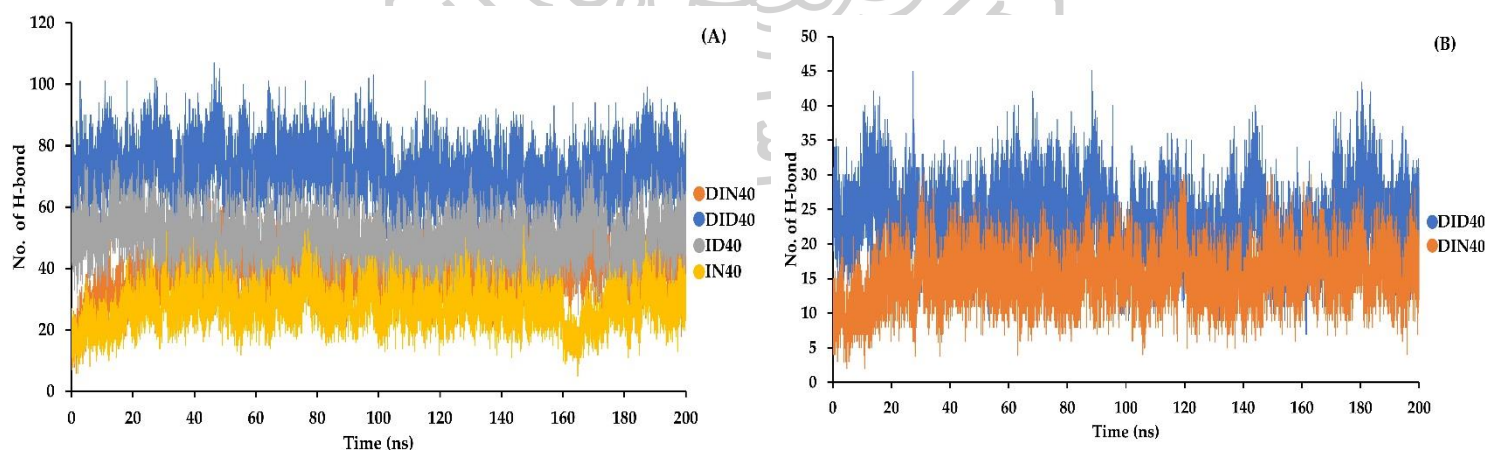


Figure 41 Change in number of hydrogen bonds of IBU-IBU (A) and DH-IBU (B) molecule of formulations box contact with water.

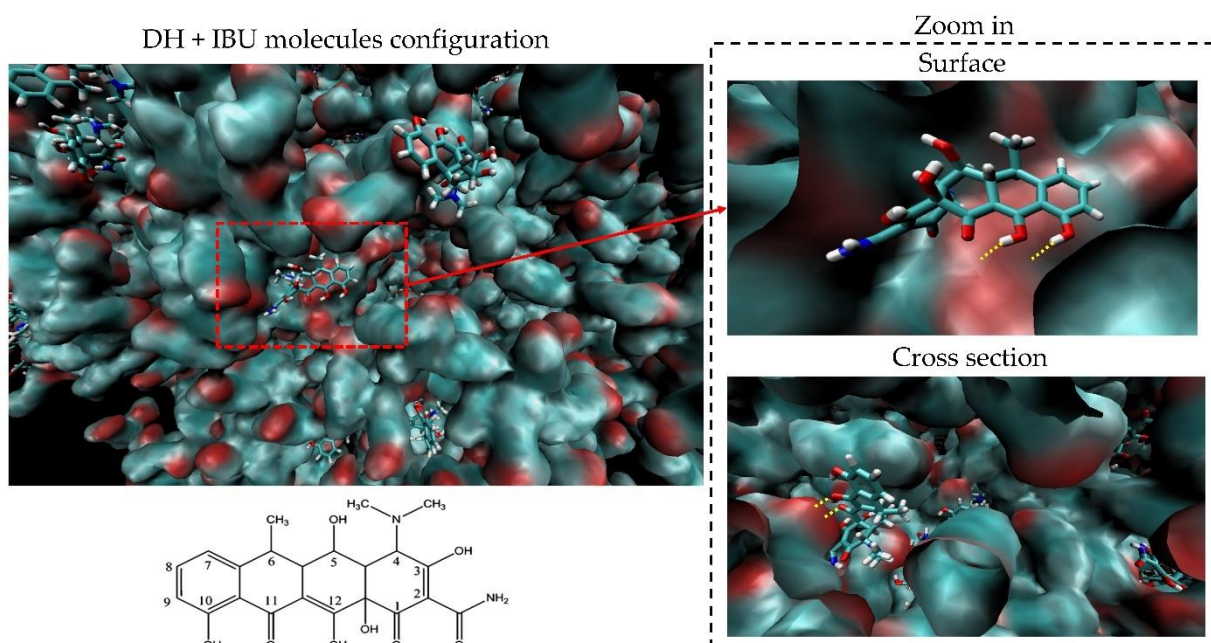


Figure 42 Configuration between DH and IBU at final stage of MD simulation in DID40 systems. (DH and IBU molecules were demonstrated as stick and rough surface mass structure, respectively. Blue, white, red and dark blue structure or surface are represented C, H, O and N atom, respectively. The yellow bond is intermolecular H - bonding of DH and IBU). A 2D-chemical structure represents C atom numbering of DH molecule.

Summary

The comprehensive investigation into the DH and IBU-loaded ISMs has illuminated their multifaceted behavior and potential therapeutic applications. A key highlight is IBU's successful integration as a matrix-forming agent, facilitated by its inherent water insolubility. This integration results in intriguing outcomes that resonate across various facets of these ISMs. Of paramount significance is the profound influence of IBU on the surface properties of the resulting ISM solutions. IBU's addition not only decrease the solution's density but also lowered its surface tension. This significant alteration not only facilitates the injectability of the ISMs but also enhances their spreadability, ensuring effective coverage and adaptation to the treatment site. An intricate aspect of these ISMs is their remarkable water tolerance property. However, a nuanced interplay emerges - while water tolerance exhibits a decreasing trend with increasing IBU content and DH addition, the NMP series showcase superior water tolerance, compared to the DMSO series. This pivotal characteristic underpins the phase transformation mechanism of these ISMs. When exposed to aqueous environments, these systems undergo a remarkable transformation from solution to matrix or matrix-like structures. This transformation is orchestrated by the delicate balance of water influx and solvent efflux, leading to droplet fluctuations around the interface. The subsequent crystallization of IBU within the matrix further substantiates the role of this interplay. A particularly fascinating

outcome emerges in the realm of drug release. Both DH and IBU exhibit sustained release profiles over a week, with DH demonstrating an initial burst release within the first day. The distinct control over DH release in the DMSO series opens avenues for tailored drug delivery strategies. This controlled release mechanism holds significant promise for localized treatment, ensuring prolonged therapeutic effect with minimized side effects. Equally noteworthy is the antimicrobial and anti-inflammatory potential demonstrated by these ISMs. The developed DID40 and DIN40 ISMs showcase substantial efficacy against an array of pathogenic strains, including *S. aureus*, MRSA, *E. coli*, *C. albicans*, *P. gingivalis*, and *A. actinomycetemcomitans*. IBU's inclusion notably enhances the antimicrobial activity, while DH and NMP synergistically contribute to the anti-inflammatory potential. This combination presents a formidable approach for periodontitis treatment, addressing both the infectious and inflammatory aspects of the disease. On the molecular front, MD simulations unveil intricate details of the initial phase transformation at the ISM-aqueous interface. Notably, IBU molecules exhibit a gradual compaction of structures over time. The choice of organic solvent significantly influences this compaction, with DMSO series manifesting a more compact arrangement driven by robust H-bonding interactions. Such computational insights into molecular dynamics provide a deeper understanding of the phase transformation process, drug release mechanisms, and intermolecular interactions within these ISMs. In summation, the DH-loaded IBU-based ISMs exhibit a dynamic interplay of properties with multifaceted potential. Their controlled drug release mechanism, antimicrobial and anti-inflammatory prowess, and molecular insights obtained through MD simulations converge to offer a promising avenue for periodontitis treatment through localized drug delivery. The study's nuanced findings lay the groundwork for advanced therapeutic approaches, setting the stage for further exploration in the realm of bioactive drug delivery systems.

Chapter 5

Conclusion

In the culmination of this thesis, an innovative exploration into the realm of small molecule-based ISMs has unfolded, shedding light on their immense potential in modern drug delivery strategies. Central to this study is the pioneering concept of "active ingredient as excipient," a conceptual framework that has been harnessed to leverage the therapeutic properties of BOR and IBU as matrix-forming agents for localized drug delivery, particularly in the context of periodontitis treatment.

Turning our focus to the LH-loaded BOR-based ISM formulations, BOR, with its natural origin and inherent matrix-forming properties, emerged as a transformative choice. The meticulous selection of solvent ratios, encompassing both DMSO and NMP, in tandem with varying BOR concentrations, orchestrated a significant reduction in viscosity and surface tension. This metamorphosis endowed the formulations with the critical attributes of injectability and uniform distribution upon application, ensuring effective coverage. Delving into the matrix formation process, a symphony of solvent exchange and hydrophobic BOR matrix creation, we unraveled its intricate impact on water tolerance and the overall matrix characteristics. Yet, the introduction of LH into the equation ushered in a fascinating twist. The dynamics of solvent exchange and matrix formation underwent a profound shift, influencing the matrix's properties. At the heart of this transformation lies the BOR matrix, displaying an unprecedented adaptability, conforming elegantly to the anatomical contours of the gum cavity, a crucial factor in enhancing patient comfort. Venturing into the molecular dynamics at the interface, the diffusion studies involving sodium fluorescein unveiled a mesmerizing choreography of water behavior and BOR matrix formation. Here, the LH-loaded ISMs took center stage, showcasing BOR matrices that were both thinner and more heterogeneous, mirroring the observed matrix formation and hardness outcomes. The drug release mechanism, fitting snugly into Fickian diffusion principles, unfolded its narrative, and formulations NBL5 and NBL7.5 emerged as frontrunners. These formulations demonstrated rapid achievement of the minimum inhibitory concentration (MIC) of LH on the first day, followed by an impressive sustained release above MIC for 8 days. The efficacy against a wide spectrum of microbial strains reinforced the formulations' potential as potent localized drug delivery systems for the comprehensive management of periodontitis.

Turning our attention to the DH-loaded IBU-based ISMs, a distinctive narrative emerged. IBU's inherent water insolubility rendered it a formidable matrix-forming agent. The addition of IBU triggered a cascade of transformations, not only lowering density but also significantly reducing surface tension. An intriguing facet surfaced as the DMSO and NMP series deftly balanced water tolerance with phase transformation. Within aqueous environments, the ISMs orchestrated a mesmerizing metamorphosis, transitioning from solution to intricate matrix or matrix structures.

This transformation was a delicate equilibrium between water influx and solvent efflux, culminating in the crystallization of IBU within the matrix. Intriguingly, the drug release profiles of both DH and IBU demonstrated sustained behavior over a week, with DH exhibiting an initial burst release within the first day. This granular control over DH release, particularly evident in the DMSO series, points to tailored drug delivery strategies. This controlled release mechanism not only promises prolonged therapeutic impact but also showcases an avenue for minimizing potential side effects. Remarkably, these ISMs also exhibited remarkable antimicrobial and anti-inflammatory potential. Formulations like DID40 and DIN40 proved their mettle against a range of pathogenic strains, spanning *S. aureus*, MRSA, *E. coli*, *C. albicans*, *P. gingivalis*, and *A. actinomycetemcomitans*. The inclusion of IBU notably heightened antimicrobial activity, while the synergistic interplay of DH and NMP added an anti-inflammatory dimension. This amalgamation presents a potent strategy for combatting the multifaceted challenges of periodontitis.

Moreover, the realm of molecular dynamics simulations has enriched our understanding of the phase transformation process, drug release mechanisms, and intermolecular interactions within these ISMs. The MD simulations have cast a spotlight on the initial phase of phase transformation at the ISM-aqueous interface, unraveling intricate structural changes and molecular dynamics. The choice of organic solvent emerged as a pivotal factor, influencing molecular arrangement and interactions. The MD simulations illuminate the complex interplay of DH and IBU within the ISMs, elucidating their roles in shaping matrix formation, influencing solvent exchange dynamics, and modulating drug release behavior.

In a comprehensive view, this thesis has not only ventured into uncharted territory but has also navigated through it with a blend of experimental and computational insights. The results bring to the fore the intricate interplay of factors influencing drug release kinetics, matrix formation, antimicrobial efficacy, and anti-inflammatory potential, collectively opening doors for innovative periodontitis treatment strategies."

This journey signifies a significant leap in the evolution of bioactive drug delivery systems, paving the way for future research in this transformative field. This amalgamation of empirical exploration and computational elucidation paints a holistic picture, offering both a promising present and a brighter future for localized drug delivery in periodontitis management.

Benefits of this study

This study offers a comprehensive exploration of the intricate realm of ISMs, with a particular focus on small molecule matrix former materials. LH-loaded BOR-based ISM and DH-loaded IBU-based ISM. It commences with meticulous material selection, harnessing active ingredients to serve as both therapeutic agents and excipients. These formulations hold promise for combating periodontitis due to their antimicrobial and anti-inflammatory properties. The research rigorously examines ISM properties, unraveling the mechanisms behind their phase transformation phenomena, which, in turn, enable precise control over drug release in pharmaceutical delivery systems. A pivotal highlight of this investigation is the integration of MD simulations, providing profound insights into the molecular mechanisms governing solvent exchange within ISM systems. By elucidating these mechanisms, this study not only advances our understanding of ISMs but also has the potential to reshape pharmaceutical science. The ramifications extend to the development of tailored drug delivery solutions, propelling advancements in personalized medicine. Furthermore, the use of biodegradable and biocompatible materials aligns with sustainability goals in pharmaceutical research. In essence, this research's impact reverberates well beyond the laboratory, with the potential to revolutionize patient care and the broader pharmaceutical landscape.

Suggestions

For future research endeavors, several promising directions beckon. Firstly, delving into the topography of BOR-ISM interfaces could be achieved through advanced imaging techniques like cryogenic-SEM, offering a closer look at their structural intricacies. Additionally, *in vitro* studies involving cell lines can provide valuable insights into the anti-inflammatory properties of the formulations. Furthermore, to translate these innovative ISMs into practical clinical applications, it would be prudent to embark on animal or *in vivo* studies to confirm their efficacy and safety in a more physiological context. Lastly, employing alternative techniques to confirm the molecular dynamics results obtained through MD simulations could further bolster our understanding of these systems. Such comprehensive investigations would not only validate the findings of this study but also offer new dimensions for enhancing localized drug delivery strategies in the context of periodontitis treatment.

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Appendices

Appendix I chromatogram and calibration curve of LH release from LH-loaded BOR-based ISM

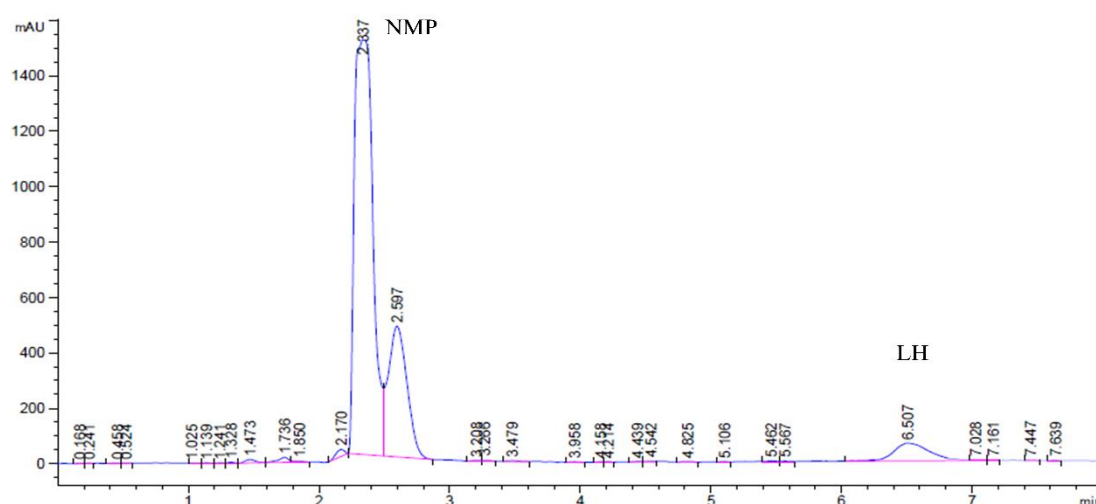


Figure 43 chromatogram of LH release from LH-loaded BOR-based ISM (NBL) (HPLC-UV-vis at 214nm).

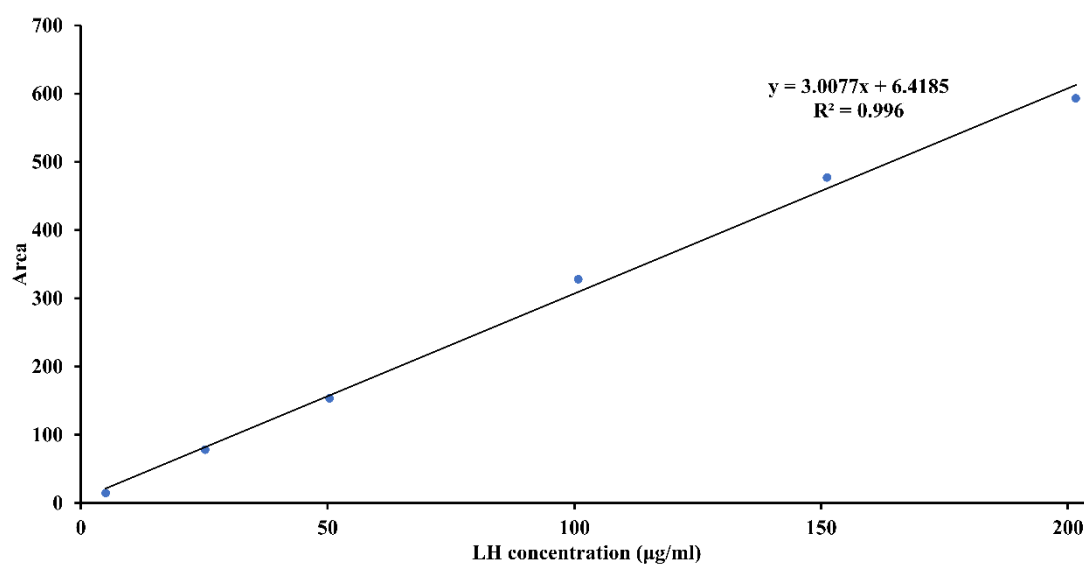


Figure 44 Calibration curve of LH in phosphate buffer pH 6.8 for drug release study.

Appendix II chromatogram and calibration curves of DH and IBU release from DH-loaded IBU-based ISM

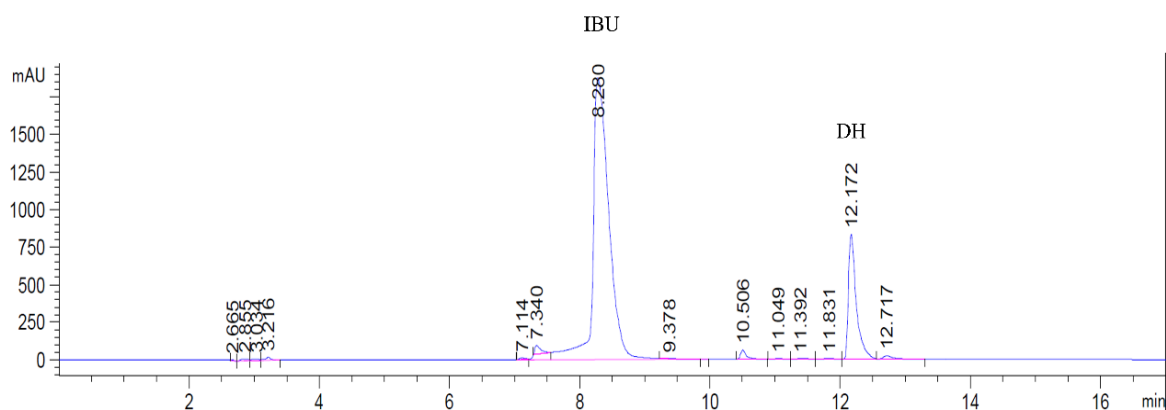


Figure 45 chromatogram of DH and IBU release from DH -loaded IBU-based ISM (DIN40) (HPLC-UV-vis at 264nm).

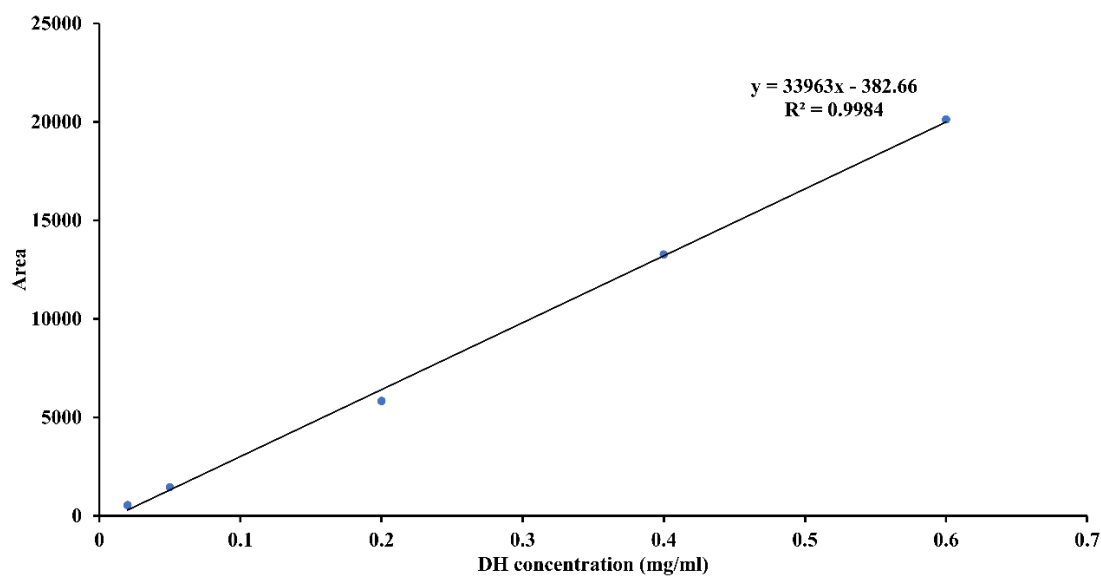


Figure 46 Calibration curve of DH in phosphate buffer pH 6.8 for drug release study.

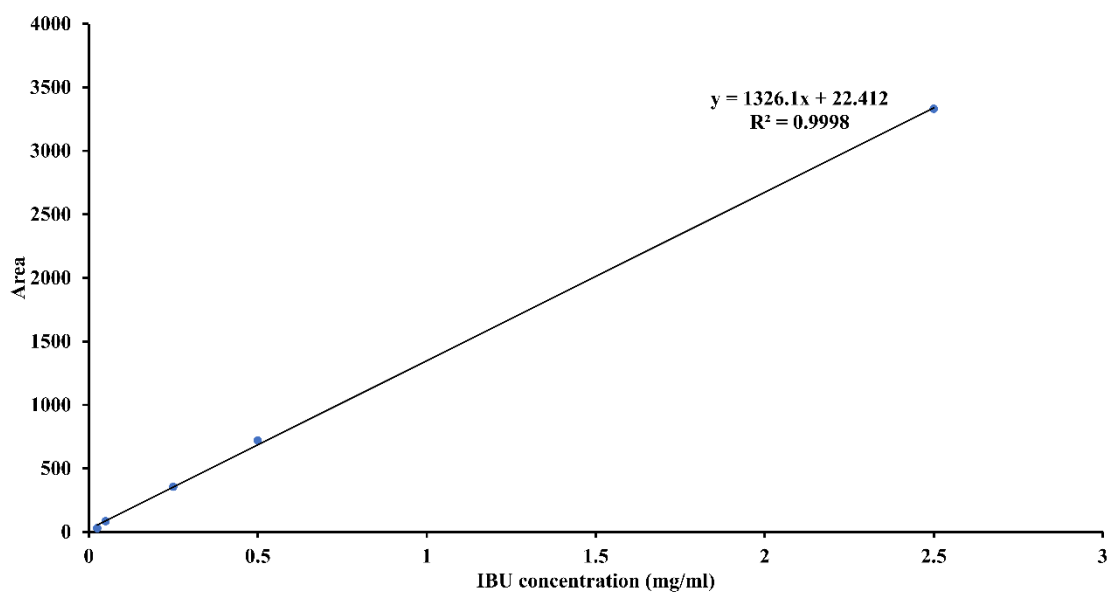


Figure 47 Calibration curve of IBU in phosphate buffer pH 6.8 for drug release study.

Appendix III script for running MD simulation through Amber20 software package.

Force field generation script

```
~/Destop/test_MD/ /home/js3566/amber20/bin/antechamber -I
filename_chage.log -fi gout -o filename.prep -fo prepi -c resp -rn ABC -at
gaff2
```

Simulation box parameter generation script

```
#!/usr/bin/perl

open (INPUTFILE,$ARGV[0]);

$countRes = 0;

while (<INPUTFILE>) {
    ($atom, $num, $type, $res, $resnum, $x, $y, $z) = split(/\s+/);
    if ($resnum != $countRes) {

        printf("%s\n", "TER");
        $countRes = $resnum;
    }
    printf("%s", $_);
}
```

```
}
```

```
close(INPUTFILE);
```

Minimization of solvent molecule in system script

```
minimization
```

```
&cntrl
```

```
  irest=0, ntx=1, ibelly=1,  
  imin=1, ntmin=1,  
  maxcyc=20000, ncyc=10000,  
  ntp=1000, ntwr=5000, ntwx=5000,  
  t=0.0, dt=0.0010, vlimit=20.0,  
  ntt=1,  
  ntb=1, ntp=0,  
  ntc=1, tol=0.0005, ntf=1,  
  cut=12.0,  
  bellymask=":NMP",
```

```
&end
```

Minimization of all molecule in system script

```
minimization
```

```
&cntrl
```

```
  irest=0, ntx=1, ibelly=0,  
  imin=1, ntmin=1,  
  maxcyc=20000, ncyc=10000,  
  ntp=1000, ntwr=5000, ntwx=5000,  
  t=0.0, dt=0.0010, vlimit=20.0,  
  ntt=1,  
  ntb=1, ntp=0,  
  ntc=1, tol=0.0005, ntf=1,  
  cut=12.0,
```

```
&end
```

Heating molecule in system script

```
Heating,
```

```
&cntrl
```

```
  irest=0, ntx=1, 21
```

```
  nstlim=50000,
```

```

ntpr=5000, ntwr=50000, ntwx=50000,
t=0.0, dt=0.0020, vlimit=20.0,
ntt=1, tautp=0.2,
ntb=1, ntp=0, ntc=2, ntf=2,
cut=12.0, iwrap=1, nmropt=1,
&end
&wt type='TEMP0', istep1=0, istep2=50000,
value1=0.0, value2=310.0, /
&wt type='END' /

```

Equilibrating system script

```

equilibrating
&cntrl
imin = 0, irest=1, ntx=5, ntr=0,
nstlim=200000,
ntpr=5000, ntwr=50000, ntwx=50000,
t=0.0, dt=0.0020, vlimit=20.0,
ntt=1, temp0=310.0, tautp=0.5,
ntb=2, ntp=1, ntc=2, ntf=2,
cut=12.0, iwrap=1,
&end

```

Running command script of minimization solvent molecule in system step to equilibrating system step

```

#!/bin/csh -f

set AMBER_DIR = '/home/js3566/amber20/bin'
set BASENAME = 'systemname'

# minimization for MMM only
set FILENAME = '1_minWAT_mon'
set INPCOORD = 'systemname.crd'
${AMBER_DIR}/sander -O \
-i ${FILENAME}.in \
-o ${FILENAME}.out \
-p ${BASENAME}.top \
-c ${INPCOORD} \
-ref ${BASENAME}.crd || goto error

```

minimization for all

```
set FILENAME = '2_minALL_mon'
set INPCOORD = 'systemname.crd'
${AMBER_DIR}/sander -O \
-i ${FILENAME}.in \
-o ${FILENAME}.out \
-p ${BASENAME}.top \
-c ${INPCOORD} \
-ref ${BASENAME}.crd \
-r ${FILENAME}.res || goto error
```

heating

```
set FILENAME = '3_heat_mon'
set INPCOORD = '2_minALL_mon.res'
${AMBER_DIR}/sander -O \
-i ${FILENAME}.in \
-o ${FILENAME}.out \
-p ${BASENAME}.top \
-c ${INPCOORD} \
-ref ${BASENAME}.crd \
-r ${FILENAME}.res \
-x ${FILENAME}.crd \
-e ${FILENAME}.en \
-v ${FILENAME}.vel \
-inf ${FILENAME}.info || goto error
```

equilibration

```
set FILENAME = '4_heat_NPT_mon'
set INPCOORD = '3_heat_mon.res'
${AMBER_DIR}/sander -O \
-i ${FILENAME}.in \
-o ${FILENAME}.out \
-p ${BASENAME}.top \
-c ${INPCOORD} \
-ref ${BASENAME}.crd \
-r ${FILENAME}.res \
-x ${FILENAME}.crd \
-e ${FILENAME}.en \
-v ${FILENAME}.vel \
-inf ${FILENAME}.info || goto error
```

```
echo "No errors reported"
exit(0)
```

```
error:
```

```
echo "--Program failed - fix problem and run this script again"
exit(1)
```

Production MD simulation step script

```
#!/bin/csh -f
set AMBER_DIR = '/home/js3566/amber20'
setenv CUDA_VISIBLE_DEVICES 0
set BASENAME = 'systemname'
set REFCOORD = '4_heat_NPT_mon.crd'
# production run
set LASTNAME = "4_heat_NPT_mon.res"
set NUM = 1
while (${NUM} <= 10)
set NUM = `printf '%02d' ${NUM}`
set FILENAME = "${BASENAME}-MD${NUM}"

cp 5_MD310K_mon.in "${FILENAME}.in"
echo "EQUILIBRATING: ${FILENAME}"
if (-f ${FILENAME}.res) then
set LASTNAME = ${FILENAME}.res
set NUM = `expr ${NUM} + 1`
continue
endif

${AMBER_DIR}/bin/pmemd.cuda -O \
-i ${FILENAME}.in \
-o ${FILENAME}.out \
-p ${BASENAME}.top \
-c ${LASTNAME} \
-r ${FILENAME}.res \
-ref ${REFCOORD} \
-x ${FILENAME}.crd \
-e ${FILENAME}.en \
-v ${FILENAME}.vel \
-inf ${FILENAME}.info || goto error
set LASTNAME = "${FILENAME}.res"
```

```

set NUM = `expr ${NUM} + 1`
end
# END
exit(0)
error:
echo " ... stop : error detected at phase ${BASENAME}"
exit(1)

```

Water box generation script

```

source leaprc.protein.ff14SB
source leaprc.gaff2
source leaprc.water.tip3p 29
loadamberprep DMSO.prepi
loadamberparams DMSO.frcmod
mol = loadpdb DMS_*.pdb
solvatebox mol TIP3PBOX {10 15 1}
addions mol Cl- 0
addions mol Na+ 0
saveAmberParm mol SystemWater.top SystemWater.crd
savepdb mol SystemWater.pdb
quit

```

RMSD analysis script

```

cpptraj filename.top << EOF

trajin filename -MD01.crd
trajin filename -MD02.crd
trajin filename -MD03.crd
trajin filename -MD04.crd
trajin filename -MD05.crd
trajin filename -MD06.crd
trajin filename -MD07.crd
trajin filename -MD08.crd
trajin filename -MD09.crd
trajin filename -MD10.crd

rms :ABC&!(@H=) out rmsd_all_ABC.agr mass

```

Diffusion constant analysis script

```
cpptraj filename.top << EOF
```

```
trajin filename -MD01.crd
trajin filename -MD02.crd
trajin filename -MD03.crd
trajin filename -MD04.crd
trajin filename -MD05.crd
trajin filename -MD06.crd
trajin filename -MD07.crd
trajin filename -MD08.crd
trajin filename -MD09.crd
trajin filename -MD10.crd
reference filename.pdb
```

```
diffusion :ABC&!(@H=) out diff_ABC_all.agr diff_ABC_all diffout
DC_SYSNAME_ABC_all.dat
```

Radius of gyration analysis script

```
cpptraj filename.top << EOF
```

```
trajin filename -MD01.crd
trajin filename -MD02.crd
trajin filename -MD03.crd
trajin filename -MD04.crd
trajin filename -MD05.crd
trajin filename -MD06.crd
trajin filename -MD07.crd
trajin filename -MD08.crd
trajin filename -MD09.crd
trajin filename -MD10.crd
```

```
radgyr :ABC out radgyr_ABC.dat mass
```

VITA

NAME

Napaphol Puyathorn

INSTITUTIONS ATTENDED

2013-2019; Doctor of Pharmacy (Pharm. D.), Faculty of Pharmacy, Silpakorn University, Nakorn Pathom Thailand.

2020-2022: Teacher assistant (TA) in "Food for Health" class at Department of Biopharmacy, Faculty of Pharmacy, Silpakorn University

2021-2022: Teacher assistant (TA) in laboratory class at Department of Pharmaceutical Technology, Faculty of Pharmacy, Silpakorn University

PUBLICATION

Puyathorn N, Lertsuphotvanit N, Chantadee T, Pichayakorn W, Phaechamud T. Lincomycin HCl-Loaded Borneol-Based In Situ Gel for Periodontitis Treatment. Gels. 2023;9(6):495. (impact factor 4.432)

Puyathorn N, Chuenbarn T, Chantadee T, Phaechamud T, Sirirak J. Computational modeling of abietic acid and Eudragit RS as matrix forming agents with antibiotic drugs for in-situ solvent-exchanged inducing phase transition. Mater. Today: Proc. (accepted).

Senarat S, Rojviriyaa C, Puyathorn N, Lertsuphotvanit N, Phaechamud T. Levofloxacin HCl-Incorporated Zein-Based Solvent Removal Phase Inversion In Situ Forming Gel for Periodontitis Treatment. Pharmaceutics. 2023;15:1199. doi:10.3390/pharmaceutics15041199 (impact factor 6.525)

Puyathorn N, Senarat S, Lertsuphotvanit N, Phaechamud T. Physicochemical and Bioactivity Characteristics of Doxycycline Hyclate-Loaded Solvent Removal-Induced Ibuprofen-Based In Situ Forming Gel. Gels. 2023;9:128. doi: 10.3390/gels9020128 (impact factor 4.432)

Puyathorn N, Limsirilak S, Pichayakorn W, Kalkornsurapranee E, Phaechamud T. Natural rubber-based pressure-sensitive adhesive as a drug carrier. Materials Today: Proceedings 2023;75(Supplement):9-14.doi:https://doi.org/10.1016/j.matpr.2022.10.096

Puyathorn N, Sirirak J, Chantadee T, Phaechamud T. Phase separation and intermolecular binding energy of ibuprofen

in some organic solvents. *Materials Today: Proceedings* 2022;65(Part 4):2303-2308.

Puyathorn N, Sukonpan C, Toungsuwan P, Narakornwit W, Phaechamud T. Effect of Arecoline, Aqueous and Methanolic Areca Nut Extracts on Rumen Fluke. *IOP Conf. Series: Mater. Sci. Eng.* 2022;1234:012019. doi:10.1088/1757-899X/1234/1/012019

Puyathorn N, Tamdee P, Sirirak J, Okonogi S, Phaechamud T, Chantadee T. Computational Insight of Phase Transformation and Drug Release Behaviour of Doxycycline-Loaded Ibuprofen-Based In-Situ Forming Gel. *Pharmaceutics*. 2023; 15(9):2315. doi: 10.3390/pharmaceutics15092315 (impact factor 5.4)

AWARD RECEIVED

Outstanding Award in the poster presentation in Chemistry and Pharmacy Sciences research field for the research project titled 'Levofloxacin HCl-loaded in situ forming matrix using myristic acid as matrix forming material for periodontitis treatment' by Chonlakan Fukasem, Nichaphat Twongkrasint, Sunisa Boonchai, Warakorn Thammasut, Napaphol Puyathorn, Setthapong Senarat, Sarun Tuntarawongsa, and Thawatchai Phaechamud presented at the 34th National Conference and the 1st International Conference on ASEAN Sustainable Development (ICASD 2023) at Thaksin University on July 20-21, 2023, at the Crystal Hotel, Songkhla Province.

Conference presentation

Naphapol Puyathorn, Tiraniti Chuenbarn, Takron Chantadee, Thawatchai Phaechamud, Jitnapa Sirirak. Computational modeling of abietic acid and Eudragit RS as matrix forming agents with antibiotic drugs for in-situ solvent-exchanged inducing phase transition. *International Conference and Exhibition on Science, Technology and Engineering of Materials (ISTEM2022)*, (November 29 - December 2, 2022), Nongnooch Garden Pattaya, Chonburi, Thailand. organized by American Ceramic Society: Thailand Chapter (ACerS-Thailand Chapter) committee and Nakhon Pathom Rajabhat University.

Napaphol Puyathorn, Sasiprapa Limsirilak, Wiwat

Pichayakorn, Ekwipoo Kalkornsurapranee, Thawatchai Phaechamud. Natural rubber-based pressure sensitive adhesive as a drug carrier. Conference on advanced materials in environment, energy and health applications. (International conference on advanced materials in environment, energy and health applications (AMEEHA 2022), Pathumwan Princess Hotel, Bangkok, Thailand (3-5 August, 2022)). organized by Department of Chemical Engineering, Khon Kaen University, Thailand and Department of Environmental Sciences, JSS Academy of Higher Education and Research, India.

Napaphol Puyathorn, Jitnapa Sirirak, Takron Chantadee, Thawatchai Phaechamud. Phase separation and intermolecular binding energy of ibuprofen in some organic solvents. The Joint International Conference on Applied Physics and Materials Applications & Applied Magnetism and Ferroelectrics (ICAPMA-JMAG-2021)., 1-4 Dec, 2021).

Naphapol Puyathorn, Chanoporn Sukonpan, Prapansak Toungsuwan, Worrakunya Narakornwit, Thawatchai Phaechamud. Effect of Arecoline, Aqueous and Methanolic Areca Nut Extracts on Rumen Fluke. The International Conference on Materials Research and Innovation (ICMARI 2021). 15-17th Dec, 2021, Chatrium hotel riverside, Bangkok, Thailand.

Wai Wai Lwin, Napaphol Puyathorn, Setthapong Senarat, Jongjan Mahadlek, Thawatchai Phaechamud. Emerging role of polyethylene glycol on doxycycline hyclate-incorporated Eudragit RS in situ forming gel for periodontitis treatment. International Conference on Materials Research and Innovation (ICMARI 2018) 17-21 December, 2018. The emerald hotel, Bangkok, Thailand. p.66.
