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Article *in* Key Engineering Materials · October 2021

DOI: 10.4028/www.scientific.net/KEM.901.55

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Key Engineering Materials
ISSN: 1662-9795, Vol. 901, pp 55-60

Submitted: 2021-03-01
Revised: 2021-04-09
Accepted: 2021-04-12
Online: 2021-10-08

Fabrication and Characterization of Azithromycin-loaded Niosomes for Periodontitis Treatment

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Keywords: niosomes, periodontitis, local drug delivery, intra-periodontal pocket administration

Abstract. Azithromycin (AZM) is a potential drug for periodontitis treatment, but its poor water solubility could be problematic for local delivery to periodontal tissues. Entrapping AZM, which is a hydrophobic drug into niosomes, could effectively deliver drugs to the target site. This study aimed to design and fabricate azithromycin-loaded niosomes (NAZ) with desirable properties for intra-periodontal pocket administration. Span 60 and cholesterol were used to prepare niosomes with modified reverse phase evaporation method. NAZ were characterized and the effects of niosome composition were investigated. *In vitro* release and cell viability were evaluated. The results of this study indicated that with the specific ratio of Span 60 and cholesterol, the particle sizes of niosomes were in nano-sized (319 nm) with optimal zeta potential (-39.57 mV). Controlled release of AZM was achieved with release kinetic followed zero order model. NAZ exhibited low toxicity as cell viability was comparable to negative control.

Introduction

Periodontitis is a bacterial infection disease of tooth supporting structure or the periodontium. The invasion of periodontal pathogens triggers defensive responses of host immunity. Although the host aims to eliminate pathogens, it also causes collateral damage to adjacent periodontal tissues such as connective tissue of gingiva and alveolar bone [1]. Destruction of periodontium initiates periodontal pocket formation. Periodontal pocket is deepened of gingival sulcus due to alveolar bone loss. This unique pathologic feature is unable to maintain health by the patient's self-cleaning leads to disease progression [2]. Severe stage of periodontitis results in multiple teeth loss which affects patient's quality of life.

Several antibiotics have been formulated as the local drug delivery for adjunctive periodontitis treatment. Direct application of drugs to the pocket would reduce undesirable side effects of systemic antibiotics [3]. Azithromycin (AZM) has been used as the adjunctive drug for periodontitis treatment because of its high susceptibility against major periodontal pathogens such as *Aggregatibactor actinomycetemcomitans, Porphyromonas gingivalis, Prevotella intermedia* [4]. However, local delivery of AZM could be problematic because of poor water solubility [5]. This would negatively affect bioavailability of drugs in the periodontal tissues.

Niosomes are bilayer vesicles self-assembled by the nonionic surfactant. Vesicular systems of niosomes are useful to entrap hydrophobic drugs and send to the target site [6]. Loading azithromycin into niosomal vesicles could efficiently deliver drugs to the periodontal tissues. It was discovered that pathogens not only colonize in periodontal pocket but also infiltrate into the connective tissue of gingiva [2]. However, there have been no studies aimed to develop niosomal formulation which deliver AZM to eliminate bacteria residing in the periodontal tissues. Therefore, the objective of this study was to design and fabricate azithromycin-loaded niosomes (NAZ) to achieve desirable properties for periodontitis treatment, evaluate the effect of surfactant and cholesterol to the particle size, zeta potential, entrapment efficiency. Release kinetic and cell viability were investigated by *in vitro* models.

Materials and Methods

Materials. Azithromycin (AZM) was kindly given from M&H manufacturing, Thailand., Span® 60 (S60) and cholesterol (CHL, Sigma-Aldrich, USA), human gingival fibroblasts (HGF, HGF-1, ATCC®, CRL-2014™, USA), Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum (FBS, Gibco, USA), antibiotics and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Bio Basic, Canada) were purchased from various suppliers and used as received.

Preparation of azithromycin-loaded niosomes (NAZ). Niosomes were prepared based on the reverse phase evaporation method [7] with slight modifications. Briefly, the exact amount of AZM, S60, CHL were dissolved with absolute ethanol in a round bottom flask. The ratio of S60 and CHL was varied as shown in Table 1. Sonicator bath was facilitated to obtain a homogenous mixture. Secondary solvent, deionized water, was added and continuously sonicated. After homogeneous emulsion was acquired, ethanol was completely removed by a rotary evaporator. Prepared niosomes were kept at 4°C and allowed for vesicle maturation until the time of experiments.

Determination of particle size and zeta potential. Niosome samples were prepared in triplicate. The particle size, polydispersity index (PDI) and zeta potential of each formulation were measured using the nanoparticle size and zeta potential analyzer (NanoPlus-3®, Particulate systems, USA).

Entrapment efficiency of niosomes. To exclude un-entrapped drugs, the formulation was placed in centrifugal filter units (Amicon® Ultra-4, Merck Millipore, Ireland) with molecular weight cut-off (MWCO) of 3000 kDa and centrifuged at 4000 g for 30 min. Un-entrapped drugs passed through the membrane filter into the filtrate and were quantified by high performance liquid chromatography (HPLC, Shimadzu LC10, Japan). Percent of entrapped drugs were indirectly calculated.

***In vitro* drug release study.** Releasing behavior of the prepared formulations was investigated with Franz cell apparatus with 12 kDa MWCO dialysis membrane (Sigma-Aldrich, USA). The niosomes formulation (1.5 ml) was injected into the upper compartment of the Franz cell above the dialysis membrane. The lower compartment was filled with 11 ml of phosphate buffer solution (PBS) pH 6.8, which was used as a release medium and set up the sink condition. The temperature was maintained at 37 °C. Medium samples contained releasing drugs were collected at time intervals and replaced with the same amount of fresh medium. The amount of AZM released was examined by HPLC. Cumulative drug releasing data were plotted and fitted to various releasing kinetic models.

Cell viability evaluation. HGF were grown in 96-well plates with culture medium consisting of DMEM, 10% FBS, L-glutamine and antibiotics at a density of 10,000 cells/well and incubated until reached confluence. In order to evaluate cytotoxic activity of each formulation, HGF were cultured with AZM, NAZ at 10 ug/ml of drug concentration. MTT assay was performed in quadruplicate. Consequently, percent of cell viability was calculated from the absorbance values examined by the microplate reader.

Table 1. Composition and characteristics of azithromycin-loaded niosomes (NAZ). Data are reported in mean ± S.D.

Formulation code	AZM [%]	S60:CHL [Molar ratio]	Size [nm]	PDI	Zeta [mV]	EE [%]
NAZ1	1	1:1	310.2 ± 5.4	0.198	-27.14 ± 0.64	92.24 ± 0.027
NAZ2	1	1:2	472.9 ± 3.0	0.273	-33.67 ± 0.97	93.79 ± 0.016
NAZ3	1	1:3	803.6 ± 51.6	0.398	-37.99 ± 0.89	94.20 ± 0.001
NAZ4	1	2:1	286.7 ± 13.9	0.285	-38.51 ± 0.26	94.72 ± 0.002
NAZ5	1	2:2	335.9 ± 3.3	0.282	-38.93 ± 0.45	92.70 ± 0.015
NAZ6	1	2:3	606.0 ± 35.9	0.346	-44.34 ± 1.60	94.47 ± 0.002
NAZ7	1	3:1	314.1 ± 1.3	0.271	-30.42 ± 1.00	95.51 ± 0.002
NAZ8	1	3:2	241.1 ± 7.1	0.273	-34.69 ± 0.88	94.54 ± 0.001
NAZ9	1	3:3	319.0 ± 3.3	0.293	-39.57 ± 0.49	94.21 ± 0.001

Results and Discussion

AZM is categorized as Biopharmaceutical Classification System (BCS) Class II due to its low solubility and high permeability [5]. Therefore, an efficient carrier system is needed to deliver AZM to target tissue. Niosomes prepared by the modified reverse phase evaporation in this study appeared in homogeneous white milky suspension. The advantages of this preparation method were reproducibility and simplicity. The formulation can be prepared with common laboratory instruments. The concentration of AZM in niosomal formulations was 1% w/v. Particle sizes of niosomes were in range of 241.1 to 803.6 nm with PDI below 0.4 as present in Table 1. Zeta potentials of all formulations were negative charge. The concentration of surfactant and CHL was found to affect niosomes characteristics. The increment of S60 in the composition was determined to decrease the particle size, while higher concentration of CHL showed a tendency to increase the size of niosomes. The group of formulations containing high concentration ratio of S60 (NAZ7, 8, 9) presented smaller particle sizes (241.1 to 319 nm).

The relationship of particle size and PDI was observed in this study (Fig. 1a). Formulations with larger particle size (NAZ3, 6) presented in higher PDI. Similar to particle size, the increase of CHL influenced the higher PDI value. Interestingly, when the concentration of S60 increased until the ratio of S60 and CHL were equal, PDI value would lower to approximately 0.2 (NAZ1, 5, 9). Zeta potential of prepared niosomes was in the range of -27.14 to -44.34 mV. The more concentration of CHL directly increased negative charge of zeta potential. The prepared niosomes had high zeta potential according to CHL concentration regardless of particle size. Zeta potential of NAZ2 - 9 expressed sufficient charge to prevent aggregation of niosomal vesicles which indicated colloidal stability [8]. However, NAZ1 possessed lower particle charge stability (-27.14 mV). This may be because the low amount of surfactant and CHL, which was noticeable less than the amount of AZM. The forming niosomes may not entrap AZM in the composition, thus the remaining of drugs dispersing in the formulation, which caused charge instability.

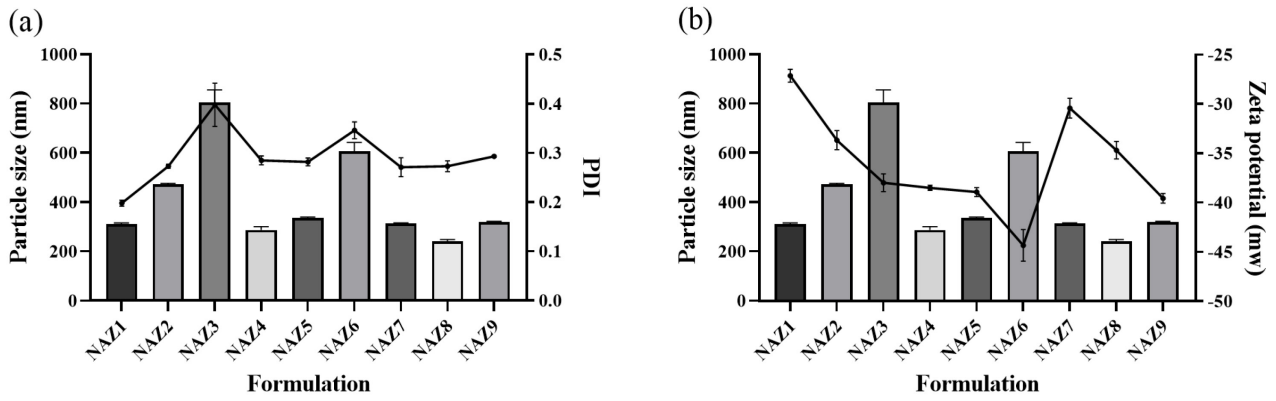

Fig. 1. Niosomes particle size of each formulation related to polydispersity index (a) and zeta potential (b). PDI and zeta potential are displayed as line graphs with scale on the right Y-axis. Data are presented in mean value, error bars signify S.D.

Entrapment efficiency of all niosomal formulations in this study was in the acceptable range of 92.24 to 95.51% (Table 1). However, formulations which included low concentration of S60 and CHL compared to the amount of drug (NAZ1, 2) showed lower %EE value. This was because there were inadequate amounts of forming niosomes to entrap drugs.

NAZ formulations were investigated for releasing characteristics in PBS pH 6.8 to simulate the inflammation environment of the periodontal pocket [9]. To ensure the sink condition, the preliminary study of AZM solubility was performed. It was found that AZM was soluble in PBS pH 6.8, which was in agreement with the other study [10]. As reported by cumulative release graph (Fig. 2), cumulative AZM releases ranged from 39.61 to 50.37% in 8 hours. It was found that at 30 mins, approximately 5% of drug in the formulation was released. This concentration reached above minimum inhibitory concentration (MIC) against major periodontal pathogens [11]. Moreover, the

release of AZM was maintained until 8 hours. This indicated controlled-release behavior of the prepared niosomes [12]. NAZ 4, 7, 8, 9 which yielded smaller particle size (286.7 – 319 nm) tended to exhibit higher drug release rate compared to the larger particle size. The membrane with 12 kDa MWCO used in this experiment allowed the permeation of both free and entrapped AZM. This may imply in clinical situations that free AZM and NAZ would release at the target site, which is the periodontal pocket. When considered in the high entrapment efficiency of this formulation, the abundance of NAZ released from the formulation may enhance the penetration of AZM into the connective tissue of the gingiva.

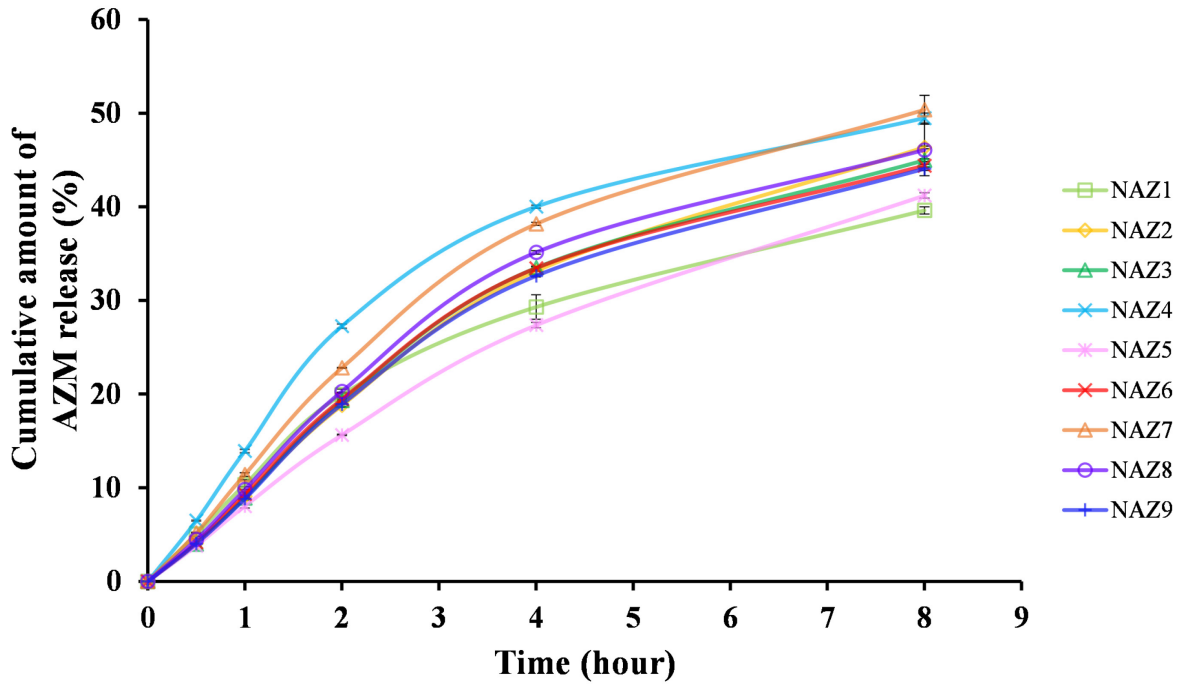

Fig. 2. *In vitro* percent cumulative release of azithromycin-loaded niosomes (NAZ). Data are presented in mean with error bars indicating S.D.

Release mechanisms of prepared niosomes were analyzed by fitting with various kinetic models. All formulations were best associated with zero order release pattern (Table 2). AZM release was independent of drug concentration. In addition, release data was also close to the Higuchi model. This indicated that the fabricated niosomes may function as the matrices and controlled release of entrapped AZM [13].

Table 2. Release kinetic models of azithromycin-loaded niosomes (NAZ), regression coefficient (r^2), equation constants (K).

Formulation code	Zero order		First order		Higuchi		Korsmeyer-Peppas	
	r^2	K	r^2	K	r^2	K	r^2	K
NAZ1	0.9635	7.3005	0.7187	0.3107	0.9552	15.309	0.7891	30.435
NAZ2	0.9946	8.3602	0.7905	0.3348	0.9182	16.918	0.8261	35.099
NAZ3	0.9930	8.4918	0.7942	0.3379	0.9158	17.176	0.8307	35.780
NAZ4	0.9633	10.0200	0.6933	0.3339	0.9511	20.969	0.7969	41.984
NAZ5	0.9938	6.8394	0.7921	0.3156	0.9288	13.925	0.8132	28.500
NAZ6	0.9923	8.4506	0.7834	0.3349	0.9218	17.154	0.8262	35.523
NAZ7	0.9889	9.5957	0.7489	0.3391	0.9318	19.617	0.8185	40.215
NAZ8	0.9931	8.8591	0.7766	0.3371	0.9221	17.979	0.8239	37.174
NAZ9	0.9927	8.2569	0.7971	0.3343	0.9186	16.729	0.8276	34.730

Niosomes of AZM and AZM solution were evaluated for cell viability with HGF. The cell viability was assessed by MTT assay. The prepared niosomes and AZM solution showed cytotoxicity at specific concentration in dose dependent manner. Data from preliminary study indicated that acceptable concentration of AZM on cultured HGF was 10 ug/ml. In addition, the effective dose against periodontal pathogens was not more than 10 ug/ml [11]. Therefore, cell viability of each formulation was then examined at this drug concentration (Fig. 3). When comparing NAZ to negative control of the experiment, it was found that niosomes induced detrimental effects to viability of HGF. There were significant differences between the prepared NAZ and negative control except in NAZ6, 9. Which were found to exhibit high cell viability comparable to negative control.

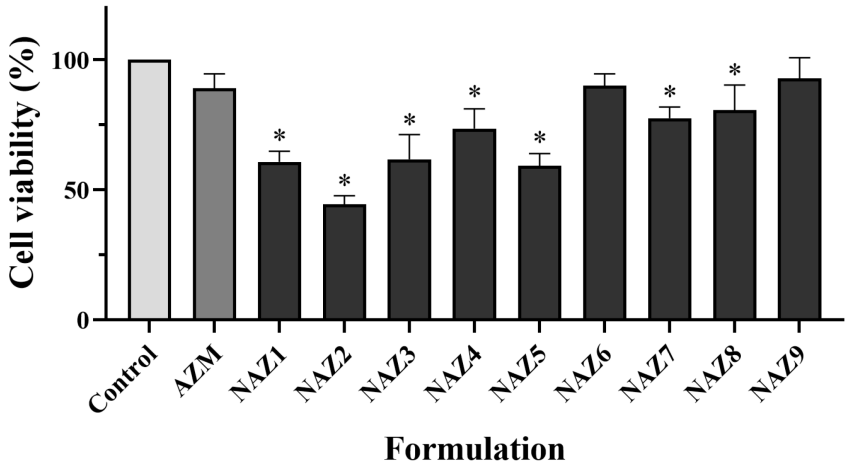

Fig. 3. Cell viability of HGF cultured with NAZ formulations and azithromycin solution (AZM) compared to culture medium (Control), which was served as negative control of the experiment. Statistical analysis was performed with ANOVA and Dunnett's multiple comparisons test. Asterisks indicating statistically significant differences compared to Control ($P < 0.01$)

The outcomes of this pharmaceutical investigation could be used to develop a potential dental material for the treatment of periodontitis. The fabricated NAZ could be further incorporated into vehicle gel as a topical delivery system for periodontal pocket administration.

Summary

NAZ formulations were successfully prepared with the modified reverse phase evaporation method. Concentration ratio of S60 and CHL was found to influence particle size and zeta potential of niosome. The niosomal formulation achieved controlled release and was associated with zero order kinetic model. NAZ9 presented proper characteristics as nano-size particles, charge stability and were proved to present biocompatibility, which could potentially deliver AZM for periodontitis treatment. However, further experiments may need to investigate for its additional efficacy.

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