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Research Article

Microfluidic Preparation of Liposomes Using Ethyl Acetate/*n*-Hexane Solvents as an Alternative to Chloroform

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Although liposomes have been used as a nutrient delivery carrier in the pharmaceutical, cosmetic, and food industries, they still suffer from the critical issue caused by the use of halogenated solvents (e.g., chloroform), which may be harmful to humans. Nonhalogenated solvents have been screened as candidate substitutes for chloroform based on their physicochemical properties. However, none of the candidates examined to date could form stable inverted micelles when used alone. Here, to obtain physicochemical properties similar to chloroform, combined mixtures were prepared using various ratios of each candidate. Based on the results of random combination trials with numerous candidates, ethyl acetate: *n*-hexane = 4 : 1(v/v) was selected as the optimum ratio because it could form stable inverted micelles and a transparent liposome solution without phase separation. The ethyl acetate and *n*-hexane mixture are a potential substitute for chloroform, which may resolve concerns regarding the toxicity of residual halogenated solvents in lipid nanovesicles.

1. Introduction

Lipid nanovesicles have been used extensively in the cosmetics, food, and pharmaceutical industries to increase stability, dispersibility, flavor or taste masking, and bioavailability of functional materials [1]. Their basic structures consist of spherical vesicles with an aqueous core surrounded by a hydrophobic lipid bilayer. Due to their inherent structures, both hydrophobic and hydrophilic bioactive materials can be simultaneously incorporated into a single lipid nanovesicle. This characteristic makes lipid nanovesicles attractive for use in various industries.

Liposomes are the best-studied lipid nanovesicles, with a lipid bilayer formed by phospholipids as the main building blocks [2]. They have also been developed for actual food applications, including encapsulation of iron (ferrous sulfate) [3], lactoferrin [4], and ascorbic acid [5] in milk. Although liposomes have potential for use as delivery systems, there are still some problems associated with the organic solvents used in their preparation [6]. Among the various organic solvents available, chloroform has often been used due to its low boiling point and high evaporation rate. However, it cannot be completely removed from final products by changing manufacturing practices because of physical or chemical barriers. In addition to having no therapeutic value, organic solvents may be associated with chronic health effects, especially halogenated solvents [7], as they affect the central nervous system, kidney, and liver and cause dermatitis and irritation of the skin, eyes, upper respiratory tract, and mucous membranes [8, 9]. In addition, encapsulated products may show accelerated decomposition due to these solvents [10].

To resolve this problem, various approaches have been reported using nonhalogenated and less hazardous solvents,

such as isopropyl alcohol [11] and ethanol [12]. Unfortunately, the methods are restricted to a small scale, such as injection methods, as their nature makes it difficult to evaporate these solvents to establish mass-production systems.

This study was performed to identify a substitute nonhalogenated solvent that could be applied to the production of liposomes from the laboratory scale to the pilot scale. The novel water/oil/water (W/O/W) emulsion method using a microfluidizer [13] was used in this study due to its advantages, including simplicity, low polydispersity, continuous flow of solvent without danger of clogging, suitability for heat-sensitive materials, and ease of scaling up to larger volumes, all of which make it well-suited to many food manufacturing processes. Finally, to assess various applications of liposomes prepared using nonhalogenated solvents, branched-chain amino acids (BCCAs) [14, 15] and curcumin [16, 17] were encapsulated as a hydrophilic material and a hydrophobic material, respectively.

2. Materials and Methods

2.1. Materials. Phosphatidylcholine (soybean lecithin) was purchased from Ilshin Wells (Seoul, Korea). β-Sitosterol (>99.0%), isopropanol, curcumin, and a ninhydrin assay kit were purchased from Sigma-Aldrich Co. (St. Louis, MO). Organic solvents, including chloroform, acetone, ethyl acetate, and *n*-hexane, were purchased from Daejung Chemical & Metals Co. (Siheung, Gyeonggi-do, Korea). Branched-chain amino acids (BCAAs, leucine : isoleucine : valine = 2 : 1 : 1 (w/w/w)) were kindly provided by Daesang Co. (Icheon, Gyeonggi-do, Korea). Ethylene glycol and stannous chloride ($SnCl_2$) were purchased from Acros Organics (Waltham, MA) and Daejung Chemical & Metals Co., respectively. All chemicals except BCAAs (food grade) were of reagent grade.

2.2. Preparation of Liposomes by the W/O/W Emulsion Method Using a Microfluidizer. Figure 1 shows a schematic diagram of the double emulsion (W/O/W) method using a microfluidizer. Lecithin (1 g) and β-sitosterol (0.125 g) were dissolved in 200 mL of each organic solvent and subsequently stirred for 5 minutes to form clear solutions. Then, 100 mL distilled water with BCAAs or curcumin was added to the mixture and sonicated with a 20 kHz probe-type ultrasonicator (ULH-700S; Jeiotech, Korea) for 5 minutes to form inverted micelles; each cycle consisted of 1 second pulse-on and 4 seconds pulse-off with 210 W sonication power at 4°C. Inverted micellar solution was injected into a microfluidizer adjusted to a pressure of 15,000 psi for 10 passage times to form small and even inverted micelles. Samples were retrieved from the microfluidizer after which two volumes of distilled water with 5 g of sucrose were added, and the mixture was sonicated. The microfluidizer was primed with distilled water. The sample mixture was microfluidized by passage five times at a pressure of 5,000 psi. After the microfluidization process, a homogeneous white opaque W/O/W double emulsion was formed with the organic phase filling the small space between the lipid layers. The W/O/W emulsion solutions were placed in glass beakers and magnetically stirred at 400 rpm to evaporate organic solvents. The emulsion solution prepared with chloroform used as the positive control was treated for 24 hours, and the emulsion solutions prepared with combinations of ethyl acetate/*n*-hexane were treated for 48 hours at room temperature. By the time evaporation was complete, the phospholipids had formed a typical liposomal bilayer and the solution was transparent.

2.3. Analysis of Liposomes

2.3.1. Dynamic Light Scattering (DLS). The size distribution and polydispersity index (PdI) of the liposomes were measured using a Zetasizer Nano ZS (Malvern Instruments, Worcestershire, UK). Samples in a volume of 0.5–1.0 mL were applied to disposable plastic cuvettes, and each measurement was conducted in triplicate. Sample measurement conditions were refractive index, 1.330; viscosity, 0.8872 conventional; equilibration time, 1 minute; measurement temperature, 25°C; and measurement angle, 173° backscattering.

2.3.2. Transmission Electron Microscopy (TEM). For visualization using TEM, uranyl acetate was used as a negative staining reagent for the liposomes [6]. The overall procedures were as follows. The vesicle sample (10 μL) was dropped onto a Formvar-coated silicon monoxide grid (200 mesh). After 1 minute, uranyl acetate solution (2%(w/v)) was loaded onto the grid for 1 minute, followed by direct washing of the grid with double-distilled water. The grid was dried completely at ambient temperature before visualization of the vesicles by TEM (120 keV; JEOL Ltd., Tokyo, Japan).

2.3.3. Encapsulation Efficiency

(1) Ninhydrin Analysis. For ninhydrin reagent preparation, ninhydrin powder (200 mg) was dissolved in 7.5 mL ethylene glycol and vortexed for 2 minutes, followed by mixing with 2.5 mL 4N sodium acetate buffer (pH 5.5). Stannous chloride solution was prepared by dissolving $SnCl_2$ powder (50 mg) in 500 μL ethylene glycol. Finally, the stannous chloride solution (250 μL) was mixed with the previous solution just before the reaction [18]. The BCAA sample (20 μL) and ninhydrin reagent (100 μL) were loaded together into 2 mL microtubes and vortexed for 5 seconds. The microtubes were heated in a boiling water bath for 10 minutes, followed by immediate cooling on ice for at least 2 minutes. Then, 1 mL 50% (v/v) ethanol solution was applied to the microtubes and vortexed for 5 seconds. The absorbance of each sample was measured spectrophotometrically at 570 nm using a UV-Vis spectrophotometer (UV-2450; Shimadzu, Kyoto, Japan).

(2) Curcumin Content. The absorbance of each sample was measured spectrophotometrically at 570 nm using a UV-Vis

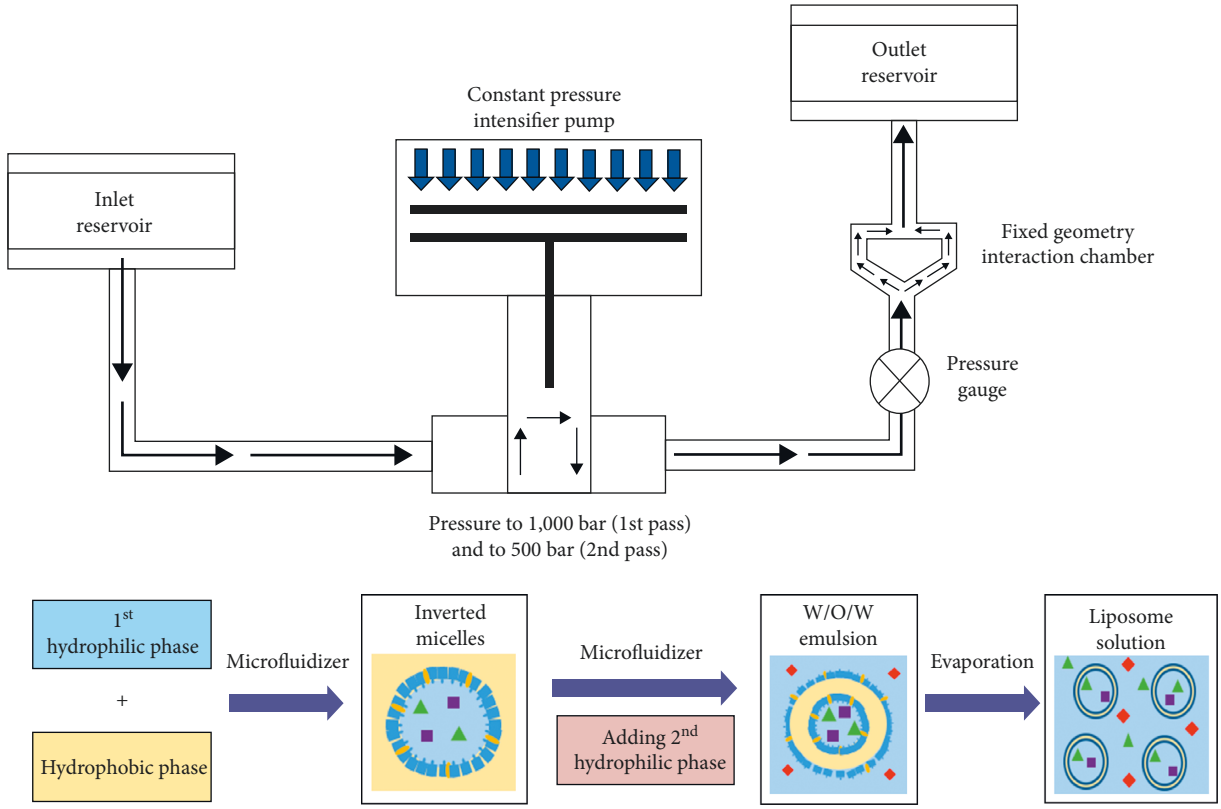


Figure 1: Schematic diagram of the double emulsion (water/oil/water) method using microfluidizer.

spectrophotometer (UV-2450; Shimadzu, Kyoto, Japan), and curcumin content was calculated based on the calibration curve of curcumin in water.

(3) Encapsulation Efficiency. The samples (approximately 3.5 mL) were loaded into ultrafiltration tubes and then centrifuged at 4,000 × g for 10 minutes. The retentant and the filtrate were collected, and their volumes were measured using a pipette. In addition, the material concentration of the retentant and the filtrate was measured using a UV-Vis spectrophotometer after treatment with Triton X-100. The encapsulation efficiency (%) was calculated using the following equation:

$$\text{encapsulation efficiency (EE)} = 100 \times \frac{\text{total material} - \text{free material}}{\text{total material}}, \quad (1)$$

where the total material is the sum of the material in the retentant and the filtrate and the free material is the amount of material in the filtrate.

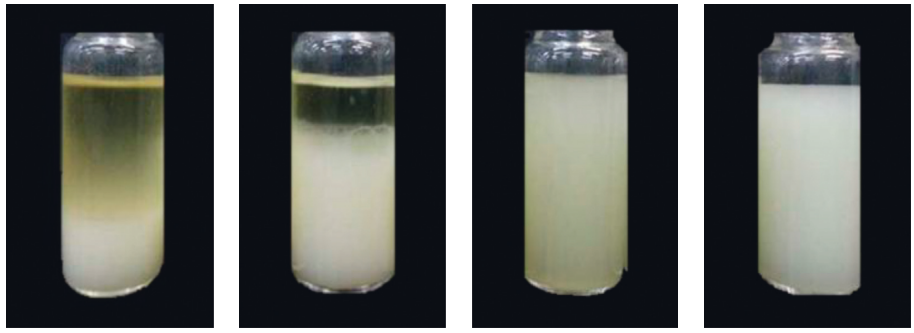
3. Results and Discussion

3.1. Selection of Nonhalogenated Solvents. Solvents were first selected from the nonhalogenated solvents that are frequently used for extraction of functional materials. They were then filtered out according to the permissible daily exposure limit, as determined by the Ministry of Food and Drug Safety (MFDS) guidelines, in milligrams. Finally, candidates (acetone, isopropyl alcohol, *n*-hexane, and ethyl acetate) were selected based on having solvent properties similar to chloroform, including high polarity and high vapor pressure. The availability of these candidates to liposomes was assessed by examining whether phase separation occurred at the stage of inverted micelle production for 24 hours at room temperature.

When *n*-hexane and ethyl acetate were used as solvents, respectively, the inverted micelle phases immediately separated (within 1 hour) into micelle-poor and micelle-rich phases that formed densely packed aggregates, and most micelles were destroyed (Figure 2) as *n*-hexane has lower polarity (polarity index = 0.1) and ethyl acetate higher solubility in water (8.7 g/100 mL) than chloroform (polarity index = 4.1 and solubility in water = 0.81 g/100 mL). When isopropyl alcohol and acetone were used as solvents, they were found to be miscible in water. This property prevented them forming a lipid bilayer for W/O/W emulsion in the second stage and dispersal like micelles.

To obtain the desired physicochemical properties, ethyl acetate and *n*-hexane were combined in various ratios based on the properties of the solvents (Table 1). Ethyl acetate: *n*-hexane = 4 : 1 (v/v) was selected as the optimum ratio because it formed stable inverted micelles without phase separation for 24 hours and produced the most transparent liposome solution (Figure 3).

3.2. Preparation of Liposomes Loaded with Hydrophilic/Hydrophobic Materials. Liposomes prepared by the double emulsion method using the mixed solvent to dissolve



(a) (b) (c) (d)

Figure 2: Inverted micelles prepared using nonhalogenated solvents to dissolve phospholipids. (a) *n*-hexane; (b) ethyl acetate; (c) acetone; (d) isopropyl alcohol.

Table 1: Physicochemical properties of chloroform and nonhalogenated solvents.

	Chloroform	*n*-Hexane	Ethyl acetate	Acetone	Isopropanol	Methanol	Diethyl ether	Methyl acetate	Benzene
Polarity index	4.10	0.10	4.40	5.10	3.90	5.10	2.80	4.40	3.00
Solubility in water (g/100 mL)	0.82	0.00	8.70	Miscible	Miscible	Miscible	6.90	24.40	0.18
Viscosity (cP)	0.57	0.33	0.45	0.32	2.30	0.55	0.22	0.36	0.61
Vapor pressure (kPa)	25.90	17.60	9.73	30.60	6.02	13.02	58.66	23.06	12.70
Lecithin solubility	S	S	S	S	S	I	S	I	S
Allowable concentration (g/kg)	Prohibited	0.01	0.05	0.03	0.05	0.05	Prohibition	Prohibition	Prohibition

S, soluble; I, insoluble.

Figure 3: Inverted micelles (a) (layer separation) and liposomes (b) (transparency) prepared using mixtures with different volumetric ratios of ethyl acetate to *n*-hexane.

lipid loaded with the hydrophilic material, BCAA, were small and monodispersed (PdI = 0.177 and diameter = 98.68 nm). TEM was used to characterize the morphology of the liposomes. The size of the liposomes was about 100 nm, which was consistent with the value determined by DLS (Figure 4). The image shows that liposomal bilayers formed from the double emulsions by solvent evaporation. However, the encapsulation efficiency of liposomes prepared using mixed solvents (23.12%) was lower than that of liposomes prepared using chloroform (42.40%). As mixed solvents have lower vapor pressure, they require a longer evaporation time than that of chloroform (48 hours vs. 24 hours, respectively). Prepared using the same method, liposomes loaded with the hydrophobic material, curcumin, were also small and monodispersed (PdI = 0.119 and diameter = 88.1 nm). TEM analysis showed the size of the liposomes to be about 100 nm, which was consistent with the value determined by DLS (Figure 5). The morphology of liposomes with curcumin produced from the double emulsion solution was similar to that of the liposomes with the hydrophilic material. In contrast to the hydrophilic material, the encapsulation efficiency of the curcumin-loaded liposomes prepared using mixed solvents (98.0%) was similar to that of liposomes prepared using chloroform (99.0%). These observations implied that

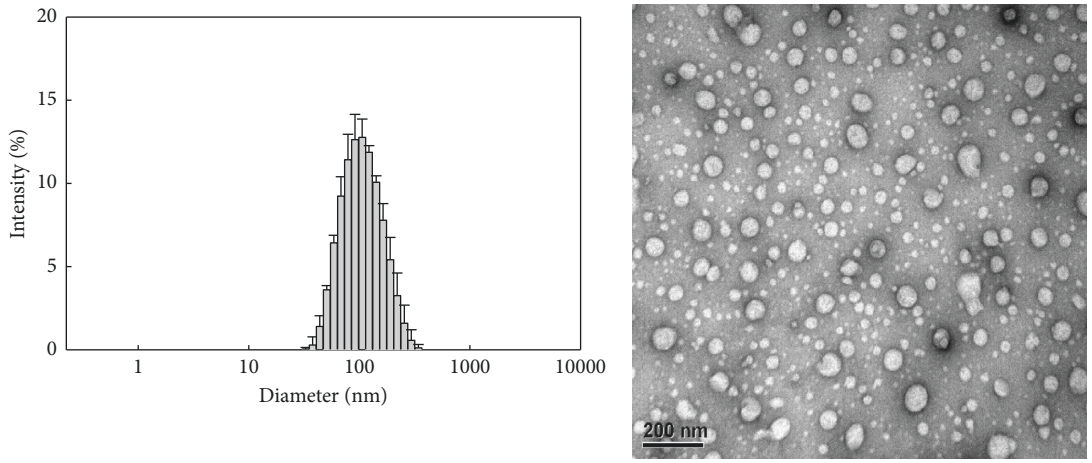


Figure 4: Size distribution (a) and TEM visualization (b) of BCAA-loaded liposomes prepared using nonhalogenated solvent (ethyl acetate : *n*-hexane = 4 : 1 (v/v)).

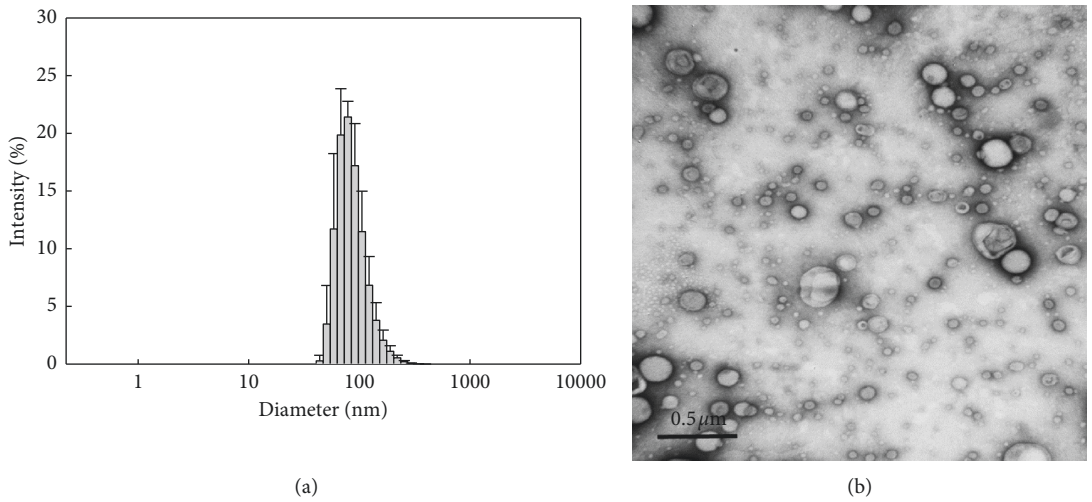


Figure 5: Size distribution (a) and TEM visualization (b) of curcumin-loaded liposomes prepared using nonhalogenated solvent (ethyl acetate : *n*-hexane = 4 : 1(v/v)).

hydrophobic materials are less affected by evaporation time than that of hydrophilic materials.

4. Conclusion

Candidate solvents (acetone, isopropyl alcohol, *n*-hexane, and ethyl acetate) were selected from among food-compatible and nonhalogenated solvents based on their solvent properties similar to those of chloroform, such as high polarity and high vapor pressure. Individual nonhalogenate solvents did not form stable inverted micelles when used alone. To obtain the desired physicochemical properties, ethyl acetate and *n*-hexane were combined in various ratios. A ratio of ethyl acetate: *n*-hexane = 4 : 1(v/v) was selected because it formed stable inverted micelles without phase separation. This study implies that combinations of nonhalogenated solvents could be promising substitutes for chloroform, which may be harmful to human health.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Eunhye Yang and Hyunjong Yu contributed equally to this work.

Acknowledgments

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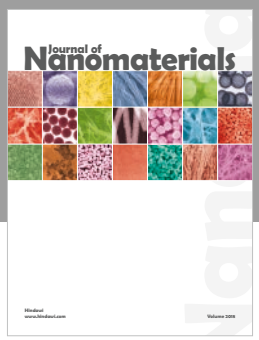

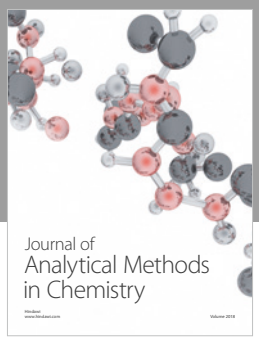

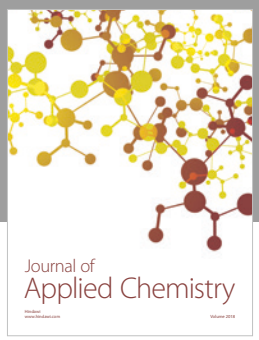

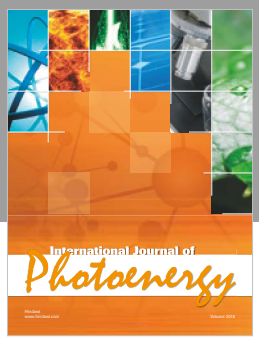

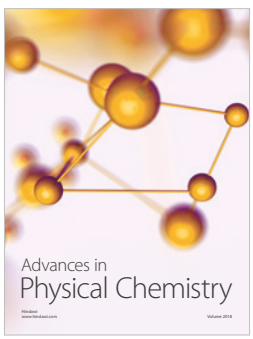

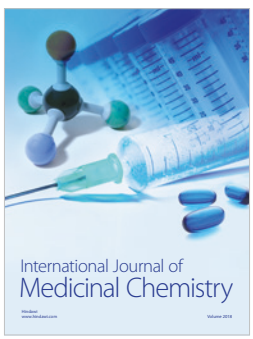

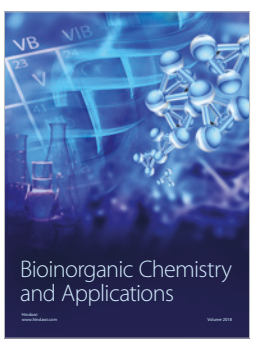

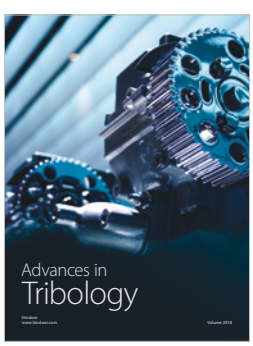

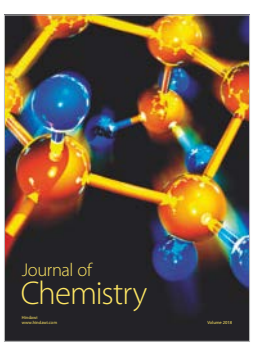

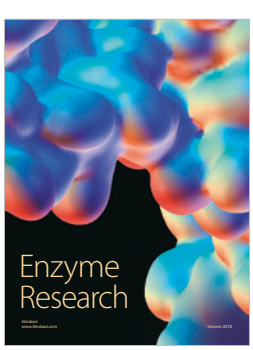

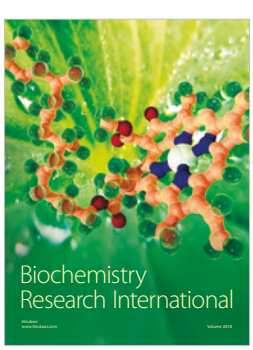

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