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Self-regulating naturally occurring microcapsules for controlled release of Levodopa

Shwan Abdullah Hamad[1]

1 *Department of Chemistry, College of Science, University of Sulaimani, Raparin Street, Sulaimaniyah, Kurdistan Region of Iraq*
Emai: shwan.hamad@univsul.edu.iq

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Abstract

Levodopa remains the gold standard treatment for patients suffering with Parkinson's disease. Patients can become sensitive to fluctuations in plasma concentration of the drug, resulting in devastating side effects. This present paper reports the encapsulation of LV into sporopollenin exine microcapsules prepared in-house from *Lycopodium Clavatum* plant. The loaded sporopollenin exine microcapsules were prepared by removing the cellulosic intine, and then filling the internal cavity surrounded by the sporopollenin exine with a concentrated solution of LV. The trapped drug was then precipitated inside the microcapsules using pH change, followed by a thorough washing. The released amount of the drug in aqueous media resembling physiological conditions of human blood plasma was then quantified using UV-Vis spectrophotometer. The results showed that LV can be successfully encapsulated within the sporopollenin exine microcapsules, and a sustained release of the drug can be achieved. This paper also shows that the microcapsules loaded with the drug can act as a self-regulating device to release the drug in aqueous medium over a longer period.

Introduction

Parkinson's disease which occurs because of dopamine deficiency in the brain [1] is incurable, but there are various therapies able to offer symptomatic relief. The gold standard of therapy is LV, which at some point during the progression of the disease, all patients suffering from Parkinson's disease will eventually undergoes its treatment [2]. LV, chemically known as 3, 4-dihydroxy-L-phenylalanine, is a precursor to the neurotransmitter dopamine which was finally recognised in 1967 as truly beneficial in the treatment of Parkinson's disease despite earlier works [3]. Following oral administration LV is rapidly decarboxylated by the enzyme AAAD to dopamine. Nutt and Fellman found that 95 % of a LV oral dose was decarboxylated peripherally, and only 1 % of the dose actually entered the brain [4].

Recently the importance of the nasal cavity in drug delivery has been realised owing to the direct transport of the drug into the systemic circulation through the highly vascularised subepithelial layer [5]. Chao *et. al*. [6] also found that following the nasal administration of water-soluble LV prodrug (butylester LV (BLED)) to rats, the bioavailability of LV increased by approximately 90 %. Biocompatible polymers have become more popular in the field of controlled delivery of active ingredient and especially therapeutic drugs [7] and [8]. One method is using microcapsules made of biocompatible polymers to encapsulate LV. Arica *et al*. [7] prepared two separate microspheres using solvent extraction technique to yield microspheres of 20-40 µm in diameter loaded with LV, whereas D Aurizio *et al*. [8] used o/w emulsion/solvent evaporation

technique to prepare microcapsules loaded with antiparkinson prodrug containing LV and natural lipoic acid moiety (LD-LA).

An ideal formulation would immediately release the drug and continues to sustain the plasma concentration over an extended period. For this purpose, using sporopollenin exine microcapsules to encapsulate and deliver the drug would be an effective method to increase its bioavailability. Sporopollenin exine microcapsules can be prepared from a spore-bearing evergreen club moss such as *Lycopodium Clavatum*, which has spores shaped like a three-sided pyramid with a rounded triangular distal face [9] and is regarded as reticulate [10]. Sporopollenin shows extraordinary resistance [12] with intact spores being found in sedimentary rock at least 500 million years old. Sporopollenin is a macromolecule composed of chains of small aliphatic organic monomers [13] and [14]. Studies show that, it is not a polymer of carotenoids and carotenoid esters as previously believed [15]. It is a biopolymer with a high proportion of carboxylic acid groups, unsaturated carbon chains, and ether bonds [15] and [11].

Sporopollenin can differ in chemical species, but Wittborn *et al*. did find similarities on a nanometric scale between the subunits of Fagus pollen grains and *Lycopodium* spores [9]. *Lycopodium Clavatum* has a continuous exine shell with nanochannels up to 40 nm in diameter, and is particularly robust. The genetic material contained within the spore can be removed through these nano-sized channels and replaced with numerous active ingredients [16]. Hamad *et al*. encapsulated living cells in sporopollenin microcapsules by forcing the trilite scars of the microcapsules to open up, by compressing the sporopollenin powder into a pellet, thus allowing the living cells to penetrate into inside the hollow capsules [17]. Although highly resistant to chemical attack, it has been shown that sporopollenin exines can be degraded in the blood stream of the circulatory system [18]; thus enabling the release of any encapsulated material, this is of great importance for the delivery of drugs. In 2009 Atkin *et al.* extracted and used Sporopollenin exines from the spores of the plant *Lycopodium clavatum* to encapsulate water, sunflower oil, and differing amounts of cod liver oil [19]. Since sporopollenin is a cheap, non-toxic, and non-allergic material; it is also capable to cross the gut wall largely intact, the microcapsules are an ideal candidate for the encapsulation and targeted delivery of LV [20]. Akyuz *et al*. used SEM and FT-IR to study panoparzole loading into sporopollenin microcapsules where high loading efficiency and drug stability were confirmed [21]. Sargin *et al.* loaded cancer therapeutic agent (imatinibmesylate) into sporopollenin microcapsules and found that the encapsulation efficiency is 21.46 %; release behaviour of the drug from microcapsules is biphasic, with an initial faster release followed by a slower rate of release [22]. Kaya *et al.* used sporopollenin microcages from the pollens of *Platanusorientalis* to encapsulate Paracetamol, and found out that the loading efficiency was (8.2-23.7) % using different techniques [23].

For the first time in the current paper the encapsulation of LV into sporopollenin exine microcapsules and its subsequent release into a medium resembling physiological conditions of human blood plasma has been reported. Continuous release may allow the encapsulated LV to maintain constant concentrations of the drug in patients suffering from Parkinson's disease. The amount of LV that can be encapsulated was investigated as well as the release of LV from the sporopollenin exine microcapsules into a medium of buffer solution at pH of 7.2, resembling physiological conditions of human blood plasma.

Materials and Methods

Materials

Lycopodium Clavatum pollen powder was purchased from Fagron, UK, LV (3, 4 –dihydroxy-L-phenylalanine) was purchased from Sigma Aldrich, UK, and general chemicals with different purity were used as purchased from either Sigma or Fisher chemicals. All experiments were undertaken in a buffer solution that resembled the physiological conditions of human blood plasma. The buffer solution contained 0.1 M NaCl, 1.5 mM triethyl ammonium acetate buffer (pH 7.2) to maintain the pH, and 0.1 mM NaN3 to prevent any bacterial growth arising from changes in temperature of the external environment. Triethyl ammonium acetate buffer (TEAA) is a buffering agent that maintains pH at approximately 7, when diluted

with water and prepared from equi-molar quantities of triethyl amine, and acetic acid. TEAA was chosen as the buffering agent since unlike phosphate buffer it will not cause precipitation by the phosphate ion.

Instrumentation

The UV-Vis Absorption Spectroscopy was performed on a Perkin Elmer UV-Vis Spectrometer Lambda Bio 10 with Lambda Bio 10 software, and quartz cuvette with a 1 cm path length. A thermocouple was used to determine the temperature of the experiments. Evo 60 field emission Scanning Electron Microscope (Carl Zeiss, GmbH) was used to take images.

Methods

a. Loading LV into sporopollenin microcapsules

Sporopollenin microcapsules were prepared in-house by extracting the cellulosic intine using procedures taken from elsewhere [17]. A saturated solution of LV was mixed by hand with Tween20 surfactant solution and the sporopollenin powder. The mixture was then subjected to vacuum at room temperature. The encapsulation process was performed using different masses of sporopollenin (0.1, 0.2, 0.3, 0.5, 0.7. and 1.0) g to determine the encapsulation efficiency. A saturated solution of LV was prepared by dissolving 2.076 g LV in 90 mL of 0.1 M HCl, (0.117 M LV). The solution was heated to 50°C, and stirred for 30 minutes to ensure maximum dissolution before it was filtered. Next 0.6 mM Tween20 surfactant was added to the acidified LV solution and stirred. The sporopollenin was immediately added, stirred and then subjected to vacuum for 30 minutes to remove air and allow the LV solution to penetrate into the exine shells. At 10 minutes' intervals, the mixture was stirred by hand to encourage homogenous penetration of the solution into the sporopollenin. The mixture was filtered and quickly washed with 5 mL of Milli-Q water (collected). 10 mL solution of 0.5 M TEAA was then poured over the sporopollenin and allowed to slowly soak the sporopollenin, to precipitate the LV inside the microcapsules by pH change. The mixture was then washed with milli-Q water (10 mL for 5 times), and every time the wash was filtered off to remove any non-encapsulated LV. Meanwhile, all the filtrates were collected (55 mL), followed by addition of 10 mL solution of 0.5 M TEAA to precipitate the entire non-encapsulated LV (to determine the mass of non-encapsulated LV). The mixture was then filtered and the precipitated LV was collected and dried with the loaded sporopollenin microcapsules overnight at 60° C, separately.

b. Efficiency of Encapsulation process

5 mL of 0.1 M hydrochloric acid solution was added into different masses of dried sporopollenin microcapsules loaded with LV, followed by stirring the mixture for 2 hours. The suspension was then filtered using a 0.22 µm syringe-driven filter. To the filtrate, 5 mL of 0.1 M NaOH was added; this solution was then diluted to 50 mL with the buffer solution. The absorbance of this solution was then measured using a UV-Vis spectrophotometer at 280 nm. The mass of experimentally encapsulated LV was calculated from the concentration of LV measured from absorbance, while the mass of theoretically encapsulated LV was calculated by subtracting the mass of non-encapsulated LV from the mass of LV used. Afterward, the encapsulation efficiency was calculated as mass of experimentally encapsulated LV / mass of theoretically encapsulated LV used x 100.

c. Release profile of LV encapsulated inside sporopollenin microcapsules

Different masses of Sporopollenin loaded with LV were added to 50 mL medium prepared to resemble the physiological conditions of human blood plasma. The suspension was left to stir at 37° C at 150 rpm for up to 12 hours. Periodically, 2 mL aliquots of suspension were removed and the sporopollenin exine microcapsules were removed by filtration, using a 0.22 µm syringe-driven filter. 2 mL of the prepared medium was added to the suspension to fix the volume at 50 mL. No adjustment is made to the amount of substance after removing the aliquots. The absorbance of the filtrate was then measured using a UV-Vis

spectrophotometer scanned at 280 nm. The value of the absorbance was then used to find the corresponding concentration and it was plotted vs. time to produce the LV release profile.

Results and Discussion

The current paper reports for the first time, the successful encapsulation of LV inside sporopollenin microcapsules using pH change precipitation method. The average size of the microcapsules is 20- 35μm (see Figure 1), therefore, they are expected to be ingested into human body and penetrate into the blood stream, where they start to disintegrate [19]. In this paper the release profile of the drug in aqueous medium resembling the physiological conditions of human blood plasma (pH of 7.2 and salt content), was investigated. The encapsulation process was aided by using 3 times CMC Tween20, which is a non-ionic surfactant and has CMC of 0.06 mM in water at 25 °C. Tween20 as a surface-active agent reduces the surface tension of the aqueous solution and should facilitate diffusion. To ensure that Tween20 has no interference with the absorbance measurement for LV, the absorption spectrum of the surfactant solution was measured and recorded between wavelengths of 200-400 nm. Using a buffer solution of Triethylammonium Acetate (TEAA), pH of 7.2 to maintain the pH of the medium at neutral, was a vital point to consider, since due to its zwiterionic structure, LV is protonated at low pH and deprotonated at higher pH. LV absorbs electromagnetic radiation in the UV-Vis region; therefore, the concentration of released LV was determined by using UV-Vis spectrophotometer. The absorbance range of the TEAA solution was also measured in 200-400 nm range to observe any interference with LV absorbance wavelength of 280 nm, and it was calculated that the buffer solution has no known interference with the drug, (See Figure 2).

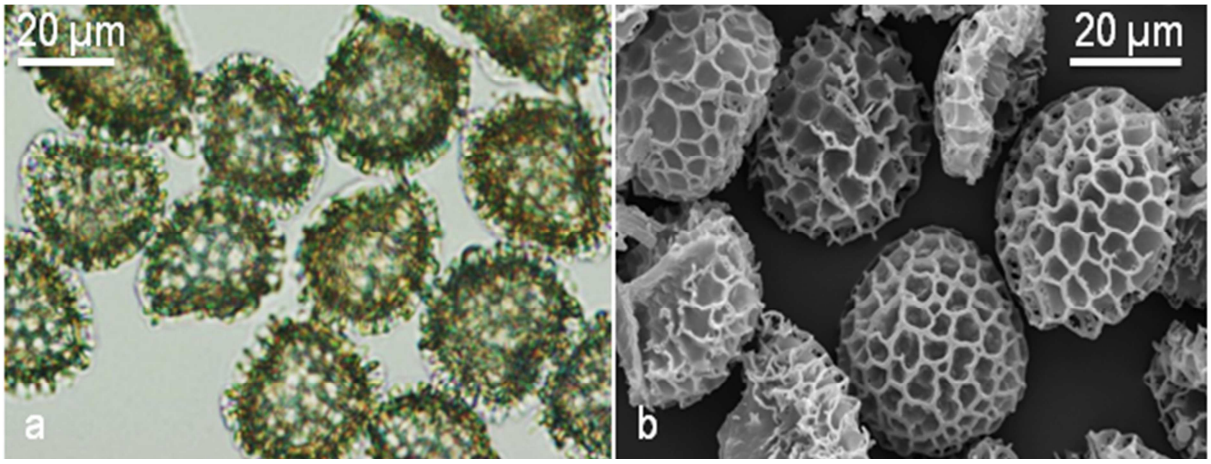

Figure 1: Optical (a), and SEM (b) images of sporopollenin microcapsules showing no existence of precipitated LV powder outside the capsules, after encapsulation process has completed. The average size of the microcapsules is (20-35μm).

a. Release Profiles of LV Encapsulated in Sporopollenin

The release of the encapsulated LV is dependent on the percentage encapsulation efficiency, i.e. it depends on the amount of drug encapsulated inside the exine shells. This in return depends on the mass of sporopollenin exine microcapsules added to the medium. The medium was prepared to resemble the physiological conditions of the human blood plasma. To monitor the release profiles, in this study three different masses (0.1, 0.3, and 0.5) g of loaded sporopollenin with LV were used. The obtained release profiles are showing LV concentration vs. time (Figure 3). The volume (50 mL), temperature (25 □C, stirring 150 rpm), and buffer composition (pH 7.2) remained constant for all the three experiments. In general the release profiles show that the maximum rate for the release of LV occurred during the first 0.5 hour of the experiments. Meanwhile, between 0.5 and 5 hours, the LV appears to be released in a continuous manner; this is illustrated by the linearity of the graph (Figure 3) during this period. After which, the rate of release decreases as the maximum concentration (2.75×10^{-3} M) is approached, this is all clearly visible from

Figure 3. In the experiment where 0.1 g of the sporopollenin was used, the release curve indicates a steadier release; while in the other two experiments where (0.3, 0.5 g sporopollenin used, respectively), the release of the drug is more profound, indicated by the shape of the curves. After 2.5 hours, the release rate in all three experiments significantly slows down (see Figure 3), and the concentration of the released drug is approximately constant, as the maximum concentration (0.705, 1.84, and 2.75) x 10^{-3} M achieved. In all three cases the percentage of drug release at 2.5 hours does not exceed 2.59 % of the drug content (see table 1). This indicates the ability of the exine shells to delay the dissolution of the drug in the prepared medium. This way a constant supply of the drug should be available to relief the illness's symptoms.

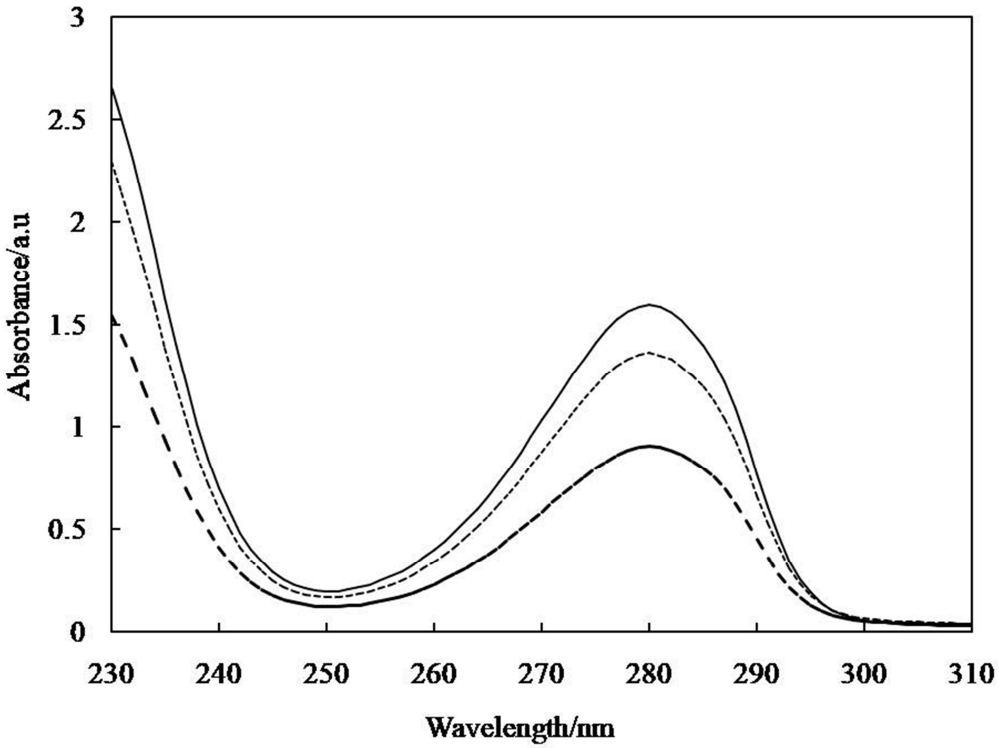

Figure 2: The absorption spectra of Levedopa dissolved in milli-Q water (top line); in solution of 10^2 mM NaCl, 1.5 mM triethyl ammonium acetate buffer pH 7.2, and 0.1 mM NaN3 (middle line); and the same solution containing 0.06 mM Tween20 represented by the (bottom line) all at 25 °C.

Table 1: percentage w/w of LV released in 50 mL medium after 2.5 hours passed.

Mass Sporopollenin/ g	**Maximum concentration of LD released/ M**	**Maximum mass of LD released/ g**	**Mass of experimental LD encapsulated/ g Table 2**	**w/w % LD released**
0.1	0.705 x 10^{-3}	0.00695	0.268	2.59
0.3	1.84 x 10^{-3}	0.0181	0.702	2.58
0.5	2.76 x 10^{-3}	0.0272	1.052	2.59

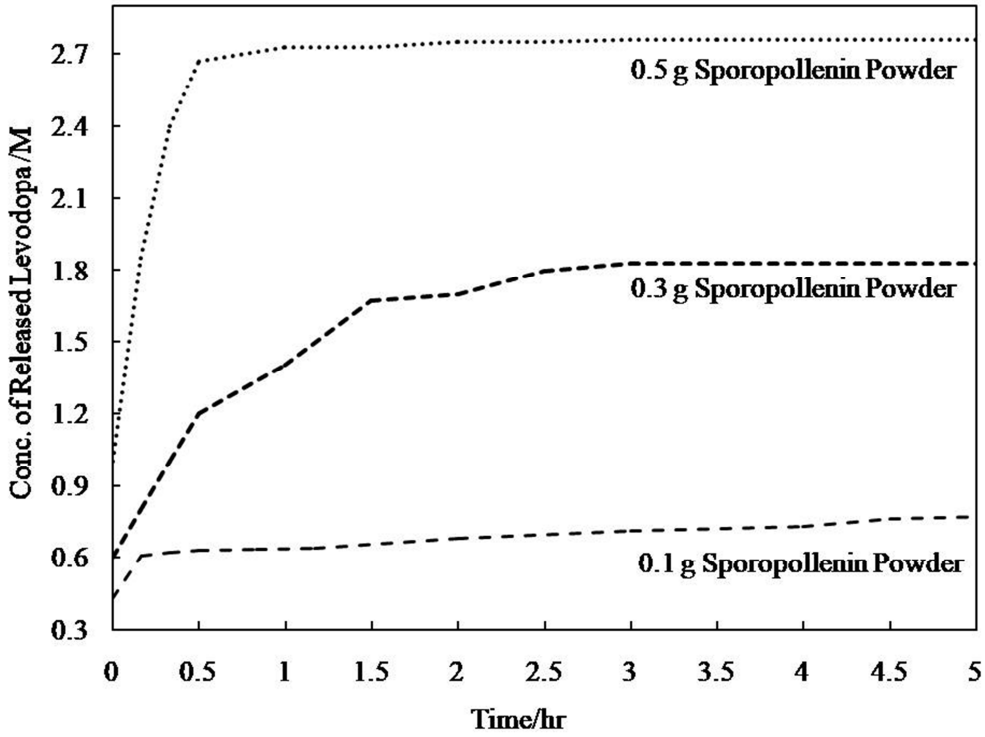

Figure 3: Release profiles of LV encapsulated inside sporopollenin microcapsules in 50 mL TEAA solution of pH 7 at 25 °C. Different amount of sporopollenin was used in the encapsulation process, while the amount of the LV was kept constant at (0.117 M). The concentrations of LV in this graph are multiplied by 10^3.

In the experiment where (0.5 g sporopollenin loaded with LV) was used, LV's kinetic release started off in the first half hour of the release process. A steady continuous release can be observed (see Figure 3) followed by a decrease in the release rate as the maximum released concentration is approached (2.76 x 10^{-3} M); however, a slower rate has been recorded between 0.5 and 1 hour. Meanwhile, in the experiment where (0.3 g sporopollenin loaded with LV) was used, the release profile shows 3 major change points. The first change occur at 0.5 hours when the amount of LV released is 0.0122 M, whereas the second change is at 1.5 hours when the amount of LV released increased to 0.0166 M, and finally the last point before the curve flattens up is at 2.5 hours when the concentration of LV released is 0.0184 M. This trend indicates a steady and organised release of the drug content of the loaded microcapsules. This means that in the experiment where (0.3 g sporopollenin loaded with LV) was used, an ideal release profile can be observed because in comparison to the other two experiments (0.1 g, 0.5 g sporopollenin loaded with LV) in this experiment the drug content has been released in a noticeable manner. Between 0.5 and 1.5 hours a further 0.62 % of the drug content has been released; whereas, this trend is not visible in the other two release profiles. From Figure 3 simple calculations were made and concluded that for the same time interval only 0.092 % for (0.1 g sporopollenin) and 0.04 % (0.5 g sporopollenin) of the drug content was released into the medium. However, in the case of (0.5 g sporopollenin loaded with LV) was used, maximum amount of the drug was released into the medium which is equal to (2.76 x 10^{-3} or 27.2 mg). This amount in comparison to many commercial LV tablets (25-50 mg) is a relatively sufficient bioavailability of the drug to maintain required drug level for the treatment of the symptoms in the patients.

b. Self-regulating sporopollenin microcapsules loaded with LV

Precipitated LV (slightly soluble salt under the influence of pH change) inside the sporopollenin microcapsules, can give the microcapsules, as a delivery vehicle, their renewable and self-regulating property. It means, the amount of LV released by the microcapsules, is dependent on the concentration gradient of the drug in the surrounding media. The LV dissolves and diffuses through the exine of the

microcapsules into the aqueous environment until equilibrium is achieved, where no more drugs dissolves. The release process arrives at steady state, until the equilibrium is disturbed by means of, pressure, temperature and/or concentration change, per Le Chatelier's principle; then more drug will dissolve and release into the surrounding medium; hence, providing a continuous supply of the drug.

To monitor the self-regulating property of the microcapsules, the equilibrium was disturbed by diluting the aqueous media. 20 mL milli-Q water was added into the aqueous suspension of the microcapsules (24 mL) loaded with LV (2.75×10^{-3} M already achieved) in equilibrium, followed by measuring the absorbance again. As it can be seen in figure 4, the concentration of the released LV has decreased, and slowly started to rise again, until it has reached back to the point before dilution. This indicates that the microcapsules have a self-regulating property in terms of controlling the amount of the drug to be released through the nanopores of the microcapsules. For this particular experiment (0.5 g sporopollenin loaded with LV), the maximum amount of released drug is (2.75×10^{-3} M) and after this concentration achieved no more drug can be released as long as the equilibrium maintained. This property is increasingly interesting as the microcapsules can be used as a reservoir of the precipitated drug, which can release exact amount of the drug required, whenever its concentration falls below equilibrium concentration (2.75×10^{-3} M). In figure 5, a close up of the dilution/release profile of the self-regulating microcapsules shows that after dilution, the concentration of the released LV has significantly fallen below the equilibrium concentration. Yet again, after a period of time has passed, the released concentration increased and reached back to exactly the maximum concentration of released LV (2.75×10^{-3} M).

Further release profiles of different mass of sporopollenin loaded with the drug i.e. (0.7, and 1.0) g were not investigated, for a few reasons: presumably ideal release profile achieved at (0.3 g sporopollenin loaded with LV) where less amount of the mircocapsules used. Although it is a fact that as the amount of the sporopollenin increased the encapsulation efficiency increased, especially when (0.5 g sporopollenin) used, the encapsulation efficiency was 65 %, which is relatively a convincing high percentage; bearing in mind that only 0.5 g of the microcapsules was used. Increasing the amount of sporopollenin to 1.0 g yields a percentage encapsulation efficiency of 81 % which is very high, but it should be noted that the amount of the microcapsules was doubled to achieve this percentage (see table 2). Nevertheless, a thorough and accurate study of the release profiles of the drug inside the microcapsules when (0.7. and 1.0 g sporopollenin loaded with LV was used) is necessary. It has been planned for the future to carry out the intended studies in a precise manner and compare the results with what have been achieved so far.

c. Efficiency of the Encapsulation Process

The efficiency of the encapsulation process was determined by completely dissolving the encapsulated LV inside the sporopollenin dry powder in 0.1 M hydrochloric acid. The absorbance of the solution was measured and recorded after the solution was filtered off to remove any solid residue. The absorbance was used to find the concentration of the encapsulated LV, which was used to calculate the mass of the encapsulated LV in grams. Fixed mass of (2.076 g) LV was used in each experiment, therefore, the mass of theoretically encapsulated LV was calculated by measuring the mass of non-encapsulated (wasted) in every experiment, and subtracting this mass from the mass used, to work out the mass theoretically encapsulated (see table 1 for the data). Using experimental mass encapsulated with theoretical mass encapsulated, the percentage efficiency was calculated as follows:

Encapsulation efficiency = experimental mass of LV encapsulated / theoretical mass of LV encapsulated x 100.

The results in (figure 6 and table 2) show that maximum encapsulation of 81 % achieved when the saturated LV solution was added into 1.0 g sporopollenin microcapsules' powder. They confirm that the increase of the mass of the sporopollenin in the encapsulation process is proportionally related to the increase

of encapsulation efficiency (see table 1 data). For further studies, one can increase the amount of the sporopollenin powder used to (1.5, 1.7, and 2) g to observe the trend and find the encapsulation efficiency, which in return has a significant effect on the amount of LV released in time.

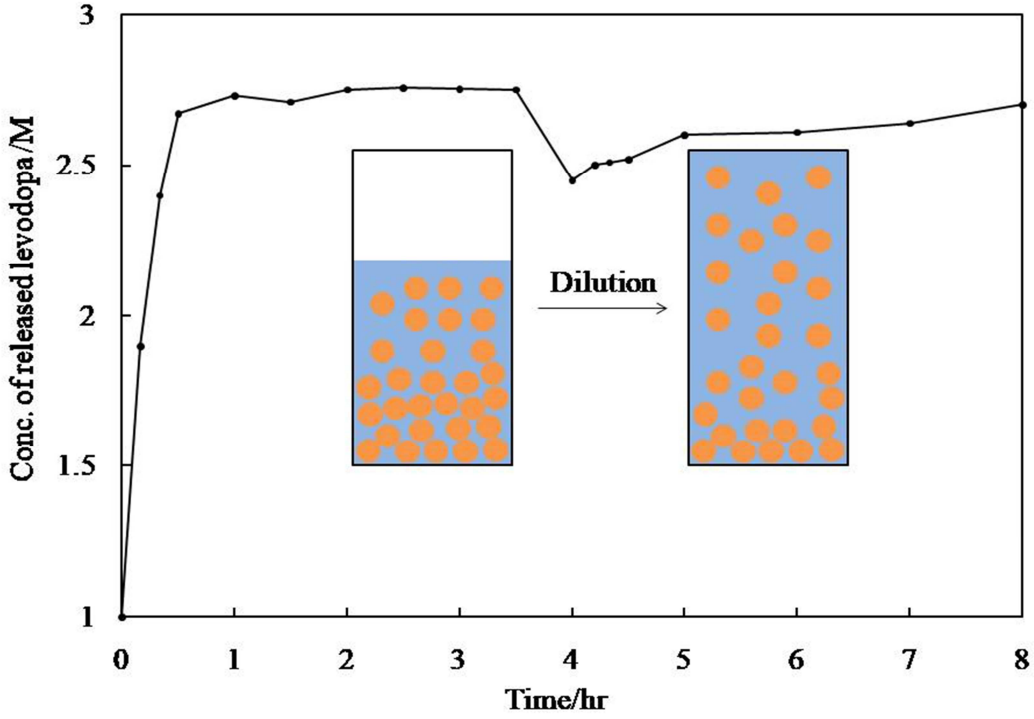

Figure 4: Self-regulating sporopollenin microcapsules loaded with LV, the concentration of released LV was monitored in time. The amount of sporopollenin used was 0.5 g; after 3.5 hours, the sample was diluted, and the release profile was monitored again.

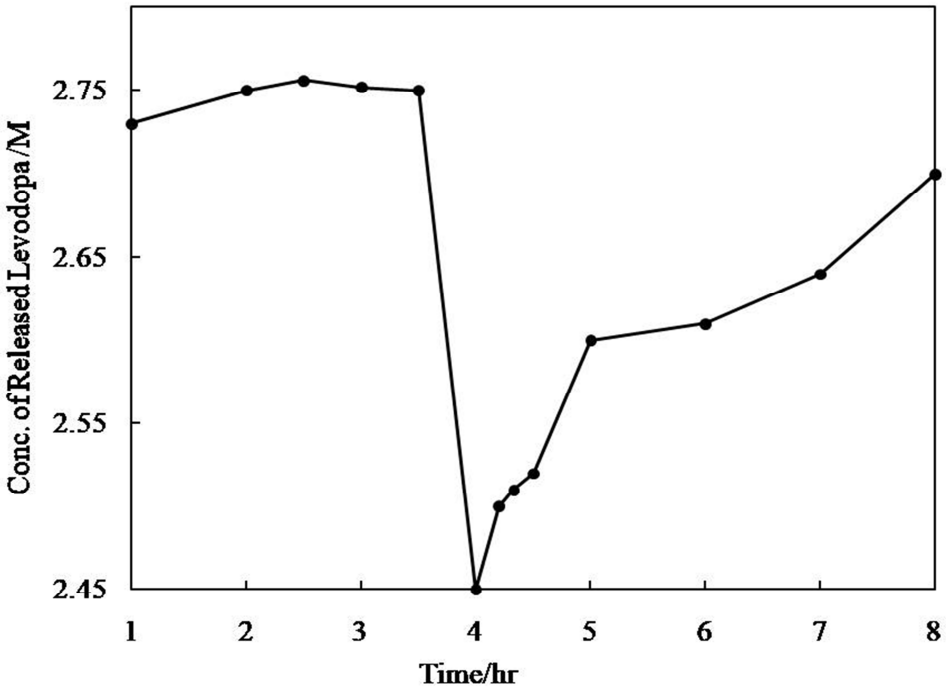

Figure 5: close up of figure 5, between 1 and 8 hours release monitoring.

Table 2: Percentage of encapsulation efficiency of different masses of sporopollenin with fixed amount of LV used (2.076 g).

Mass Sporopollenin used/ g	Mass of LD used/ g	Mass of non-encapsulated LD/ g	Theoritical Mass of LD Encapsulated/ g	Experimental Mass of LD Encapsulated/ g	% Encapsulation Efficiency
0.1	2.076	1.12	0.956	0.268	28
0.2	2.076	0.798	1.278	0.422	33
0.3	2.076	0.612	1.464	0.702	48
0.5	2.076	0.458	1.618	1.052	65
0.7	2.076	0.233	1.843	1.345	73
1.0	2.076	0.141	1.935	1.567	81

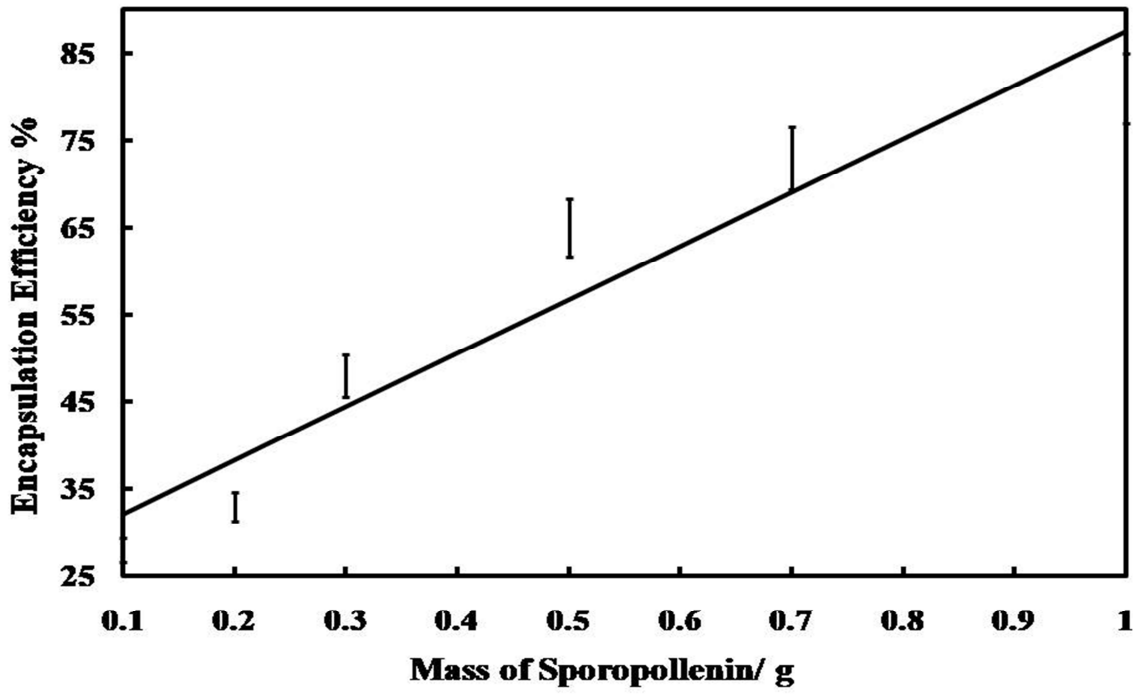

Figure 6: Encapsulation efficiency of constant amount of LV in sporopollenin microcapsules at pH 7 and 25 °C, dependent on different amount of sporopollenin used in the process.

Conclusions

The current paper reports for the first time, the successful encapsulation of LV inside sporopollenin microcapsules using pH change precipitation method. It can be concluded that the release of the encapsulated LV is dependent on the percentage encapsulation efficiency, i.e. it depends on the amount of drug encapsulated inside the exine shells, which itself depends on the mass of sporopollenin exine microcapsules used for the encapsulation process. In all the experiments the maximum release rate of the LV observed after the first half hour passed, although, LV appears to be released in a continuous manner even after 5 hours elapsed, followed by decrease in the rate of release. Different masses of sporopollenin were used in each experiment; however, it was observed that in all three cases the percentage of drug released at 2.5 hours does not exceed 2.59 % of the drug content. Here the ability of the exine shells to delay the dissolution of the drug

in the prepared medium is obvious. This can be another technique to maintain a constant supply of the drug in the blood stream to relief the symptoms in patients suffering from Parkinson's disease.

In another part of this paper, it was concluded that sporopollenin microcapsules loaded with precipitated LV can act as a delivery vehicle for the encapsulated drug by providing a continuous supply of the drug. This will give the microcapsules their renewable and self-regulating properties, which highly important in the treatment of disease such as Parkinson's. The percentage encapsulation efficiency of the microcapsules was also calculated to optimise the amount of sporopollenin to be used with fixed amount of the drug. It was concluded that as the amount of the sporopollenin increased the encapsulation efficiency also increased. It was especially noted that where 0.5 g sporopollenin used with fixed amount of the drug, the encapsulation efficiency was 65 %, which is relatively a high percentage. Nonetheless, increasing the amount of sporopollenin to 1.0 g resulted in a 16 % increase in the percentage encapsulation efficiency. It is obvious that 81 % encapsulation efficiency is very high, however, it should be noted that obtaining this percentage requires to double the amount of sporopollenin i.e. 1.0 g of the biopolymer is needed.

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