Controlled release of huperzine-A from biocompatible copolymer microspheres

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Abstract. Huperzine-A (Hup-A) is a reversible acetylcholinesterase inhibitor used for patients suffering from Alzheimer's disease. Hup-A has to be administered daily because of its short half-life time and narrow therapeutic range, making it less suitable for clinical use. In the present study, we studied its controlled drug delivery using biodegradable microspheres to avoid frequent dosing. Hup-A-loaded microspheres were prepared by an emulsion crosslinking method using a mixture of water and oil. Calcium chloride was used as a crosslinker. Primarily, we prepared sodium alginate (NaAlg) microspheres and investigated the *in vitro* release. The *in vitro* release studies were carried out successively at three different pH (1.2, 6.8 and 7.4) values for 2 h. To increase the durability of the NaAlg microspheres, NaAlg was blended with acrylamide (AAm)-grafted poly(vinyl alcohol) (PVA). It was determined that the presence of the PVA-g-AAm increased the physical resistance of the microspheres and also increased the amount of Hup-A loading and release. Additionally, the effect of the NaAlg/PVA-g-AAm (w/w) blend ratio, drug/polymer (w/w) ratio, crosslinker concentration and crosslinking period on the release of Hup-A was investigated. The prepared microspheres were characterized using Fourier transform infrared spectroscopy, differential scanning calorimetry and scanning electron microscopy.

Keywords. Huperzine-A; drug-delivery systems; controlled release; Alzheimer's disease; graft copolymer.

1. Introduction

Alzheimer's disease (AD) is the most common cause of dementia in elderly persons, and thus is a social, economic and medical problem. Although there is no approved exact treatment for AD, some treatments have been developed to delay the progression of the disease and cause symptomatic improvement. Recently, approved agents in the treatment of AD are acetyl cholinesterase inhibitors (tacrine, rivastigmine, donepezil, galantamine and huperzine-A (Hup-A)) [1,2]. Hup-A has been proven to be one of the most promising agents for treatment of AD based on its high bioavailability and minimal side effects [3,4]. It is a natural alkaloid isolated from the Chinese herb *Huperzia serrata*, a reversible acetyl cholinesterase inhibitor and its half-life is about 288 min; therefore, it is given 2–3 times per day [5,6]. Daily repeated oral administration is the convenient way for the most patients; however, this treatment is difficult for Alzheimer's patients because of their decreased memory [7,8]. In addition, long-term therapy with Alzheimer's patients is difficult in terms of the need to reach therapeutic levels. Over-dosing or under-dosing can cause adverse effects. Thus, a controlled-release dosage form for the use over a long

period of time is the best way to improve the treatment of AD. Recently, many biodegradable polymers containing various drugs have been used as controlled release systems [9–25]. For this purpose, researchers have attempted to develop the controlled release of Hup-A. Encapsulation of Hup-A using biodegradable polymers has also been widely investigated as a technique to produce controlled formulations. For instance, Cai *et al* [1] studied the Hup-A-loaded biodegradable poly lactic-co-glycolic acid (PLGA)–poly ethylene glycol (PEG)–PLGA polymer gel as an injectable implant system. Gao and coworkers [7] designed biodegradable microspheres for sustained release of Hup-A using poly(D,L-lactide-co-glycolide). Chu *et al* [8] prepared Hup-A-loaded PLGA microspheres using an O/W method. They reported that the release of Hup-A from the polymeric matrix was diffusion-controlled and larger particle size slowed the diffusion of Hup-A from the microspheres and increased the effect of PLGA degradation on the release of Hup-A.

Sodium alginate (NaAlg) and poly(vinyl alcohol) (PVA) are both biodegradable and nontoxic polymers; each has been widely used in controlled-release applications [9,10,12,26–32]. However, it is difficult to prepare microspheres and to study controlled release from these polymers because of

Table 1. Definition of the preparation of microspheres.

Formulation code	Polymer	Drug/polymer ratio (w/w)	Crosslinker concentration (M)	Crosslinking time (min)	Entrapment efficiency (%)	Drug loading (%)
A	NaAlg	—	0.1	10	—	—
B	NaAlg/PVA-g-AAm (2/1)	—	0.1	10	—	—
C	NaAlg/PVA-g-AAm (1/2)	—	0.1	10	—	—
A1	NaAlg	1/1	0.1	10	4.70	4.15
B1	NaAlg/PVA-g-AAm (2/1)	1/1	0.1	10	7.77	6.80
C1	NaAlg/PVA-g-AAm (1/2)	1/1	0.1	10	6.98	5.86
B2	NaAlg/PVA-g-AAm (2/1)	1/2	0.1	10	24.02	22.97
B3	NaAlg/PVA-g-AAm (2/1)	1/4	0.1	10	22.03	19.65
B4	NaAlg/PVA-g-AAm (2/1)	1/8	0.1	10	62.10	60.54
B5	NaAlg/PVA-g-AAm (2/1)	1/8	0.05	10	26.23	25.14
B6	NaAlg/AAm-g-PVA (2/1)	1/8	0.025	10	22.07	18.32
B7	NaAlg/PVA-g-AAm (2/1)	1/8	0.1	15	55.67	52.20

their poor stability and bead-forming propensity in aqueous medium; therefore, we propose here the use of blending and grafting techniques as useful tools for the preparation of new NaAlg and PVA microspheres. The goal was to prepare NaAlg and NaAlg/PVA-*graft*-acrylamide (NaAlg/PVA-g-AAm) blend microspheres containing Hup-A to achieve a controlled-release profile suitable for oral administration.

2. Materials and methods

2.1 Material

NaAlg (medium viscosity) and Hup-A were purchased from Sigma Chemical Co. Ltd. (USA). PVA, AAm, dimethylsulphoxide, acetone, ethanol, Span-85 (non-ionic surfactant, sorbitan trioleate), light liquid paraffin oil (emulsifier), hexane, benzophenone, Na₂HPO₄, NaH₂PO₄ and CaCl₂ were supplied by Merck (Germany). The average molecular weight of the PVA was 72,000 g mol⁻¹.

2.2 Methods

2.2a Synthesis of graft copolymer (PVA-g-AAm): Graft co-polymerization of AAm onto PVA was carried out in an aqueous medium using a benzophenone solution in ethanol (1 M) as a photosensitizer for 3 h under nitrogen. The graft polymerization medium was irradiated by UV light. Initially, aqueous PVA (10% m/v) was stirred with AAm (6 M) at 25°C. Oxygen-free nitrogen was purged through the solution for 30 min. At this stage, the benzophenone solution was added to the reaction mixture and the stirring was continued for 3 h under UV irradiation. Then, saturated hydroquinone solution was added to terminate the reaction. The obtained copolymers were precipitated using an excess amount of acetone and filtered, and the copolymer was treated with dimethylsulphoxide to remove the remaining AAm

completely. The grafted polymer was dried at 60°C for 48 h under vacuum. The graft percentage of the copolymer was calculated using equation (1):

$$\text{Graft percentage (\%)} = [(M_g - M_o)/M_o] \times 100, \quad (1)$$

where M_o and M_g are the mass of the original (ungrafted) and grafted PVA, respectively. The graft percentage of AAm in the PVA-g-AAm copolymer was found to be nearly 35%.

2.2b Preparation of microspheres: NaAlg/PVA-g-AAm and NaAlg microspheres were prepared by an emulsion-crosslinking method using calcium chloride (0.1 M) as the crosslinker. Briefly, NaAlg (2.5%) was dissolved in distilled water and various amounts of Hup-A were added to the reaction solution and mixed. The drug-loaded polymer solution was emulsified into light liquid paraffin oil to form a water/oil emulsion using a high-speed stirrer in a beaker containing paraffin oil, Span-85 (2% (w/v)) and CaCl₂ (0.1 M) for 10 or 15 min. The microspheres formed were filtered, washed several times with *n*-hexane and water, and oven-dried at 40°C before further analysis. The size of the microspheres was measured using scanning electron microscopy (SEM) (JSM-6400, JEOL Ltd., Tokyo, Japan). The compositions, crosslinking periods and labels of the various microspheres prepared are listed in table 1.

To determine the practical loading of the microspheres, a known mass of microspheres (0.05 g) was placed in a flask. Then, water (50 ml) was added and the mouth of the flask was closed by a reflux-column to prevent probable water evaporation from the system. The temperature of the system was maintained at 37°C for an optimized period of 1 h and the mixture was stirred magnetically during this period to ensure the complete extraction of Hup-A from the microspheres. Then, the solution was filtered and the filtrate was analysed for Hup-A spectrophotometrically at 305 nm, by using

a UV spectrophotometer (Unico 4802, United Products & Instruments Inc., USA).

The entrapment efficiency (%) and drug loading were calculated by the following equations:

$$\begin{aligned} \text{Drug loading (\%)} &= (\text{Weight of Hup-A in microspheres}) / \\ &[(\text{Weight of Hup-A in microspheres}) \\ &+ (\text{Weight of Hup-A-loaded microspheres})], \quad (2) \end{aligned}$$

$$\begin{aligned} \text{Entrapment efficiency (\%)} &= (\text{Practical loading} / \text{Theoretical loading}) \times 100, \quad (3) \end{aligned}$$

where the theoretical loading is the weight of the Hup-A added into the polymer solution from which microspheres were prepared and the practical loading was the value determined by the UV measurement.

2.2c Equilibrium swelling measurements: To determine the equilibrium swelling degree of the empty, crosslinked microspheres, they were allowed to swell in different buffer solutions (pH 1.2, 6.8 and 7.4) at 37°C for 48 h and then, they were weighed to determine M_s of the swollen microspheres. The excess surface-adhered liquid drops were removed by blotting, and the swollen microspheres were weighed using an electronic balance (Precisa XB 22A, USA). Then, they were dried in an oven at 40°C until constant weight was reached. The equilibrium swelling degree (%) of the microspheres was calculated by equation (4):

$$\begin{aligned} \text{Equilibrium swelling degree (\%)} &= [(M_s - M_d) / M_d] \times 100, \quad (4) \end{aligned}$$

where M_s and M_d are the mass of the swollen microspheres and dry microspheres, respectively.

2.2d Characterization: Fourier transform infrared (FTIR) spectra of the microspheres and polymer samples was obtained with a Mattson 1000 FTIR spectrometer (Welwyn Garden, England).

The glass transition temperature (T_g) of the microspheres was determined by using a V4.1C 2000 differential scanning calorimeter (DuPont general Perkin Elmer, USA). The measurements were performed over the temperature range from 0 to 300°C at a heating rate of 10°C min⁻¹.

SEM photographs of the microspheres were taken with JSM 6400 SEM (Quanta 400F field emission, JEOL Ltd., Japan) to examine their morphology. The microsphere samples were first dried and then sputtered with gold under vacuum before SEM imaging.

2.2e In vitro Hup-A release: *In vitro* drug release from the Hup-A-loaded microspheres was carried out at pH 1.2, 6.8 and 7.4 to provide the pH of gastric, input intestinal and intestinal pH conditions, respectively. For this aim, known mass of

Figure 1. FTIR spectra of (a) A1, (b) Hup-A, (c) NaAlg, (d) PVA-g-AAm and (e) B1.

microspheres (0.05 g) was placed in 50 ml HCl solution (pH 1.2) and then incubated in a shaking water bath (BS-21, Medline Scientific, Korea) at 37°C at a speed of 50 rpm. A 5 ml of the sample was withdrawn at specific time intervals, and the Hup-A content was determined by using a UV spectrophotometer at 305 nm. An equal volume of fresh buffer solution was replaced into the release medium to maintain constant volume. After a release period of 2 h, the microspheres were filtered and the filtrate was transferred to the same volume of buffer solution at a pH of 6.8 and 7.4. In the same way, 5 ml of the sample was withdrawn at specific time intervals, and the Hup-A content was determined by using a UV spectrophotometer at 305 nm. From the spectrophotometric analysis, the cumulative released amount (%) was determined by using a calibration curve. All experiments were performed in triplicate to minimize the variation error. Standard deviations from the average values were calculated.

3. Results and discussion

3.1 Microsphere characterization

FTIR spectra of Hup-A, NaAlg, copolymer and A1- and B1-formulated microspheres are shown in figure 1. The broad band between 3000 and 3500 cm⁻¹ is attributed to the O–H stretching vibrations of Hup-A, NaAlg and A1-formulated microspheres. The peaks at 1662, 1625 and 1650 cm⁻¹ in the spectra of Hup-A, NaAlg and A1-formulated microspheres, respectively, were due to the C=O groups. The C–H stretching bands appearing at 2925, 2912 and 2875 cm⁻¹, correspond

Figure 2. DSC results of (a) Hup-A, (b) NaAlg, (c) A1 and (d) B1.

to C–H groups of the Hup-A, NaAlg and A1-formulated microspheres, respectively. A sharp band at 1240 cm^{-1} corresponds to the C–O–C symmetrical stretching (in acetyl group) present on the PVA backbone due to the non-hydrolysed acetate groups of poly(vinyl acetate). This band is also seen in the spectra of B1 at 2920 cm^{-1} . The peak due to an asymmetric N–H stretching vibration of the primary amide appears at 3400 cm^{-1} . In the spectra of B1, this band overlaps with the O–H stretching vibration. From the analysis, it was concluded that Hup-A was successfully loaded into the microspheres and did not affect the characteristics of the polymers used for the microspheres.

The differential scanning calorimetry (DSC) thermograms of Hup-A, NaAlg and A1- and B1-formulated microspheres are illustrated in figure 2. From the thermograms, the glass transition temperature (T_g) values of NaAlg and A1- and B1-formulated microspheres were found to be 59, 64 and 104°C , respectively. The intercept points of the slopes were taken as T_g . T_g of a polymeric material is one of the most important thermophysical properties of amorphous polymers. In the highly viscous region above T_g , polymeric materials are soft and rubbery, whereas below T_g , polymers are hard and brittle. From this point of view, the greater T_g value for the B1-formulated microspheres indicates that it remained

Figure 3. SEM images at $2400\times$ magnifications of the B1.

rubbery to a considerably higher temperature than A1. As it is reflected from figure 2 that temperature of the end point of the endotherm peak shifted from 74 to 80°C on loading Hup-A. The exothermic decomposition point for NaAlg is shown to be 218°C , while that of Hup-A-loaded NaAlg is shown to be 270°C , respectively. However, the exothermic decomposition point for Hup-A-loaded NaAlg/PVA-g-AAm is shown to be 283°C . The hydroxyl groups of NaAlg in Hup-A-loaded NaAlg/PVA-g-AAm were isolated partially by bonding monomers. Therefore, the thermal decomposition of NaAlg occurred easily than the NaAlg/PVA-g-AAm.

In figure 2, the melting peak of Hup-A appeared at 306°C . This means that the studied temperature of 37°C is a suitable temperature at which there should not be any risk for the thermal degradation of Hup-A.

Additionally, no characteristic peak of Hup-A was observed in the DSC thermograms of the A1- and B1-formulated microspheres. This supports that the drug was molecularly dispersed in the polymer matrix.

SEM image of one of the B1 microspheres is shown in figure 3. Figure 3 shows the prepared microspheres are almost spherical in shape.

The effects of microsphere composition, drug/polymer ratio and the crosslinker concentration on the percentage of entrapment efficiency were also investigated and are shown in table 1. The formed microspheres had entrapment efficiencies ranging from 4.70 to 62.10%. The entrapment efficiency of the microspheres changed with the NaAlg/PVA-g-AAm ratio, drug/polymer ratio, crosslinker concentration and crosslinking time.

The entrapment efficiency values were slightly affected with changes in the NaAlg/PVA-g-AAm ratio; the entrapment efficiency of the microspheres changed in the range of 4.70–7.77%. Since, the NaAlg/PVA-g-AAm with a polymer

Table 2. Equilibrium swelling degree for the empty microspheres at different formulations.

Formulation code	pH		
	1.2	6.8	7.4
A	242.5 ± 1.9	682 ± 3.4	Microspheres eroded
B	207.7 ± 2.9	838.6 ± 7.0	1014 ± 4.1
C	202.1 ± 1.0	634.2 ± 4.1	863.4 ± 3.2

ratio of 2/1 (B1) had the greatest entrapment efficiency, this polymer ratio was kept constant for the rest of the study.

To determine the effect of drug/polymer ratio on the entrapment efficiency of Hup-A, the drug/polymer ratio was changed as 1/1; 1/2; 1/4 and 1/8 (B1, B2, B3 and B4, respectively). It was determined that although the B2 and B3 formulations had almost the same efficiency percentages, for 1/8 drug/polymer ratio (B4), the maximum percentage of Hup-A entrapment was obtained. For further optimization studies, this ratio was accepted as the preferred drug/polymer ratio to determine the crosslinker concentration and period. As shown in the table, the suitable crosslinker concentration was determined as 0.1 M from the comparison of the entrapment efficiencies of B4, B5 and B6; additionally, by the comparison of B4 with B7, the suitable crosslinking time was determined as 10 min for the highest entrapment efficiency percentage.

Equilibrium swelling experiments were performed in buffer solution of different pH values (1.2, 6.8 and 7.4) for various empty microsphere formulations and the results are presented in table 2; for NaAlg/PVA-g-AAm ratios of 2/1 (B1) and 1/2 (C1). The equilibrium swelling degree increased with the increased NaAlg content of the microspheres for all the three pH values. Furthermore, an increase in pH increased the equilibrium swelling degree values of the microspheres.

3.2 Effect of NaAlg/PVA-g-AAm ratio on the release of Hup-A

The *in vitro* release of Hup-A from the crosslinked NaAlg/PVA-g-AAm microspheres was studied under, successively, gastric (pH 1.2), input intestinal (pH 6.8) and intestinal pH (pH 7.4) conditions at 37°C. As shown in figure 4, the cumulative drug release from the B1-formulated microspheres was higher than that of the A1- and C1-formulated microspheres. Although the presence of PVA-g-AAm increased the pH resistance of the microspheres for pH 7.4, the increase in the amount of graft polymer in the blend decreased the cumulative release of Hup-A. The A1-formulated microspheres do not contain graft copolymer. For this reason, it is non-resistant to pH 7.4. As shown in table 2, an increase in the amount of graft copolymer (B1 to C1), caused a decrease in the swelling percentage which led to a decrease in release percentage. The highest cumulative Hup-A release obtained for a release period of 6 h was 99.4% for the B1-formulated microspheres.

Figure 4. Effect of NaAlg/PVA-g-AAm ratio on Hup-A release (◆: A1, ▲: B1, ■: C1). **Figure 5.** Effect of drug/polymer ratio on Hup-A release (■: B2, ◆: B3, ×: B4).

3.3 Effect of the Hup-A/polymer ratio on the Hup-A release

Effect of the drug/polymer ratio on the release of Hup-A is shown in figure 5. It shows that the % cumulative release of Hup-A from the microspheres decreased as the drug/polymer ratio was decreased from 1/2 to 1/8. This means that the % release (and total release) of Hup-A was much higher for the higher amount of drug in the microspheres compared to the higher amount of polymers. This is most probably due to the decrease in the available-free void space, through which a smaller number of drug molecules will transport, when the formulation has a lower drug/polymer ratio. Similar results have been described in the literature [12,33,34]. Babu *et al* [12], for instance, studied the release of nifedipine-loaded NaAlg-methyl cellulose blend microspheres with various amounts of drug loadings (5, 10 and 15 wt%). The release data showed that the formulations containing the highest amount of drug (15 wt%) displayed faster and higher release rates than those formulations containing smaller amounts of nifedipine.

As shown in figure 5, it is understood that Hup-A release for pH 1.2 is low and close to each other. The microspheres

Figure 6. Effect of crosslinker concentration on Hup-A release (▲: B4, ◆: B5, ■: B6).

are pH-sensitive and resistant to pH 1.2. For this reason, the Hup-A release is very low at pH 1.2 for all microspheres.

3.4 Effect of the crosslinker concentration and the crosslinking period on the Hup-A release

To examine the effect of crosslinker concentration on the Hup-A release from the microspheres, the release study was performed with the B4-, B5- and B6-formulated microspheres; the release results are presented in figure 6. As shown, although B5 and B6 microspheres essentially have almost the same cumulative release percentages at pH 1.2, increase in the crosslinker concentration essentially decreased the release from B4 microspheres for the same pH. Similar results have been reported in previous studies. Kurkuri and Aminabhavi [35] studied the release of diclofenac sodium from PVA and poly(acrylic acid) interpenetrating polymer network microspheres and used glutaraldehyde as a crosslinker. They determined that as the glutaraldehyde concentration was increased from 2.5 to 7.5 ml, the release of diclofenac sodium decreased from the microspheres. Bajpai *et al* [36] prepared KNO₃-loaded alginate/carboxymethyl cellulose beads and found that increasing the concentration of CaCl₂ from 0.05 to 0.15 M caused a decrease in the release of KNO₃. Since B5 and B6 microspheres were prepared with 0.05 and 0.025 M crosslinking concentrations, the lower crosslinker concentration decreases the physical resistance of the microspheres at pH 6.8 and 7.4 and therefore, with increase in pH, no cumulative release was observed from the B5 and B6 microspheres as shown in figure 6. The close cumulative release of B5 and B6 microspheres was probably due to their close entrapment efficiency percentages as stated in table 1. Therefore, the microspheres crosslinked with 0.1 M crosslinker concentration were used for the rest of the study.

Effect of crosslinking period on the release of Hup-A was investigated for two crosslinking times using the B4- and B7-formulated microspheres. As can be seen from figure 7, the release of Hup-A decreased with the increase in crosslinking period from 10 to 15 min. As the crosslinking period increased

Figure 7. Effect of exposure time on Hup-A release (■: B4, ▲: B8).

from 10 to 15 min, the Hup-A release decreased from nearly 90 to 80% at pH 7.4 after 2 h each at pH 1.2, 6.8 and 7.4. Increasing the crosslinking period gave rise to a higher degree of crosslinking of the macromolecular chains and reduced the free volume; therefore, the penetration of solvent molecules and diffusion of Hup-A molecules became more difficult [37].

4. Conclusions

In this study, NaAlg and NaAlg/PVA-*g*-AAm microspheres were prepared to control the release of Hup-A. It was determined that the presence of PVA-*g*-AAm increased the physical resistance (resistance to erosion) of the NaAlg microspheres to pH 6.8 and 7.4. However, increasing the amount of graft polymer in the blend decreased the Hup-A release. An increase in the amount of NaAlg in the blend increased the swelling degree of the microspheres. By this study, it was concluded that the release of Hup-A from the NaAlg/PVA-*g*-AAm microspheres was pH-sensitive for pH values of 1.2, 6.8 and 7.4. The Hup-A release was found to be higher at higher pH values than at lower pH values. The highest Hup-A release was found to be 99.4% for the B1-formulated microspheres at a pH of 7.4.

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