International Journal of Pharmaceutics 384 (2010) 120–127

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Novel microencapsulation of potential drugs with low molecular weight and high hydrophilicity: Hydrogen peroxide as a candidate compound

Sing-Muk Ng[a,c,d], Jeong-Yeon Choi[a,d], Hyung-Soo Han[b,d], Jeung-Soo Huh[c,**], Jeong Ok Lim[a,d,*]

[a] Bio-Medical Research Institute, Kyungpook National University School of Medicine, Daegu, Republic of Korea
[b] Department of Physiology, Kyungpook National University School of Medicine, Daegu, Republic of Korea
[c] Department of Material Science and Metallurgy, Kyungpook National University, Daegu, Republic of Korea
[d] Joint Institute for Regenerative Medicine, Kyungpook National University Hospital, Daegu, Republic of Korea

ARTICLE INFO

Article history:
Received 17 July 2009
Received in revised form
25 September 2009
Accepted 2 October 2009
Available online 9 October 2009

Keywords:
Microencapsulation
PLGA microspheres
Hydrogen peroxide
Oxygen releasing polymeric materials
Water–oil–water (W_1/O/W_2) emulsion
Hydrophilic drugs

ABSTRACT

Microencapsulation of drugs into solid biodegradable polymeric microspheres via solvent evaporation technique remains challenging especially with those having low molecular weight and high hydrophilicity nature. This paper presents an efficient encapsulation protocol for this group of drugs, demonstrated using hydrogen peroxide as a model compound that is encapsulated into poly(lactic-co-glycolic acid) microspheres. Hydrogen peroxide can be employed as antiseptic agent or its decomposed form into oxygen can be useful in various pharmaceutical applications. The new encapsulation technique was developed based on the modification of conventional double emulsion and solvent evaporation protocol with a backward concentration gradient of hydrogen peroxide. This was achieved by adding and controlling the concentration of hydrogen peroxide at the continuous phase during the solidification stage of the microspheres. Parameters involved in the production and the formulation aspect were optimized to achieve the best protocol having controlled efficiency of encapsulation that is simple, safe, practical, and economical. Evaluation on the encapsulation efficiency and the release profile has been made indirectly by monitoring the dissolved oxygen level of the solution where the microspheres were incubated. Morphology of the microspheres was investigated using scanning electron microscopy. This proposed method has successfully used to prepare batches of microspheres having different encapsulation efficiencies and its potential applications have been demonstrated accordingly.

© 2009 Elsevier B.V. All rights reserved.

1. Introduction

The ability to deliver and have a controlled release of therapeutic agents at injured or targeted disease sites is an important aspect in drug development and regenerative medicine. Such system avoids unnecessary health side effects due to burst effect or overdose, ensuring optimum supply of drug that is required by the biological system for a prolonged period, and cutting down wastage of expensive drugs. Encapsulating the active agents within a polymeric matrix microsphere is a good option to achieve the objective as the polymer can act as the rate-controlling membrane to obtain the desired controlled release (Soppimath et al., 2001; Freiberg and Zhu, 2004). Besides encapsulation minimizes the deactivation of drugs during the delivery process due to the protection from the polymer shell and this ensures sufficient amount can reach the targeted area. Various therapeutic drugs have been investigated and proven to portray such controlled release manner, which also include peptides and proteins (Dai et al., 2005). Although so, modern microencapsulation of bioactive substances still continues to be an important area and effort mostly concentrates on formulation and protocol optimization strategies.

Various encapsulation techniques are readily available for microencapsulation of drugs and one of the most commonly employed is the solvent evaporation method. The method can be performed via various protocols and the selection for best option readily depends on the property of the compounds that are intended to be encapsulated (Li et al., 2008). Particularly for water-soluble compounds, the most often employed method is the double emulsion type, in which aqueous phase containing the dissolved compounds is entrapped into water insoluble matrices. The state of art falls on the unique inter-phase formation between immiscible aqueous and organic layers. Emulsion occurs when aqueous solution containing the dissolved compounds is suspended within organic solvent containing dissolved polymer. This emulsion mixture is then dispersed into a secondary aque-

* Corresponding author at: Bio-Medical Research Institute, Kyungpook National University Hospital, 50 Samduk 2-ga, Jung-gu, Daegu, Republic of Korea. Tel.: +82 53 420 5447; fax: +82 53 427 5447.
** Co-corresponding author at: Department of Material Science and Metallurgy, Kyungpook National University, Daegu, Sangyeok-dong, Buk-gu, Daegu, Republic of Korea. Tel.: +82 53 950 5562; fax: +82 53 950 6559.
E-mail addresses: jshuh@knu.ac.kr (J.-S. Huh), jolim@knu.ac.kr, findgracekr@yahoo.co.kr (J.O. Lim).

0378-5173/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.ijpharm.2009.10.005

ous solution forming secondary emulsion; commonly known as water–oil–water ($W_1/O/W_2$) double emulsion. Under such process, the first aqueous solution will be entrapped in the core of the polymer while physically the polymer is shaped into small fine spheres due to the secondary dispersion. The microspheres are usually stabilized using suitable surfactant and harden by continuously stirring in second aqueous solution to evaporate the organic solvent. In drug delivery system, different kinds of hydrophilic drugs have been successfully encapsulated in biodegradable polymers such as poly(lactic-co-glycolic acid) (PLGA) using this method (Fernández-Carballido et al., 2004; Fude et al., 2005).

Although the working concept behind microencapsulation by double emulsion solvent evaporation technique seems to be straight forward, its practicality during real development often found to be sophisticated. Such phenomenon is not surprising as this method involves lots of effecting parameters (e.g. materials and amount used, synthetic steps, and operation conditions) that may vary the properties of the microspheres such as the encapsulation efficiency and its releasing profile (Li et al., 2008). As a result, efforts have been initiated to harvest a better understanding between the effecting parameters of $W_1/O/W_2$ process on the morphology and the release profile of the microspheres (Kim and Park, 2004; Ito et al., 2007; Mao et al., 2007, 2008). This type of information is useful especially to give advance prediction, besides as planning guidelines to integrate some predetermined criteria onto the microspheres. Some efforts have also been devoted to construct mathematical models that describe the connection of parameters within the complex microencapsulation system and the controlled release profile (Halder and Sa, 2006). Nevertheless, such referencing only applies to systems having close similarity, as only chemically alike compounds behave in the same manner under controlled condition. As far as concerned in this aspect, there is still no investigation reported on the microencapsulation of small volatile water-soluble molecules (MW $<100\,\text{mol g}^{-1}$) using this conventional method. This group of compounds may also pose its own potential in various applications including as effective drugs for certain diseases or in facilitating the development of a biological system. Considering this, there will be a need in some cases to encapsulate the compound into having a controlled release mechanism and therefore performing a study to review the insight of such process will be necessary. Besides, microencapsulation can reduce the volatility and enhance the stability of such small molecules, which makes handling and transportation more convenient.

In this work, an investigation has been performed to review the effect on process-engineering factors in direct encapsulating small volatile water-soluble molecules into PLGA microsphere via double emulsion and solvent evaporation method. Hydrogen peroxide (H_2O_2) has been chosen as the model compound due to its small size and highly water-soluble property. Besides, H_2O_2 has the potential to act as antiseptic to kill bacteria, which can be used in areas such as agricultural for pest control (Gaikowski et al., 1999). When decomposed, oxygen will be produced and it is extremely useful in bio-medical applications. The possibility of having biodegradable microspheres that produces oxygen in a continuous and controlled manner can be the key to solve a lot of the problems currently faced in heath-care discipline. For instance, the microspheres can be used as medicine to oxygenate tissue that faces failure due to diseases such as stroke. This avoids damages to tissue especially those important part like brain tissue while measure is taken to recover back the normal respiratory system. Besides, the microspheres can also be incorporated into scaffolds for tissue engineering, in supplying oxygen to newly regenerated tissue and avoid necrosis due to oxygen diffusion limitation. PLGA was employed as the main encapsulation matrix due to its well-known properties and the approval by Food and Drug Administration (FDA) to be used in human. Different formulations, encapsulation protocols, and synthetic conditions were tested during the development. The presence of H_2O_2 was monitored indirectly by monitoring the level of dissolved oxygen in the solution, where the microspheres were incubated. Morphology of microspheres was observed using scanning electron microscopy (SEM). Research has been performed using cell culture study to demonstrate some potential applications of the microspheres in biological related areas.

2. Experimental

2.1. Reagents and materials

All chemicals used are analytical grade unless otherwise stated. Poly(lactic-co-glycolic acid) (PLGA, Boehringer Ingelheim, Germany), lactic acid/glycolic acid (LA:GA, 50:50, MW = $11{,}000\,\text{g mol}^{-1}$), methoxy polyethylene glycol (m-PEG, Sigma, USA), and polyvinyl alcohol (PVA, Sigma, USA, MW = $9000–11{,}000\,\text{g mol}^{-1}$) were used as purchased. PLGA ratio of 50:50 was chosen due to its intermediate physical property (Wu, 1995). Higher ratio of LA is found to increase the hydrophobic nature of the polymer, which directly reduces the encapsulation efficiency for hydrophilic drugs, while lower ratio of LA may causes difficulties in microspheres producing process due to poor solubility of the polymer in organic solvent. In this study, lower molecular weight PLGA was chosen in order to produce microspheres having denser shell in avoiding leaching of potential drugs with low molecular weight and high hydrophilicity. Hydrogen peroxide (H_2O_2, 30 and 50 wt%, Aldrich, USA) was used as the encapsulation compound and potassium iodide (KI, Aldrich, USA) was employed as a catalyst. Alginate microbeads were formed using alginic acid sodium salt from macrocystis pyrifera (kelp) (Sigma) that was gelled using calcium ion from calcium chloride dihydrate (Kanto Chemical Co., Inc, 99%). Catalase was obtained from Bovine Liver (Sigma, 2950 units/mg solid). Dichloromethane (DCM, Junsei Chemical, Japan) and triple distilled deionised water were used as solvents throughout the study.

In biological related study, Clone Neuro-2a (N2a, ATCC No. CCL-131) established from a spontaneous tumor of a strain A albino mouse cell was used for cell culture study. Cells were cultivated using standard DMEM media in a standard 6 well plate. PBS buffer was used thought out the study.

2.2. Preparation methodology of microspheres

H_2O_2 loaded PLGA microspheres were prepared by $W_1/O/W_2$ microencapsulation method. Briefly, 200 mg of PLGA was dissolved in 1.5 ml DCM and into this organic phase (O), 200 μl of aqueous H_2O_2 (W_1) was emulsified using a high speed homogenizer (Scientific Industries, INC, model G-560) for 30 s to form the first W_1/O emulsion. The mixture was then added with 1 ml of PVA (1.0, w/v%) as a stabilizer and re-homogenized for 30 s. Resulting emulsion then was poured slowly into a 200 ml aqueous phase (W_2) containing PVA (0.3%, w/v) and H_2O_2, which was constantly stirred using a magnetic bar. pH was adjusted to a predetermined value and the final emulsion was continuously stirred at ambient condition for a minimum period of 4 h to evaporate the DCM. The microspheres were sieved, collected by filtration, and washed three times with triple distilled deionised water. The microspheres were then frozen at $-70\,^{\circ}\text{C}$ overnight and followed with freeze-drying for 6 h. Final collected microspheres were kept at low temperature while not being used to avoid evaporation or decomposition of H_2O_2. Reference microspheres were produces using the same protocol but loaded with triple distilled deionised water replacing H_2O_2.The above given protocol was varied concerning the concentration of ingredients used, mixture of H_2O_2 in W_2 phase, and the removal of certain steps for investigation purposes.

2.3. Instrumentation and measurements

Dissolved oxygen (DO) concentration was recorded using a portable oxygen meter (Thermo Orion Series, 3 Star). The electrode of the meter (Thermo, ORION 081010 MD) was attached with an in-house designed seal, which can be screwed directly to commercial 10 ml borosilicate vial. This allowed the measurements of DO to be carried out under a closed system, avoiding unnecessary interferences from the environment.

All measurements were carried out using clean bench. A fixed amount of microcapsules (30 mg) was incubated in 3 ml of standard KI solution (0.01 M) that was sealed within a 10 ml borosilicate vial. After a predetermined duration of incubation, the cap of the vial was removed and replaced with the electrode to obtain the DO measurement. After each measurement, the vial was resealed for the next measurement. All incubations of microsphere batches were done in triplet. In order to minimize and eliminate the fluctuation of readings caused by environmental and instrumentation factors, the readings for KI solution in the absence of microspheres were taken as reference and all subsequent readings of samples were corrected against the reference readings. All the data points collected were depicted as mean ± standard deviation from the triplet measurements performed.

2.4. SEM images of microspheres

The prepared microspheres were dispersed on a double faced tape that is place on top of the metal sample stage. The gold sputtering was performed with ion sputtering device for a thin layer coating. The observation of microspheres was carried out with field emission scanning electron microscope (FE-SEM, Hitachi, Japan).

2.5. Cell culture

N2a cells were seeded to a 6 well plate having the density of 1.0×10^5 cells/well before treatment. The cells were incubated in the cell culture incubator for 24 h under controlled condition before further studies were carried out. Total volume of cell culture media in each well was fixed at 3 ml. For the study of microspheres, a known amount of microcapsules obtained from a fixed protocol and formulation were first added with 1 ml of culture media and mixed homogenously. Using the mixture as stock, a predetermined volume of the solution was, respectively, added into the wells that have been first plated with cells. The proliferation of the cells was monitored under microscopes and images were recorded after a fixed time of incubation. Each experimental condition was repeated at least 3–4 times for each batch of sample tested.

3. Results and discussion

3.1. Blending of polymer matrices

In encapsulating H_2O_2, series of modifications and improvements on the standard technique are required. Although microencapsulation of water-soluble compounds was commonly reported, to our best knowledge, no studies have pursued the similar thought on molecules having the molecular weight of 34 g mol^{-1}. Theoretically, the idea seems to give no significant variations in achieving the final encapsulation efficiency as those reported for larger compounds, but however practical study using H_2O_2 reviews that the result was not even near to the initial prediction. No significant existence of H_2O_2 was recorded from the solution that was incubated with the PLGA microspheres prepared using the conventional standard method. This may be caused by the high diffusion coefficient of H_2O_2 out from the polymer shell during the hardening process, when the microspheres were continuously stirred in aqueous media to evaporate the organic solvent. Immediate measures have been taken by reducing the stirring time from 4 to 2 h under moving air environment and increased the concentration of H_2O_2 at the core from 30 to 50 wt%. However, no clear improvement on the encapsulation efficiency was observed from those batches of microspheres (results not shown).

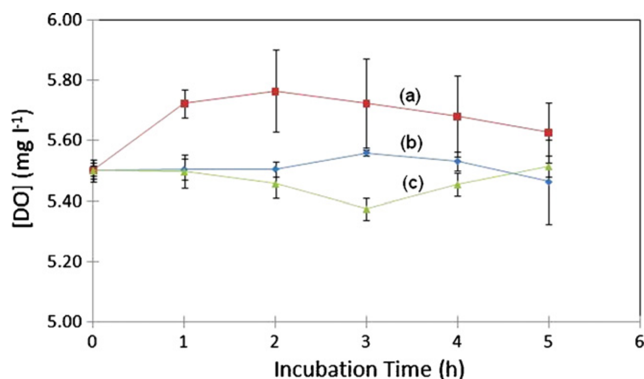

Fig. 1. DO concentration for incubation solution having microspheres produced (a) with the addition of PVA (2.5%, w/v) in the W_1, (b) with the addition of 60 μl of m-PEG into PLGA, and (c) without the addition of secondary matrices in any of the phases.

Li et al. (2008) have reviewed that choice of materials used in the microencapsulation by double emulsion solvent evaporation method can alter the encapsulation efficiency. Therefore, initial studies based on materials blending have been performed, in search for the key to effectively microencapsulate H_2O_2. First, PVA was added to W_1 (core phase) with the final concentration of 2.5% (w/v), before W_1 was emulsified in O phase. The intentions were to stabilize the primary W_1 emulsion by creating a hydrophilic microenvironment within the hydrophobic O phase and utilizing PVA to generate hydrogen bonding with H_2O_2 that can minimize leaching occurring from the microspheres. Results (Fig. 1) show better encapsulation was achieved when compared to microspheres prepared in the absence of PVA in W_1, agreeing with the expected outcomes. However, increment of DO concentration was still considerable low.

Second approach was taken to increase the hydrophilicity of the main PLGA matrix via blending the polymer with m-PEG. PEG segment is a hydrophilic part that has been reported widely used for changing the physicochemical properties of hydrophobic and biodegradable PLGA block segments (Kim et al., 2005; Jackson et al., 2007). Increase in hydrophilicity of matrices and resulting conformational change in polymer matrices was assumed to allow the deeply entrapped H_2O_2 in the polymer to release more easily during the incubation process. Although that is the initial idea, Fig. 1 shows no significant improvement in the encapsulation efficiency as indicated with no sign of increment in DO readings over the incubation period. This proves the failure in adopting such concept for this system. Such assumption may be appropriate for larger hydrophilic compounds but not with small molecule ones. In this case, H_2O_2 can easily escape due to its small size through the more hydrophilic polymer blend to W_2 solution during the preparation stage. As the result, the end obtained microspheres will be left without or low in H_2O_2 during the incubation tests.

3.2. Backward concentration gradient

In order to avoid the leaching, equilibrium factor was considered to create a condition where the diffusion will occur back to the microsphere instead of out or at least stabilized during the *meta stable* stage. *Meta stable* stage is defined as the period when the

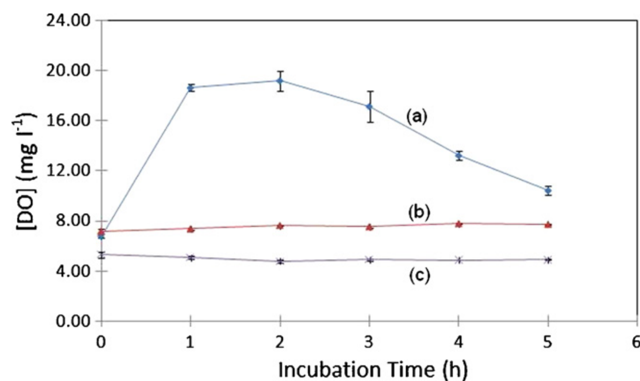

Fig. 2. DO concentration of incubation solution having microspheres produced (a) with 30 wt% H_2O_2 at W_1 and W_2, (b) with only 30 wt% H_2O_2 at W_1 and none at W_2, and (c) without H_2O_2 at W_1 and W_2. All volume of W_1 was fixed at 200 μl and W_2 fixed at 200 ml.

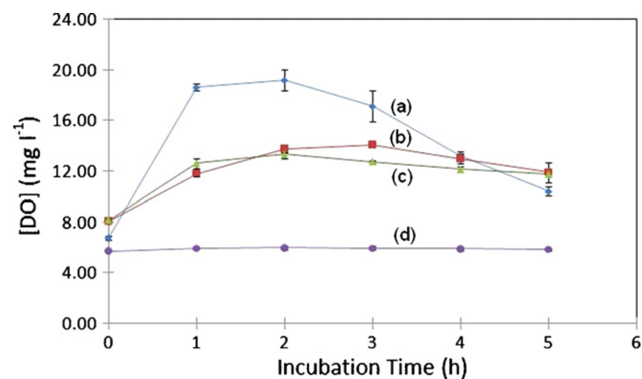

Fig. 3. DO concentration of incubation solutions having microspheres produced with (a) 30 wt% H_2O_2 at W_1 and W_2, (b) 30 wt% H_2O_2 at W_1 and 15 wt% H_2O_2 at W_2, (c) 50 wt% H_2O_2 at W_1 and 15 wt% H_2O_2 at W_2, and (d) none at W_1 and 15 wt% at W_2.

polymer is in dissolved form and physically soft during the early stage of the solvent evaporation process. In other words, the polymer is still in emulsion droplets rather than solid microspheres. It is aware that long stirring time for at least 4 h is required in this technique to completely remove of toxic organic solvent from the polymer. During this time, diffusion of H_2O_2 will occur within the polymer matrix and the direction is dependent to the physical conditions of the system. In fact, such diffusion mechanism is employed in creating a controlled release of drug during application, where the slow diffusion of encapsulated drugs occurs at targeted site when the favored conditions such as pH, temperature, or concentration gradient are met. Therefore, the conditions during production stage are usually altered to avoid having the favored conditions for leaching that is needed during application. To achieve such criteria in this study, a *backward concentration gradient* was applied to the system where H_2O_2 is added into the continuous phase and the concentration is controlled above or the same as the one added in the microsphere. Under such circumstance, H_2O_2 diffuses from high concentration region of the continuous phase toward the low concentration region of the microsphere core or in dynamic equilibrium when both the concentrations of the two phases are the same. This theoretically can avoid the outleaching of H_2O_2 from microsphere and Fig. 2 shows clearly that efficient microencapsulation has been achieved by such technique.

Leaching of H_2O_2 from polymer can only be avoided if the concentration in W_2 is greater or the same than W_1. In such a condition, there is no compromise to reduce the concentration for W_2 when higher concentration of encapsulation is required. Therefore, as far as cost, safety, and handling precaution are of concern, the volume of W_2 containing H_2O_2 needs to be reduced as a counter-balance measure. Reducing volume can cut down the amount of H_2O_2 need to achieve a certain level of concentration. This will be useful as well for other future works when the candidate drug is of an expensive one. In order to investigate this, original volume for stirring solution from 200 ml (H_2O_2, 15 wt %) was cut to 10 and 5 ml, respectively. Under such drastic volume changes, inhomogeneous distribution of particle size was obtained, having the largest in the millimeter range (result not shown). This was the resultant of the increased activity of coalescence under small volume effect, when two or more identical droplets collide and forming a larger one. Measure has been taken to overcome this by increasing the PVA concentration gradually in a serial of study from the initially set 0.3% (w/v). Higher concentration of surfactant can better stabilize emulsion droplets and avoid coalescence (Mao et al., 2007). It was found that 2.5% (w/v) of PVA concentration manage to produce fine particles that are similar to those previously obtained when observed under a standard optical microscope. When the microspheres were sieved and separated into a range of sizes, it was found that almost 60% (w/w) of the microspheres produced in stirring solution with the volume of 5 ml have the size smaller than 250 μm compared to 85% (w/w) when stirred in 10 ml, while almost 100% when stirred in 200 ml.

To avoid wastage of drugs especially those expensive one that were added in the continuous phase, recycling it via various methods such as solvent evaporation or standard separation technique is a possible approach. As the volume of the stirring solution has been successfully cut to its minimal, such effort became easier and practical to be carried out.

The effect of concentration for H_2O_2 in both the W_1 and W_2 phases was investigated by first fixing the H_2O_2 concentration at W_1 with varied concentration at W_2, following the fixing of concentration at W_2 and varied the concentration at W_1. Three sets of DO profile were obtained (Fig. 3), and the results show that the concentration factor in W_2 has greater influence rather compared to W_1. In this case, dynamic equilibrium between the concentration of H_2O_2 in both W_1 and W_2 separated by O phase was expected during the stirring stage when polymer shell has yet fully hardened. Due to the fact that the volume of W_2 is far excess compared to W_1, any gradient in concentration between the phases will equilibrate toward the concentration of W_2. W_1 concentration did not have the strength in sustaining its initial level due to the small volume and the high diffusion rate. In this sense, the end encapsulated H_2O_2 concentration should be similar to the concentration at W_2 and this is also maybe the reason why low or no H_2O_2 was observed when the microspheres were produced in W_2 without having H_2O_2 added.

3.3. Effect of pH

During the synthesis of microspheres, pH effect has been studied by controlling the stirring solution at pH 6.85 and 8.85. SEM micrographs (Fig. 4) show that surface morphology of the microcapsules was directly influenced by the pH. When stirred at lower pH, surface of the microcapsules was not smooth and has tiny holes. Most of the microspheres were not round in shape rather more to donut-shaped particles. However with higher pH, the surface became smooth and uniformly round in shape. Although H_2O_2 is reported to be most stable at neutral pH and usually decomposes at high alkaline media, this observation was not seen in this work. Rather lower pH shows greater decomposition as evidenced from the SEM micrograph. The possible reason for this observation may be related to the presence of PVA in W_2. When pH was increased, deprotonation of the hydroxyl group on the PVA chains can occur

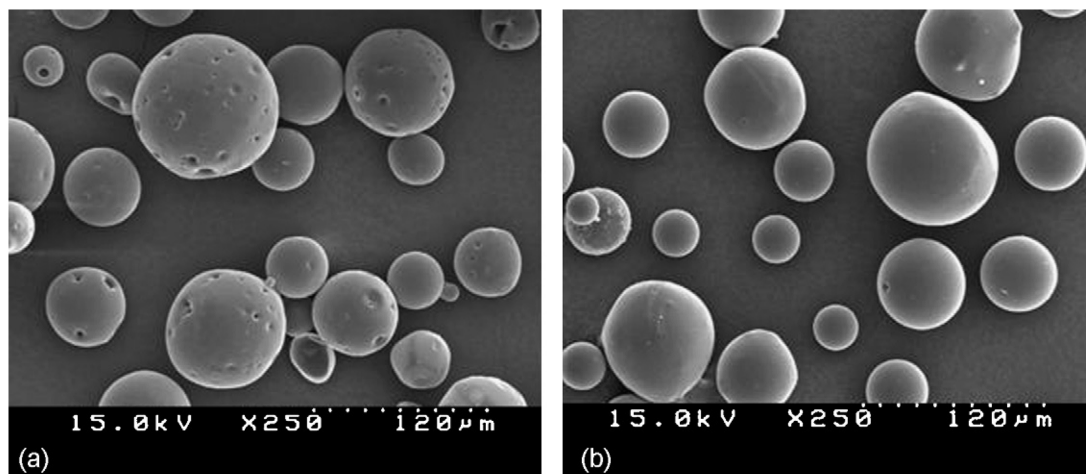

Fig. 4. SEM micrograph of microspheres synthesized at a controlled pH of (a) 6.85 and (b) 8.85.

leaving negatively charged terminal that can form cheating complexes with impurities. Thus this can avoid the decomposition of H_2O_2 by those impurities which can act as a catalyst. Opposite scenario will happen when pH was lowered. This causes protonation of PVA and subsequently the dissociation of possible impurities complexes that are present in the system. As this occurs, the impurities will be freed and diffuse through the polymer layer causing H_2O_2 being decomposed. As a result, all subsequent batches of microspheres were prepared in the pH range of 8–9.

3.4. Dissolved oxygen profile and diffusion rate

In this study, direct determination of H_2O_2 residue in the microspheres was not performed although it can be done by various methods such as the one suggested by Bae et al. (2009). Those methods are lengthy and therefore a relatively easier indirect method was employed by monitoring the release of oxygen in the incubation solution as H_2O_2 decomposes. As the decomposition process was chemically known and analytically balance, a direct analogy can be used to relate the trend of profile for the presence of H_2O_2 using the DO measurement values. KI was added as a catalyst to ensure complete decomposition of H_2O_2 occurs, besides increasing the kinetic rate of decomposition to speed up the study. In allowing the comparison of profiles to be made more accurately between different batches of microspheres and the one recorded from naked H_2O_2 (without microencapsulation), all the data points were converted into normalized percentage scale to eliminate the factor of concentration. The results shown in Fig. 5 indicate that the microencapsulation process has successfully reduced and prolonged the release profile of H_2O_2. The profile of Fig. 5(a) was obtained from sample that is prepared with 30 wt% H_2O_2 at both the W_1 and W_2 phases and having the average size in the range of 25–250 μm. Naked H_2O_2 added to KI solution reaches its maximum production of oxygen within the first 2 h and the DO concentration drops drastically after that. This can be rationalized as the H_2O_2 is readily excess by the catalyst to produce maximum amount of oxygen at the initial period of incubation causing serious burst effect. However within short time, all available H_2O_2 will be consumed and continuous generation of oxygen is no longer possible. Therefore, drastic drop in the DO profile with weak DO sustaining strength was observed when DO equilibrates with the environment. In all cases when H_2O_2 was encapsulated, lower initial burst effect was obtained besides more controlled and prolonged DO profiles (not all results were shown to avoid confusion and over-crowded graphs). Diffusion needs to occur first either H_2O_2 out or iodide ion into the polymer phase before decomposition can take place to generate oxygen. Although DO equilibrates with the air phase causing a net drop in readings after 2–3 h of incubation for all batches of microspheres, the degree of decrease was far lower compared to the naked H_2O_2 profile. This slower rate of release can provide sufficient time to ensure complete decomposition of H_2O_2 occurs once diffused out of the PLGA shell. This factor is important as H_2O_2 decomposition rate is considerably slow in the absence of catalyst and this can reduce the possibility of naked H_2O_2 polluting the surrounding environment in excess, which can be very harmful especially if the surrounding is of a biological one.

3.5. The effect of microspheres size

In this study, the effect of microspheres size toward the release of H_2O_2 has also been investigated. Microspheres produced under same protocol were separated into three groups of sizes: smaller than 25 μm, between 25 and 250 μm, and larger than 250 μm using a sieve. The result recorded (Fig. 6) shows a dependent trend of DO profiles with the microspheres physical sizes. Microspheres with larger size sustain better DO concentration level compared to the smaller size microspheres after a fixed incubation time. Besides, lower initial burst was also observed. This observation was expected as larger microspheres have smaller surface area ratio and thicker polymer shell, which reduces diffusion rate. This open the opportunity to group the microspheres into categories of different release rates via size range discrimination. Therefore, better control of dosage that is required for specific applications can be made pos-

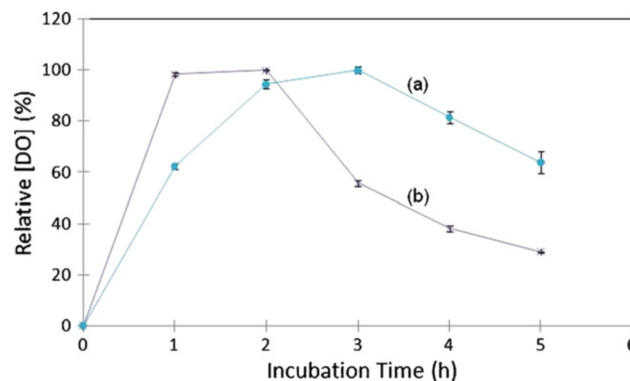

Fig. 5. DO profile recorded for (a) microspheres encapsulated with H_2O_2 that is prepared with 30 wt% H_2O_2 at both the W_1 and W_2 phases and having the average size in the range of 25–50 μm and (b) naked H_2O_2 without encapsulation.

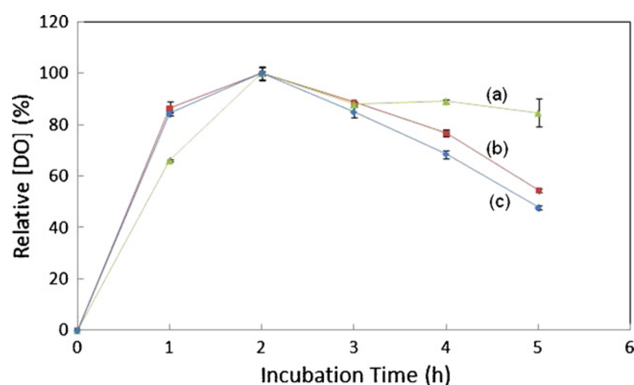

Fig. 6. DO profile recorded for solutions having microspheres encapsulated with H_2O_2 with particle size of (a) larger than 250 μm, (b) between 25 and 250 μm, and (c) smaller than 25 μm.

sible and standardize. Currently, study is still on going in obtaining optimum synthetic protocol that can precisely produce uniformly microspheres within certain narrow range of size. In parallel, further studies are also intended to establish the DO profile *of* the incubation solution for a longer duration without or minimum use of catalyst.

3.6. Effect of freeze-drying

Freeze-drying process is normally performed in many microencapsulation works for drugs to remove excess water content under low temperature and pressure. It is also been reported by Kim and Park (2004) as having a critical effect toward the sustained release of drugs. The freezing process can cause micro-cracks on the polymer matrix while the removal of water produces micro-channels, which both favor drugs release. However in this study, H_2O_2 was chosen as the encapsulation molecule that has almost similar physical properties like water. Under such circumstance, it is predicted that H_2O_2 will be removed along with water molecule during the drying process. In the same report by Kim and Park, freeze-drying process has been demonstrated to remove almost 98% of water content out of microspheres. This result was also observed in this study with microspheres that have been freeze-dried for 6 h, when total loss of H_2O_2 was evidenced with no increment of DO level in the incubation solution. To avoid such lost of H_2O_2, preparation of all consecutive batches of microspheres skips the freeze-drying process and instead just dried overnight under flowing air on clean bench. The freezing process was found to have no significant effect toward the encapsulated amount of H_2O_2. Therefore, all microspheres samples were frozen under −70 °C during storage.

Table 1
Preparation descriptions for microspheres samples used for the investigation for some potential applications.

Sample	H_2O_2 in W_1 (%, v/v)	H_2O_2 in W_2 (%, v/v)
A	30.0	15.0
B	6.0	1.0

3.7. Potential application for the microspheres

In this study, H_2O_2 was treated as drug and encapsulated within PLGA microspheres. Two batches of microspheres (high and low encapsulation) were prepared having the detail as summarized in Table 1. All other parameters (synthetic procedures, ingredients, synthetic conditions, etc.) have been fixed to be equivalently identical for comparison purpose. The size of the microspheres for both the batches was controlled in the range of 25–250 μm using a sieve. Cell culture study using neural Clone Neuro-2a cell was performed on these samples and the outcome of the study has lead to the suggestions of its potential applications. Observations from the initial works will be presented to demonstrate the relevancy of possible applications, but however details of those studies were out from the scope of this paper and will not be included in the discussion.

When Sample A was incubated in well having the cell for 24 h under controlled normal condition, serious cellular damage such as granule formation in the cytoplasm was observed as shown in Fig. 7. Such observation was expected as H_2O_2 is a strong oxidizing agent that can harm cell and cause serious damages. This directly opens the potential to use such system as a medicine for the application in fighting against cancer cell. Mesiwala et al. (2003) have reported in their in vitro and in vivo studies that H_2O_2 has clear antitumor effects and is dependent on the time of exposure and the concentration. It is demonstrated that longer exposure and higher concentration of H_2O_2 can kill cells effectively, but however the effect was not selective. In other words, all in contact cells are damaged including healthy one. Therefore, encapsulating the H_2O_2 in high concentration as proposed here can be the solution for this. Having more advancement on the delivery system, there is the possibility to deliver the H_2O_2 to the targeted site without harming the surrounding, and released controllably to kill cell identified.

Sample B having lower concentration of H_2O_2 however shows a total opposite result from Sample A as shown in Fig. 8. The cells with the microspheres incubated were observed to be extremely healthy and can proliferate and maintain the viability well. Although quantitative counting of cell is not been carried out, the images obtained under microscope can significantly show the difference in the cell amount. The number of cells when incubated with microspheres

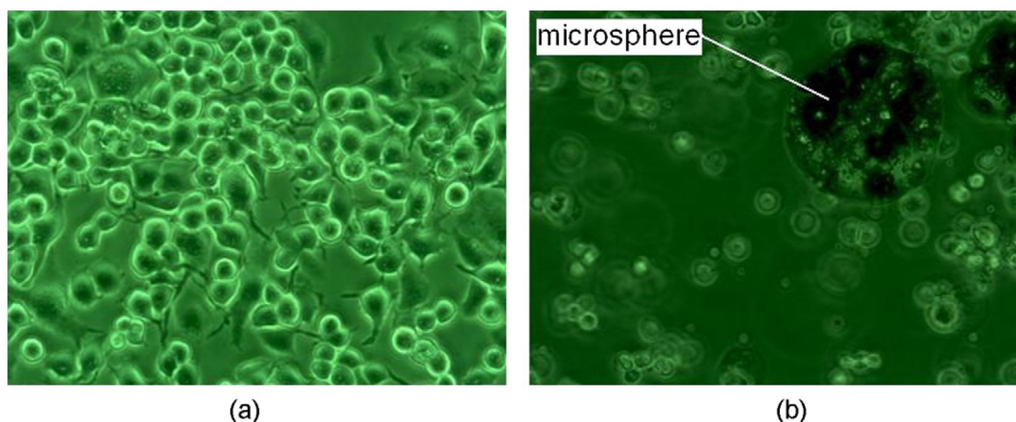

Fig. 7. Image of cells taken after incubated 24 h with 3 ml of DMEM consisting (a) no microspheres and (b) 0.2 mg of microspheres Sample A.

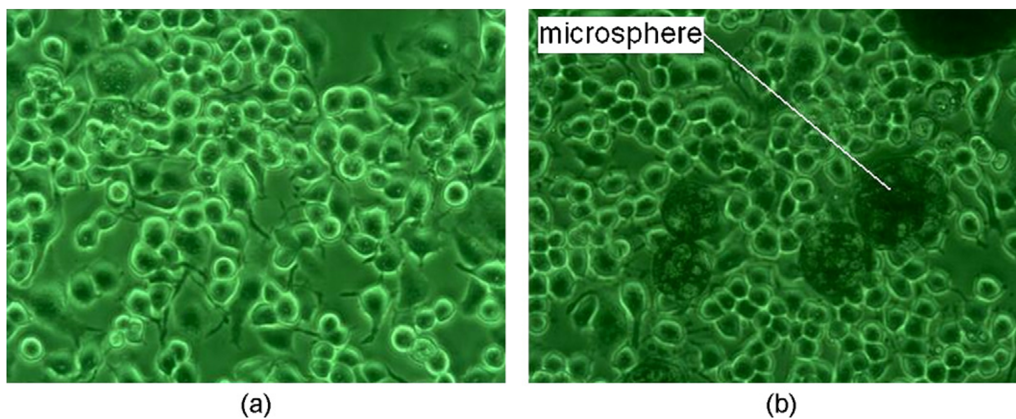

Fig. 8. Image of cells taken after incubated 24 h with 3 ml of DMEM consisting (a) no microspheres and (b) 0.2 mg of microspheres Sample B.

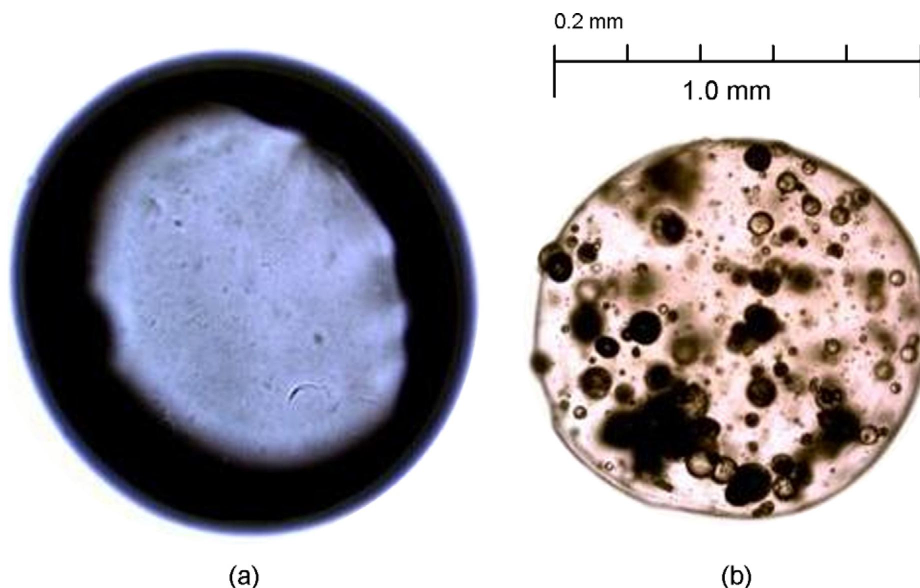

Fig. 9. Optical microscope image for alginate microbead having catalase grafted (a) without PLGA microspheres and (b) with PLGA microspheres incorporated.

was observed to be more in amount compared to the culture well in the absence of microspheres. This observation confirms that the materials and modified method produce biologically safe products and the harming effect shown by Sample A was solely caused by the H_2O_2. Possible explanation for such observation may be on the nature of H_2O_2 that is thermodynamically unstable and spontaneously decomposes into oxygen at a very slow rate. As this occurs, oxygen is generated which turns the harmful affect of H_2O_2 into useful oxygen for cell survival. Encapsulation has been the key success here as it managed to separate the direct contact of H_2O_2 with cells, besides creating as slow release that allows sufficient decomposition time for the H_2O_2 into oxygen. Having this unique feature, the microspheres can be used to generate oxygen for applications such as artificial blood, medicine to oxygenate tissues under hypoxia condition, and oxygen micro-reactor in tissue engineering. In fact, the use of H_2O_2 for generating oxygen in tissue engineering scaffolds has been demonstrated using peroxide salts and gave promising results for further clinical trials (Harrison et al., 2007; Oh et al., 2009). However system using direct H_2O_2 as proposed here can eliminate the presence of cations to the surrounding as the decomposition produces just water and oxygen. Higher level of oxygen can be achieved by encapsulating more concentrated H_2O_2 and then coated with catalyst to increase the decomposition rate. This also can ensure no leakage of naked H_2O_2 to the surrounding. One of such attempts carried out in this work was by producing a blend of PLGA microspheres encapsulated with H_2O_2 into alginate beads, in which the alginate has been grafted with catalase (Fig. 9). Catalase is a biological enzyme that specifically decomposes H_2O_2 with fast turnover. The alginate beads were produced via dropping small droplet of alginate solution into solution containing calcium ion.

4. Conclusions

This work has successfully demonstrated a direct microencapsulation for small, volatile, and water-soluble compounds via double emulsion solvent evaporation technique, represented by the model molecule of H_2O_2. Various engineering factors affecting the microencapsulation process of H_2O_2 were revealed and discussed accordingly. Key finding in this work was the equilibrium aspect consideration of having a higher concentration of H_2O_2 in the stirring solution ($[W_2] \geq [W_1]$) during the production process that ensures effective microencapsulation. The potentials have been certainly demonstrated by the initial biological related studies that prove the biocompatibility of the microspheres under controlled conditions. Further optimizations and refinements for sure can facilitate the development to utilize the microspheres in real clinical application.

Acknowledgement

This work was supported by the National Research Foundation (NRF) grant funded by the Korea government (MEST No. 2009-0083753). Thanks are also forwarded to those who provided direct contribution on the experimental works, comments, and supportive materials that were used in this paper.

References

Bae, S.E., Son, J.S., Park, K., Han, D.K., 2009. Fabrication of covered porous PLGA microspheres using hydrogen peroxide for controlled drug delivery and regenerative medicine. J. Control. Release 133, 37–43.

Dai, C., Wang, B., Zhao, H., 2005. Microencapsulation peptide and protein drugs delivery system. Colloids Surf. B: Biointerfaces 41, 117–120.

Fernández-Carballido, A., Herrero-Vanrell, R., Molina-Martínez, I.T., Pastoriza, P., 2004. Biodegradable ibuprofen-loaded PLGA microspheres for intraarticular administration: effect of Labrafil addition on release in vitro. Int. J. Pharm. 279, 33–41.

Freiberg, S., Zhu, X.X., 2004. Polymer microspheres for controlled drug release. Int. J. Pharm. 282, 1–18.

Fude, C., Dongmei, C., Anjin, T., Mingshi, Y., Kai, S., Min, Z., Ying, G., 2005. Preparation and characterization of melittin-loaded poly (DL-lactic acid) or poly (DL-lactic-co-glycolic acid) microspheres made by the double emulsion method. J. Control. Release 107, 310–319.

Gaikowski, M.P., Rach, J.J., Ramsay, R.T., 1999. Acute toxicity of hydrogen peroxide treatments to selected life stages of cold-, cool-, and warmwater fish. Aquaculture 178, 191–207.

Halder, A., Sa, B., 2006. Preparation and in vitro evaluation of polystyrene-coated diltiazem–resin complex by oil-in-water emulsion solvent evaporation method. AAPS Pharm. Sci. Technol. 7, E105–E112.

Harrison, B.S., Eberli, D., Lee, S.J., Atala, A., Yoo, J.J., 2007. Oxygen producing biomaterials for tissue regeneration. Biomaterials 28, 4628–4634.

Ito, F., Fujimori, H., Makino, K., 2007. Incorporation of water-soluble drugs in PLGA microspheres. Colloids Surf. B: Biointerfaces 54, 173–178.

Jackson, J.K., Hung, T., Letchford, K., Burt, H.M., 2007. The characterization of paclitaxel-loaded microspheres manufactured from blends of poly(lactic-co-glycolic acid) (PLGA) and low molecular weight diblock copolymers. Int. J. Pharm. 342, 6–17.

Kim, M.S., Seo, K.S., Hyun, H., Kim, S.K., Khang, G., Lee, H.B., 2005. Sustained release of bovine serum albumin using implantable wafers prepared by MPEG-PLGA diblock copolymers. Int. J. Pharm. 304, 165–177.

Kim, T.H., Park, T.G., 2004. Critical effect of freezing/freeze-drying on sustained release of FITC-dextran encapsulated within PLGA microspheres. Int. J. Pharm. 271, 207–214.

Li, M., Rouaud, O., Poncelet, D., 2008. Microencapsulation by solvent evaporation: state of the art for process engineering approaches. Int. J. Pharm. 363, 26–39.

Mao, S., Shi, Y., Li, L., Xu, J., Schaper, A., Kissel, T., 2008. Effects of process and formulation parameters on characteristics and internal morphology of poly(D,L-lactide-co-glycolide) microspheres formed by the solvent evaporation method. Eur. J. Pharm. Biopharm. 68, 214–223.

Mao, S., Xu, J., Cai, C., Germershaus, O., Schaper, A., Kissel, T., 2007. Effect of WOW process parameters on morphology and burst release of FITC-dextran loaded PLGA microspheres. Int. J. Pharm. 334, 137–148.

Mesiwala, A.H., Farrell, L., Santiago, P., Ghatan, S., Silbergeld, D.L., 2003. The effects of hydrogen peroxide on brain and brain tumors. Surg. Neurol. 59, 398–407.

Oh, S.H., Ward, C.L., Atala, A., Yoo, J.J., Harrison, B.S., 2009. Oxygen generating scaffolds for enhancing engineered tissue survival. Biomaterials 30, 757–762.

Soppimath, K.S., Aminabhavi, T.M., Kulkarni, A.R., Rudzinski, W.E., 2001. Biodegradable polymeric nanoparticles as drug delivery devices. J. Control. Release 70, 1–20.

Wu, X.S., 1995. Synthesis and Properties of Biodegradable Lactic/Glycolic Acid Polymers. Marcel Dekker, New York.