ENCAPSULATION AND RELEASE PROFILE OF PROTEIN CAGE FROM A POLYMERIC MATRIX

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Protein cages have been widely investigated as molecular drug carrier. E2 protein from Bacillus stearothermophilus forms a dodecahedral cage structure of approximately 24 nm in diameter. To formulate a sustainable release profile, E2 protein was further encapsulated into poly(lactic-co-glycolic acid) (PLGA) microparticles to form a composite structure using water-in-oil-in-water (W/O/W) double emulsion method. The influence of fabrication parameters on microparticle morphology and E2 protein release profile were investigated. The microparticle size increased when the stirring speed of the second emulsion decreased. Decrease in the volume of external aqueous phase led to the reduction of microparticle size without affecting its porosity. The higher ionic concentration of external aqueous phase in the presence of surfactant resulted in microparticles with closed pores on surface. Increase in polymer concentration also led to the formation of less porous microparticles. The E2 protein was not dissociated upon encapsulation into PLGA microparticles based on the unchanged particle size of E2 protein. E2 protein release was studied in phosphate-buffered saline solution at 37°C. The initial burst and release rate were lowered as the surfactant concentration in external water phase during the fabrication process was increased from 0.1% to 1% (w/v). After 14-day incubation, no observable polymer degradation was found while the surface of microparticles appeared to be smoother than before incubation.

Keywords: Protein cage; nanocage; microparticles; double emulsion.

1. Introduction

The E2 protein, derived from Bacillus stearothermophilus pyruvate dehydrogenase, has been investigated as a potential drug carrier.1,2 Sixty subunits self-assemble into a hollow dodecahedral cage structure of approximately 24 nm in diameter with twelve 5-nm openings.1,3 These openings result in porous nanosphere appearance of the E2 protein. The E2 protein can maintain its integrity when foreign molecules, such as dye or drugs, are introduced to the internal surface.1,2 The E2 protein is also amenable to various modifications.1 Recently, Dalmau et al. has shown that a pH-sensitive switch can be engineered onto the E2 protein to facilitate the release of molecular contents in the acidic environment.2,4

To fabricate a reservoir for localized sustainable release, the E2 protein was encapsulated into...
poly(lactide-co-glycolide) (PLGA) microparticles in this investigation. PLGA was used due to its biocompatibility and biodegradability. The encapsulated substances in PLGA microparticles have been reported to be released at a controlled rate over a prolonged period of time, allowing less frequent administrations. Additionally, microparticles encapsulation provides an advantage of localized release. The E2 protein was engineered to have a pH-sensitive linker, resulting in 90% drug release within 72 h at pH 5.0. The fluorescence microscopy result indicated that E2 protein was efficiently internalized into breast cancer cells and only the drug demonstrated cytotoxic effect on the cancer cells in vitro. Hence, the drug-loaded PLGA/E2 composite microparticles may demonstrate a potentially efficient treatment outcome.

In this investigation, water-in-oil-in-water (W/O/W) double emulsion method was used to fabricate the protein-loaded PLGA microparticles. Bovine serum albumin (BSA) was chosen as a model protein during the optimization of the fabrication parameters. The influence of fabrication parameters on the morphology of microparticles was extensively studied using light microscope and field emission electron microscope (FESEM). The integrity of E2 protein after the encapsulation process was monitored by hydrodynamic size measurement using dynamic light scattering (DLS) technique. The in vitro E2 protein release study was carried out in phosphate buffered saline (PBS) solution at 37°C and the morphology of microparticles after protein release was observed under FESEM.

2. Materials and Methods

2.1. Materials

PLGA was from Boehringer Ingelheim with lactide:glycolide molar ratio of 75:25 and inherent viscosity of 0.68 dl/g. Poly(vinyl alcohol) (PVA) (87–89% hydrolyzed, molecular weight of 13,000–23,000) and PBS solution were from Sigma-Aldrich. Dichloromethane (DCM) was from Analar. GF buffer solution (50 mM K2HPO4, 150 mM NaCl, 5 mM EDTA, 0.02% w/v Na3, pH 7.4) was used to dissolve the protein. BSA, supplied by Calbiochem, was used as a model protein to achieve the best fabrication parameters. E2 protein was produced in E. coli following the published method. Briefly, E. coli strain BL21 (DE3) was transformed with pET-11a plasmid containing the E2 protein gene (pE2, a generous gift from Prof. Szu-Wen Wang at University of California, Irvine). Protein production was induced with 1 mM IPTG for 3 h at 37°C. The cells were harvested, broken, and the insoluble fraction was removed by ultra centrifugation. The soluble fraction was heat treated at 72°C for 10 min and subjected to ion exchange chromatography to obtain the pure E2 protein. Pierce BCA™ Protein Assay Kit was purchased from Thermo Scientific.

2.2. Optimization of fabrication parameters

The fabrication process of W/O/W double emulsion was first optimized using BSA as the model protein. The fabrication parameters were varied, including the stirring speed of the second emulsification, volume of the external aqueous phase (W2), type of solutions as W2, concentration of surfactant in W2, polymer concentration in the oil phase (O) and protein concentration in the inner aqueous phase (W1). Throughout the optimization process, stirring speed for the first emulsification was kept constant at 10,000 rpm for 30 s since it was reported that there was no significant difference in surface morphology when the homogenization speed was at 13,500 and 20,500 rpm. To minimize the possible mechanical damage on the encapsulated protein, homogenization at 10,000 rpm for 30 s was used in this investigation.

2.3. Preparation of E2-loaded PLGA microparticles

E2-loaded PLGA microparticles were fabricated as follows. GF buffer solution (1 ml) containing E2 protein (6.1 mg/ml, the internal aqueous phase, W1), was emulsified in 10 ml PLGA-DCM solution (5% w/v, the oil phase, O) using a high-speed homogenizer (Heidolph Silent Crusher M) at 10,000 rpm for 30 s. The first emulsion (W1/O) was then injected into 200 ml 90% concentrated GF buffer solution containing 0.1% or 1% w/v PVA (the external aqueous phase, W2) at 400 rpm using a magnetic stirrer for 1 h. The emulsion was continuously stirred at 200 rpm for another 3 h in a fume hood to allow DCM evaporation and micro-particles solidification. The E2-loaded microparticles were then filtered, washed with deionized (DI) water, and dried overnight at room temperature (25°C). The microparticles were stored at −20°C.
2.4. **Characterization of microparticles**

2.4.1. **Microparticles size analysis**

Microparticle shape and size were examined using a light microscope (Olympus BX51). After microparticles solidified, small amount of the final emulsion was placed onto a glass slide and observed under the light microscope. Pictures were digitally taken and analyzed using the microscope software to calculate the microparticle size (averaged over 200 particles).

2.4.2. **Surface morphology observation**

The surface morphology of microparticles was examined using a FESEM (JEOL JSM-6700F). Small amount of microparticles were mounted onto a metal stub using double-sided carbon adhesive tape. Samples were coated with a thin layer of platinum at 10 mA for 200 s using a sputter (JEOL JFC-1600).

2.4.3. **Confirmation of E2 protein integrity**

The integrity of E2 protein after W/O/W double emulsion process was examined using DLS technique after extracting the E2 protein from PLGA microparticles. The microparticles (20 mg) were first dissolved in 1.2 ml of DCM. GF buffer solution (2.4 ml) was added and the mixture was shaken for 7 h using a New Brunswick Scientific shaker at 200 rpm at 37°C. The mixture was then centrifuged at 500 x g for 5 min to separate the DCM from the GF buffer solution containing the extracted E2 protein. The size of E2 protein was determined using DLS (Malvern Zetasizer Nano ZS90).

2.4.4. **Determination of protein encapsulation efficiency**

The amount of protein encapsulated in PLGA microparticles was determined by measuring the protein quantity after hydrolyzing PLGA microparticles to water-soluble monomers. The hydrolysis method was used because some protein precipitated at the interfacial layer between DCM and aqueous solution in the extraction method, which subsequently lowered the measured protein loading. Protein-loaded microparticles (20 mg) were hydrolyzed in 1.2 ml of 0.3 M NaOH. The solution was diluted five times with GF buffer solution, and analyzed using Pierce BCA™ Protein Assay Kit. Each sample was measured in triplicate.

The protein loading and encapsulation efficiency of protein in PLGA microparticles were calculated using the following equations:

\[
\text{Theoretical protein loading} = \frac{W_{\text{added protein}}}{W_{\text{added polymer}} + W_{\text{added protein}}} \times 100\% \quad (1)
\]

\[
\text{Actual protein loading} = \frac{W_{\text{protein}}}{W_{\text{microparticles}}} \times 100\% \quad (2)
\]

\[
\text{Encapsulation efficiency} = \frac{\text{actual protein loading}}{\text{theoretical protein loading}} \times 100\% \quad (3)
\]

2.5. **In vitro E2 protein release study**

*In vitro* E2 protein release was carried out in triplicate. PLGA microparticles (20 mg) was suspended in 2 ml PBS (pH 7.4) and incubated in water bath at 37°C without agitation to simulate *in vivo* conditions. At predetermined time points, 1 ml of the supernatant was analyzed for protein concentration using Pierce BCA™ Protein Assay Kit and the same volume of fresh PBS was refilled. On the 14th day, samples were washed with DI water and dried in a vacuum oven overnight at room temperature (25°C). The samples were then observed under FESEM to examine the morphological changes of the microparticles.

3. **Results and Discussion**

3.1. **Investigation on fabrication parameters**

3.1.1. **Stirring speed of the second emulsification**

Stirring speed of the second emulsification is important in determining microparticle size because it provides shear energy to disperse the first emulsion (W1/O) into the external aqueous phase (W2).\(^{11,12}\) In this investigation, the second emulsion was produced by stirring at 8,000 rpm for 30 s, 1,000 rpm for 30 min, and 400 rpm for 60 min.

Figure 1 shows the light microscope and FESEM images of microparticles fabricated at stirring speed...
of 8,000 rpm. When the stirring rate for the second emulsification was close to the first emulsification (8,000 rpm to 10,000 rpm), the resulting diameter of the final emulsion droplets was close to the inner aqueous droplets; hence the inner aqueous phase was not encapsulated completely. Similar observation was confirmed through light microscope images that showed most of microparticles did not have inner hollow core, indicating that the internal aqueous phase (W1) was not well encapsulated within the microparticle matrix. On the FESEM image, open pores were observed on the surface of microparticles, which might result from the fact that both inner W1 droplets and the final emulsion droplets were of similar size. This was similar to the results reported by Rosca et al. The microparticles size was determined to be 11 ± 3 µm.

Decreasing the stirring speed from 8,000 rpm to 1,000 rpm resulted in an increase of microparticle size from 11 ± 3 µm to 101 ± 28 µm and a shape alteration. Some of the microparticles were not spherical but tablet in shape. Shape alteration of microparticles indicated that during microparticle solidification, the emulsion droplets were exposed to excessive shear energy, thus the distorted microparticle shape. The microparticles had open pores on the surface.

At the stirring speed of 400 rpm, the microparticle size was close to that at 1,000 rpm, which was 108 ± 33 µm to 101 ± 28 µm (at 1,000 rpm). The microparticles were more uniform and less distorted in shape when compared with those produced at 1,000 rpm and were also porous. When the second emulsion was conducted at 400 rpm for 60 min, the inner droplets were much smaller than the final emulsion droplets as observed by light microscope (data not shown) indicating that the inner droplets were successfully encapsulated. Stirring speed at 1,000 rpm may impose excess shear energy on microparticles during solidification process, and thus the microparticles morphology was distorted to be pellet like. As 400 rpm was much lower than 1,000 rpm, the solidified microparticle shape was well defined.

### 3.1.2. Volume of external aqueous phase (W2)

The removal rate of solvent was reported to affect the microparticles properties. The solvent, here DCM, was eliminated by extraction followed by evaporation process. To vary the extraction rate, the volume of W2 was varied while other conditions were kept constant. When W2 volume was reduced from 400 ml to 200 ml, the microparticles size decreased from 108 ± 33 µm to 83 ± 26 µm (Table 1). This was possibly due to the increase in removal rate of DCM at 400 ml of W2, where microparticles were formed faster and harder to break down. The surface morphology of microparticles at W2 of 200 ml and 400 ml showed little difference (Fig. 2), where both had open pores. The morphology of microparticles produced at 200 ml of W2 appeared to be better defined as the removal rate of DCM was slower than at 400 ml of W2. As microparticles with well-defined morphology were preferred and small volume of W2 was easier to manipulate during the fabrication process, W2 volume of 200 ml was subsequently used to prepare the microparticles.

![Fig. 1. Microparticles prepared at stirring speed 8000 rpm for 30 s, 200 ml of DI water with 1% PVA as external phase and polymer concentration of 5% w/v. (a) Light microscope image and (b) FESEM image.](image-url)
3.1.3. Effect of ionic concentration in the external aqueous phase (W2)

Since high concentration gradient between W1 and W2 decreased loading efficiency,\textsuperscript{10} two types of solutions, DI water with PVA and GF buffer with PVA, were used as the external aqueous phase, in this work to study the effect of concentration gradient on microparticles properties. Microparticles fabricated using DI water with PVA as external aqueous phase (W2) were more porous than those prepared using GF buffer with PVA (Fig. 2). When DI water with PVA was used as W2, there was a large concentration gradient between the inner droplets (protein-GF buffer solution as W1) and the external aqueous phase W2. Due to this concentration gradient, water may diffuse into the internal phase (W1) from external aqueous phase through the organic layer during the elimination of organic solvent (DCM). Water diffusion may result in the swelling of emulsion droplets due to the increase of W1 volume, which would lead to the formation of pores on microparticles surface. This highly porous condition was minimized when GF buffer solution with PVA was used as the external aqueous phase W2, where microparticles with closed pores on surface were formed (Fig. 2). This was probably due to the reduction of the concentration gradient between

![Microparticles surface morphology](image)

**Table 1. Fabrication parameters for PLGA microparticles using W/O/W double emulsion method and the size of the solidified microparticles.**

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Microparticle size (μm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stirring speed of second emulsification (rpm)</td>
<td></td>
</tr>
<tr>
<td>400</td>
<td>108 ± 33</td>
</tr>
<tr>
<td>1000</td>
<td>101 ± 28</td>
</tr>
<tr>
<td>8000</td>
<td>10 ± 3</td>
</tr>
<tr>
<td>Volume of external aqueous phase (ml)</td>
<td></td>
</tr>
<tr>
<td>200</td>
<td>83 ± 26</td>
</tr>
<tr>
<td>400</td>
<td>108 ± 33</td>
</tr>
<tr>
<td>Types of solution used as external aqueous phase</td>
<td></td>
</tr>
<tr>
<td>DI water</td>
<td>83 ± 26</td>
</tr>
<tr>
<td>GF buffer</td>
<td>67 ± 13</td>
</tr>
<tr>
<td>Polymer concentration in organic phase (DCM) (w/v)</td>
<td></td>
</tr>
<tr>
<td>1%</td>
<td>45 ± 11</td>
</tr>
<tr>
<td>2.5%</td>
<td>53 ± 10</td>
</tr>
<tr>
<td>5%</td>
<td>73 ± 15</td>
</tr>
<tr>
<td>PVA concentration in external phase (w/v)</td>
<td></td>
</tr>
<tr>
<td>0.1%</td>
<td>71 ± 16</td>
</tr>
<tr>
<td>1%</td>
<td>62 ± 13</td>
</tr>
</tbody>
</table>

**Fig. 2.** Surface morphology of microparticles fabricated at external aqueous phase of (a) 200 ml and (b) 400 ml of DI water with 1% PVA, (c) 200 ml of GF buffer solution with 1% PVA. Stirring speed: 400 rpm for 60 min, polymer concentration: 5% (w/v).
inner W1 and external W2 when GF buffer solution was used as W2. Surface pores might reduce the encapsulation efficiency of protein in microparticles since these pores act as channels for protein to diffuse into W2 during the solidification of microparticles. Furthermore, pores might also influence the release kinetics of microparticle system.\textsuperscript{14,15} Hence, microparticles with closed pores on surface were preferred in this investigation.

The microparticle size was smaller when GF buffer was used as W2, $66 \pm 13\, \mu m$ compared to $83 \pm 26\, \mu m$ when DI water was used as W2. This also supported the speculation that emulsion droplets were swollen due to water diffusion driven by large concentration gradient when DI water was used as the external phase. In this work, GF buffer with PVA as W2 was used for the fabrication of protein-loaded microparticles.

### 3.1.4. Polymer concentration in DCM

Viscosity of organic phase is one of the important factors influencing the properties and release profiles of microparticles.\textsuperscript{16,17} In this investigation, the viscosity was determined by polymer concentration in organic solvent. Three concentrations of PLGA in DCM (w/v), 1\%, 2.5\%, and 5\% were studied. The sizes of microparticles formed at these polymer concentrations were $45 \pm 11\, \mu m$, $53 \pm 10\, \mu m$, and $65 \pm 16\, \mu m$, respectively. This was consistent with published data that the size of microparticles increased as the polymer concentration increased.\textsuperscript{10,18}

Keeping other fabrication parameters constant, polymer solution with higher viscosity was dispersed into larger droplets when the same stirring speed was applied during the second emulsification step; hence the size of the resulting microparticles was larger.

The surface porosity of microparticles decreased when polymer concentration increased. Figure 3 shows that microparticles produced at polymer concentration of 5\% (w/v) had condensed surface. During the elimination of organic solvent, polymer precipitated as a gel-like layer on the surface of the microdroplets,\textsuperscript{11} which would inhibit water diffusion between the external and internal phase. The water
permeability through this layer decreased with polymer concentration increased. Hence, less swelling of microdroplets was expected at higher polymer concentration. Additionally, the higher polymer concentration resulted in a thicker gel-like layer on microdroplets surface. Thus fewer pores were formed on the microparticles surface produced at higher polymer concentration.

3.1.5. Protein concentration in internal aqueous phase (W1)

To investigate the effect of protein concentration in internal W1, fabrication parameters determined in previous experiments were kept constant while the protein concentration in internal W1 was varied. Similar surface morphology was observed for microparticles produced at two protein concentrations, 25 mg/ml and 40 mg/ml, where both had condensed surface (Fig. 4). Higher protein concentration reduced the encapsulation efficiency (Table 2). High protein concentration in W1, led to the increase of protein concentration gradient between the inner W1 and external W2. As the concentration gradient was higher, protein was easier to diffuse out of the droplets towards the external W2, causing more protein loss during the solidification of microparticles. Thus, the encapsulation efficiency was reduced at higher protein concentration in W1.

3.1.6. PVA concentration in external aqueous phase (W2)

At higher PVA concentration, smaller microparticles were produced since PVA stabilized the emulsion droplets against coalescence. When the PVA concentration (w/v) increased from 0.1% to 1%, the microparticle size decreased slightly from 71 ± 16 μm to 62 ± 13 μm. The morphologies of microparticles produced at these different PVA concentrations were almost identical (Fig. 5). All microparticles were spherical in shape and had closed pores on the surface.

To fabricate E2-loaded microparticles, the optimized parameters were used: PVA concentrations (w/v) 0.1% or 1% in GF buffer solution, stirring speed: 400 rpm for 60 min, external phase: 200 ml, polymer concentration: 5%, E2 protein concentration in W1: 6.1 mg/ml. The protein loading of the E2-loaded microsphere was determined to be 1.2% and 1.3% for fabrication using 0.1% and 1% PVA, respectively. The protein loading agreed well with the theoretical value of 1.2%, indicating that there was minimal protein lost during the fabrication process.

Table 2. Comparison of encapsulation efficiency of microparticles produced at different protein concentrations in the internal W1 phase.

<table>
<thead>
<tr>
<th>Protein concentration in W1 (mg/ml)</th>
<th>BSA-loaded microparticles</th>
</tr>
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<tbody>
<tr>
<td>25</td>
<td>40</td>
</tr>
<tr>
<td>Theoretical protein loading (%)</td>
<td>5</td>
</tr>
<tr>
<td>Actual protein loading (%)</td>
<td>1.72 ± 0.02</td>
</tr>
<tr>
<td>Encapsulation efficiency (%)</td>
<td>34.5 ± 0.3</td>
</tr>
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</table>
3.2. Characterization of E2-loaded PLGA microparticles

3.2.1. Surface morphology of E2-loaded PLGA microparticles

E2-loaded PLGA microparticles were fabricated using W/O/W double emulsion method with two different PVA concentrations in the external aqueous phase (W2). The size of microparticles was in the range of 50 to 80 µm, regardless of the PVA concentration. The microparticles had closed pores distributed on the surface (Fig. 6).

3.2.2. E2 protein integrity in PLGA microparticles

Integrity of the E2 protein upon encapsulation in polymeric matrix is important for sustained release

Fig. 5. Surface morphology of microparticles fabricated in 200 ml GF buffer solution with (a) 0.1% PVA and (b) 1% PVA. Stirring speed: 400 rpm for 60 min, polymer concentration: 5% (w/v).

Fig. 6. Surface morphology of microparticles fabricated at different PVA concentrations (w/v) of (a) 0.1% and (b) 1% in GF buffer before incubation and after 14-d incubation. Stirring speed: 400 rpm for 60 min, external phase: 200 ml, polymer concentration: 5% (w/v), E2 protein concentration in the internal W1 phase: 6.1 mg/ml.
of molecules from the E2 protein. To examine the integrity of E2 protein after encapsulation in PLGA microparticles, the E2 protein was extracted from microparticles for DLS analysis. The hydrodynamic size of intact E2 protein has been reported to be in the range of 24–28 nm. The observed size of the extracted E2 protein was determined to be 27 ± 6 nm, indicating that it was stable during the microparticles fabrication process and that the specified conditions were suitable for embedding E2 protein in the microparticle matrix.

3.2.3. In vitro E2 protein release study

The release profiles of microparticles produced at different PVA concentrations (w/v) of 0.1% and 1% were studied. After the initial burst release, a sustainable protein release was followed up to 14 days for both samples (Fig. 7). The initial burst release was possibly resulted from the expulsion of E2 protein located near the surface of the microparticles. Larger amount of protein was released for the 0.1% PVA microparticles than the 1% PVA microparticles during the burst period. The release rate of E2 protein appeared to be faster from 0.1% PVA microparticles than from 1% PVA microparticles. The 0.1% PVA microparticles had completed release on the 14th day of incubation, while the 1% PVA microparticles released 95% of the total protein encapsulated. These results suggested that higher PVA concentration in external W2 during the fabrication of microparticles not only reduced the initial burst but also the release rate. A high PVA concentration hindered the transfer of E2 protein from microparticles to the surrounding. Hence, the E2 protein in 0.1% PVA microparticles was easier to diffuse out compared to that in 1% PVA microparticles.

The 14-day release range was similar to the results reported by Feczkó et al. that ~65% of the loaded BSA was released from the porous PLGA microparticles within 7 days. Here, the microparticles had condensed surface which contributed to the smaller initial burst than the reported results.

3.2.4. Morphology change of microparticles after protein release

After 14-day incubation, the microparticles had smoother surfaces compared to before incubation for both 0.1% and 1% PVA microparticles (Fig. 6). This might arise from the presence of water molecules during the incubation of microparticles in PBS at 37°C for protein release study. Water molecules diffused into polymer matrix, acted as a plasticizing agent and increased the flexibility of polymer chains. Thus, during the incubation in PBS at 37°C, polymer chains were relatively more mobile than during storage at −20°C, resulting in the reduction of surface roughness. This observation was consistent with the published data where the size of external pores on microparticles reduced after incubation.

4. Conclusion

The E2-loaded PLGA microparticles were spherical and had closed pores on the surface. Increase in the volume of external aqueous phase decreased the microparticle size, whereas increase in polymer concentration in the oil phase increased the microparticle size and reduced the surface porosity. When GF buffer with PVA was used as the external aqueous phase, the surface porosity of the microparticles was minimized compared to those prepared in DI water with PVA. This was due to the reduction of concentration gradient between the inner and external aqueous phase when GF buffer solution with PVA was used. Increasing protein concentration in inner aqueous phase enlarged the concentration gradient, thus decreased the encapsulation efficiency. PVA concentration in the external phase during the fabrication of microparticles influenced the microparticle size and the

**Fig. 7.** The influence of PVA concentration in the external W2 phase (w/v) during the fabrication process on the in vitro release of E2 protein from PLGA microparticles (protein loading 0.44%). (●) 0.1% PVA, (▲) 1% PVA.
release rate of E2 protein from microparticles. Increase in PVA concentration decreased the size of microparticles. Higher PVA concentration also resulted in lower burst release and slower release rate of E2 protein from microparticles. The sustained E2 protein release lasted for 14 days. The surface roughness of microparticles was reduced upon incubation in PBS at 37°C due to the presence of water as plasticizer. As confirmed by size measurement, the integrity of E2 protein was not compromised by the encapsulation process.

References
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