Preparation and Optimization of Chitosan-hEGF Nanoparticle Using Ionic Gelation Method Stabilized by Polyethylene Glycol (PEG) for Wound Healing Therapy

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ABSTRACT
The administration of human Epidermal Growth Factor (hEGF) for diabetic ulcer treatment has been known to have high effectiveness because hEGF has mitogenic properties and has been proved to increase epithelial cell proliferation both in vitro and in vivo. However, hEGF in environmental conditions of diabetic ulcers is known to have low stability, so it is necessary to repeat the administration or protect the hEGF using suitable preparation. This study aims to produce a new drug delivery system in the form of chitosan nanoparticle as a therapy for diabetic ulcers. The nanoparticle formulation was carried out by varying hEGF concentrations using the ionic gelation method with sodium tripolyphosphate (Na-TPP) as a crosslinker and Polyethylene glycol (PEG) as a stabilizer. Chitosan-hEGF nanoparticle formed were characterized using particle size analysis, polydispersity index, zeta potential, SEM and TEM, pH, and FTIR to observe the functional groups. Chitosan-hEGF nanoparticle-containing 0.1% chitosan, 0.15% sodium tripolyphosphate (Na-TPP), 2% polyethylene glycol 400 (PEG 400), and 75 ng/mL hEGF has the smallest particle size with an average of 600.6 nm and D90 value of 135.7 nm. Nanoparticle formed were relatively stable with zeta potential reaching +41.29. The results of in vitro testing showed that hEGF 50 ng/mL had an optimal cell viability percentage with a value of 192%.

INTRODUCTION
The management and therapy of diabetic ulcers have so far been deemed ineffective in guaranteeing adequate wound healing. We still found a large enough of the recurrence rate so that the treatment given is considered ineffective, especially health care costs, which are increasingly expensive and increase the total medical expenditure of patients worldwide (Gainza et al., 2015). The increasing care of chronic wounds has now become a significant need, so the focus of the scientific community is not only on finding new treatments for wound healing
but also on increasing the effectiveness of therapy. In this case, significant efforts have been made by developing a new drug delivery system to release active compounds in a controlled manner, so that the management of ulcers is expected to be cheaper and more straightforward (Gainza et al., 2015).

Repeated treatment of diabetic ulcer patients with hEGF preparations has been shown to increase dose-dependent epithelial cell proliferation in accelerating the wound healing process (Maksum et al., 2017). However, the chemical reaction, which is common in diabetic ulcers, the physical instability of hEGF has limited the efficacy of its treatment (Laiva et al., 2018). One way to stabilize hEGF in these wound conditions is by coating hEGF called nanoencapsulation (Dong et al., 2008; Gainza et al., 2015).

Biodegradable protein encapsulation has many advantages, such as increasing pharmaceutical stability, extending effectiveness, avoiding excessive drug administration, and making sustainable drug release manageable (Dong et al., 2008). The strategy of formulating hEGF into the chitosan nanoparticle dosage forms to improve the stability of hEGF has become a very rational and affordable approach (Değim et al., 2011; Gainza et al., 2015). The strategy of formulating hEGF into the in chitosan nanoparticle dosage forms to improve the stability of hEGF has become a very rational and affordable approach (Değim et al., 2011; Gainza et al., 2015).

This nanoparticle research has been carried out by utilizing natural chitosan polymers as one of the most widely used biopolymers (Agarwal, 2013), and hEGF used in this research was recombinant hEGF obtained from expression of E. coli BL21 pD886-hEGF-PelB cells in previous studies (Maksum et al., 2017; Sriwidodo et al., 2017; Melati et al., 2019).

MATERIALS AND METHODS

Preformulation

Identification of functional group using FTIR

Chitosan with 95.2% deacetylation degree (molecule weight 50,000-80,000) (Biotech Surindo, Indonesia), hEGF (Sigma Aldrich, Germany), Na-TTP (Wako, Germany) Polyethylene glycol 400 (Brataco, Indonesia), and the mixtures were homogenized with 200 mg KBr (Merck, Germany), then compressed to form pellets. Pellets were analyzed by FTIR (Jasco-4200) and observed at wavenumbers 4600-400 cm \(^{-1}\).

Formula optimization of chitosan nanoparticle

Optimization was done by varying the concentrations of Na-TTP (0.05%, 0.1%, and 0.15%) and PEG 400 (1%, 1.5%, and 2%). Formula optimized can be seen in Table 1.

First of all, the chitosan solution was put into the beaker glass on a magnetic stirrer. Then Na-TPP was added dropwise to chitosan solution and stirred for 30 minutes. PEG 400 was added dropwise while stirring for 15 minutes. The mixed solution was then sonicated using an ultrasonic (NEY) for 25 minutes.

Characterization

Particle size, polydispersity index, and size distribution of chitosan-hEGF nanoparticle

The particle size distribution of chitosan nanoparticle formulations containing hEGF and negative control was measured using the DELSATM Nano C Particle Size Analyzer (PSA) (Beckman Coulter, USA). Polydispersity index of chitosan-hEGF nanoparticle formulations containing hEGF was also measured using PSA.

Particle surface charge

Particle surface load on chitosan nanoparticle-containing hEGF formulations was measured using zeta sizer (Beckman Coulter, USA).

pH measurement

The pH of chitosan nanoparticle formulations containing hEGF was measured using a pH meter (Eutech Instruments, Singapore).

Morphology of nanoparticle

The nanoparticle solution was first converted into powder form by the lyophilization method using a freeze dryer (IHANIL VAC 8), for 24 hours, to obtain nanoparticle powder. Chitosan-hEGF nanoparticle powder was placed on copper tape on an aluminum stage, then the sample was coated using gold, and the sample was scanned with light, which can emit electrons in a SEM SU3500 (HITACHI) device. For the morphological observation procedure using TEM HT7700 (HITACHI), the solution of the nanoparticle was diluted using distilled water. Then, the solution was pipetted and dropped on the TEM grid. Samples were dried by settling at room temperature for 1 hour. After drying, the sample was placed in a TEM holder and then analyzed.

The entrapment efficiency analysis

Preparation of standard curve and sample analysis were based on protocol measurements of hEGF using ELISA (ELISA kit (AbCam, R &D Systems, USA). The solution of chitosan-hEGF nanoparticle was centrifuged, then the supernatant was extracted. The supernatant was inserted into a separating funnel then added extracting solvent to form 2 phases.
Figure 1: The infrared spectrum of chitosan standard.

Figure 2: The infrared spectrum of hEGF sample
Table 1: Formula optimization with variation in ingredient concentration

<table>
<thead>
<tr>
<th>Formula</th>
<th>Chitosan (%)</th>
<th>Na-TPP (%)</th>
<th>PEG 400 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FA</td>
<td>0.1</td>
<td>0.05</td>
<td>1</td>
</tr>
<tr>
<td>F</td>
<td>0.1</td>
<td>0.1</td>
<td>1.5</td>
</tr>
<tr>
<td>FC</td>
<td>0.1</td>
<td>0.15</td>
<td>2</td>
</tr>
</tbody>
</table>

Table 2: The mean diameter of chitosan nanoparticle.

<table>
<thead>
<tr>
<th>Formula</th>
<th>Na TPP (%)</th>
<th>PEG 400 (%)</th>
<th>Mean diameter (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FA</td>
<td>0.05</td>
<td>1</td>
<td>2515.6</td>
</tr>
<tr>
<td>FB</td>
<td>0.1</td>
<td>1.5</td>
<td>878.9</td>
</tr>
<tr>
<td>FC</td>
<td>0.15</td>
<td>2</td>
<td>550.0</td>
</tr>
</tbody>
</table>

Table 3: The formula of chitosan-hEGF nanoparticle

<table>
<thead>
<tr>
<th>Formula</th>
<th>hEGF (ng/mL)</th>
<th>NaTPP (%)</th>
<th>PEG 400 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FI</td>
<td>25</td>
<td>0.15</td>
<td>2</td>
</tr>
<tr>
<td>FII</td>
<td>50</td>
<td>0.15</td>
<td>2</td>
</tr>
<tr>
<td>FIII</td>
<td>75</td>
<td>0.15</td>
<td>2</td>
</tr>
</tbody>
</table>

Then the new supernatant was measured using a Plate reader (Biorad) at the maximum wavelength. The amount of hEGF absorbed in the polymer was measured by the following formula:

Entrapment percentage of hEGF = (CA-CB)/CA x 100%

CA = Initial active substance levels
CB = Free active substances levels

In Vitro Evaluation (Cell Proliferation)

First of all, NIH3T3 cells (1 x 105 cells/well plate) are grown in 24 well plates for 24 hours. Furthermore, chitosan-hEGF nanoparticle with concentrations of 25, 50, and 75 ng / mL was inoculated into the well plate (6 replications for each concentration). PBS was inoculated into one culture cell group as a negative control. 30 μL of WST-8 reagent was added to 24 well plates and incubated at 37 °C for 3 hours.
4 hours. The absorbance of the solution was measured at a wavelength of 450 nm using the Tecan Infinite 2000 Spectrophotometer and a reference wavelength at 650 nm.

RESULTS AND DISCUSSION

Preformulation

Identification of functional group using FTIR

Testing using FTIR was carried out to observe the functional groups on each material used. The infrared spectrum provides information about the changes that occur in the material before and after it was formulated into a nanoparticle.

Standard chitosan will show peaks in the area of 2850-3000 cm\(^{-1}\) for CH groups, 3400-3500 cm\(^{-1}\) for primary amine groups, 3200-3600 cm\(^{-1}\) for OH groups, and 1100 cm\(^{-1}\) for C-O groups as seen in Figure 1. (Singh et al., 2009; Govindasamy et al., 2013). Chitosan sample showed the peak at 3367.1 cm\(^{-1}\), which is a stack of O-H and N-H groups. Chitosan sample has O-H stretch, N-H bend, C-H stretch, and C-O groups in the same wavenumber area as standard chitosan.

Standard Na-TPP shows the specific peak at wavenumber 1150 cm\(^{-1}\) for the aliphatic P = O group and 870-1000 cm\(^{-1}\) for the P-O-P group (Govindasamy et al., 2013). Na-TPP sample has a peak corresponding to the range shown in the standard Na-TPP spectrum at 883,238 cm\(^{-1}\), indicating the presence of a P-O-P group, and at 1145.51 cm\(^{-1}\), indicating the presence of a P = O group.

Standard PEG has a specific peak that indicates the presence of an ether group in the wavenumber area of 1320-1000 cm\(^{-1}\), indicating the presence of the C-O-C group. PEG also shows peaks in the wavenumber area 3000-2850 cm\(^{-1}\) and 1470-1450 cm\(^{-1}\) due to the C-H stretch and C-H bend groups in the alkane (Shameli et al., 2012).

PEG 400 sample shows that the absorption is in accordance with the range of the standard PEG at the wavenumber 1295.93 cm\(^{-1}\); 1249.65 cm\(^{-1}\); 1103.08 cm\(^{-1}\) which shows C-O stretch vibrations in the ether group, and absorption at 2911.99 cm\(^{-1}\) and 1461.78 cm\(^{-1}\) shows vibrations of C-H stretch and C-H bend that indicate the presence of alkane groups.

The structure of hEGF has many amine groups, aliphatic amines, and aromatic rings, as seen in Figure 2. (Ogiso et al., 2002).

Based on Figure 3, the mixture still has the same functional group when the substance is still single, which shows that the mixture of materials does not produce bonds or new functional groups. So there is no chemical interaction between Chitosan-PEG-TPP and hEGF.

Formula optimization of chitosan nanoparticle

The optimization of chitosan nanoparticle was performed by varying the ingredient concentration. Chitosan nanoparticle was formed by dissolving chitosan in a beaker glass on a magnetic stirrer, then adding Na TPP dropwise while stirring using a magnetic stirrer with a rotational speed of 1500 rpm for 30 minutes. After that, PEG 400 was added dropwise into the mixture while still stirring for 15 minutes. This mechanical stirring is an essential technique in the ionic gelation method and plays a vital role in nanoparticle formation when Na-TPP was added into the chitosan solution. The mixture was sonicated to reduce the particle size.

Chitosan nanoparticle formed were then subjected to particle measurement using a Particle Size Analyzer (PSA). The mean diameter of the chitosan nanoparticle is shown in Table 2.

Based on the measurement of particle size with variations in Na-TPP and PEG 400 concentration, the smallest particle size was produced using 0.15% Na-TPP and 2% PEG 400 with particle size was equal to 550 nm. The mean diameter of chitosan nanoparticle made by ionic gelation generally has a size range of around 20-900 nm (Mohammed et al., 2017).

Formulation of chitosan-hEGF nanoparticle

Based on the optimization result done, the optimal formula was obtained in a solution containing 0.15% TPP Na and 2% PEG 400. Then chitosan-hEGF nanoparticle formulations were prepared using the ionic gelation method. Chitosan solution was put into the beaker glass on top of the magnetic stirrer, hEGF solution was added dropwise while stirring with a rotating speed of 1500 rpm for 10 minutes. Na-TPP 0.15% was added dropwise to the mixture while stirring for 30 minutes. Chitosan-hEGF nanoparticle will form and be stronger when Na-TPP was added to the solution. Then PEG 400 2% was added dropwise while stirring for 15 minutes. The chitosan-hEGF nanoparticle formula with variations in hEGF concentration is shown in Table 3.

Characterization

Organoleptic observation of chitosan-hEGF nanoparticle

Organoleptic observation is a physical depiction presented from the preparation made. Visually, each formula looks slightly misty (transparent
Table 4: The results of particle size testing and polydispersity index on the chitosan-hEGF nanoparticle.

<table>
<thead>
<tr>
<th>Formula</th>
<th>hEGF (ng/mL)</th>
<th>Mean diameter (nm)</th>
<th>Polydispersity index</th>
<th>D value (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blancko</td>
<td>0</td>
<td>550.0</td>
<td>0.427</td>
<td>27.4 30.6 37.9</td>
</tr>
<tr>
<td>Fi</td>
<td>25</td>
<td>1135.3</td>
<td>0.425</td>
<td>459.4 525.8 625.7</td>
</tr>
<tr>
<td>FII</td>
<td>50</td>
<td>833.5</td>
<td>0.275</td>
<td>12.8 14.4 18</td>
</tr>
<tr>
<td>FIII</td>
<td>75</td>
<td>600.6</td>
<td>0.259</td>
<td>80.5 94 135.7</td>
</tr>
</tbody>
</table>

Table 5: Zeta potential of chitosan-hEGF nanoparticle

<table>
<thead>
<tr>
<th>Formula</th>
<th>Zeta Potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blancko (without hEGF)</td>
<td>+46.55</td>
</tr>
<tr>
<td>Fi</td>
<td>+33.42</td>
</tr>
<tr>
<td>FII</td>
<td>+39.36</td>
</tr>
<tr>
<td>FIII</td>
<td>+41.29</td>
</tr>
</tbody>
</table>

Table 6: The result of pH measurement of chitosan-hEGF nanoparticle

<table>
<thead>
<tr>
<th>Formula</th>
<th>Mean pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blancko (without hEGF)</td>
<td>5.23</td>
</tr>
<tr>
<td>Fi</td>
<td>6.95</td>
</tr>
<tr>
<td>FII</td>
<td>6.96</td>
</tr>
<tr>
<td>FIII</td>
<td>7.01</td>
</tr>
</tbody>
</table>

Figure 4: Chitosan-hEGF nanoparticle (A) FI, (B) FII, dan (C) FIII.
Figure 5: Morphology of Formula 3 (hEGF 75ng/mL shows hEGF trapped in chitosan. (A) Scanning Electron Microscopy (B) Transmission Electron Microscopy

Figure 6: Percentage of hEGF trapped in chitosan (entrainment efficiency)
transparency), and there was no sediment. According to Rajam et al. (2013), nanoparticle should not have floating particles that indicate it was stable, and the distribution of particles in the solution was quite evenly distributed. Chitosan-hEGF nanoparticle can be observed in Figure 4.

**Particle size, polydispersity index, and size distribution of chitosan-hEGF nanoparticle**

The measurement results of particle size using PSA are shown in Table 4. The formula I, II, and III have an average particle size distribution of 1135.3 nm, 833.5 nm, and 600.6 nm. From F1 to F11, the particle size obtained was getting smaller. The determination of particle size distribution can be done using the values of D10, D50 and D90. D10 states that there are 10% of the total particle size distribution having a size below the D10 value and 90% having a size above the D10 value. D50 is the median value, where 50% of the total particle size distribution has a size below the D50 value, and 50% has a size above the D50 value. Likewise, the D90 value, there are as many as 90% of the overall particle size distribution that has a size below the D90 value, and 10% has a
size above the D90 value.

FIII has an average particle size distribution of 600.6 nm. As many as 10% of particles have sizes below 80.5 nm, and 90% have sizes above 80.5 nm. Then 50% of the particles are below 94 nm, and 50% are above 94 nm. And there are as many as 90% of particles below 135.7 nm in size and 10% having sizes above 135.7 nm.

The precondition for the polymer to be applied to the pharmaceutical field is a polydispersity index. All three formulas show a decrease in the polydispersity index along with decreasing particle size of each formula. Based on the requirements, the polydispersity index value of the three formulas is appropriate for the smaller or near zero polydispersity index value of an ingredient, the better and homogeneous the mixture.

The role of PEG is significant in the formation of nanoparticle preparations with low polydispersity. PEG itself has a low polydispersity value and shows high solubility in organic solvents. So that the dissolution of the polymer can be increased by mixing with PEG. The biological application of PEG is very suitable because it has low toxicity and soluble in water. The solubility of a drug or hydrophobic carrier can be increased by PEG because it has a high hydrophilic nature. This is very beneficial because it increases the physicochemical stability of the drug and prevents drug aggregation. This is as the result of steric resistance or covering the charge, which results in a cloud covering the particle (Kadajji and Betageri, 2011).

The polydispersity results showed that the formula which had the smallest polydispersity index was in FIII with hEGF concentration of 75 ng/mL. Of the three formulas tested, FIII has high homogeneity.

**Zeta potential of chitosan-hEGF nanoparticle**

The zeta potential value of chitosan-hEGF nanoparticle was determined using zeta sizer. Chitosan-hEGF nanoparticle was taken as much as 1.5 mL using a pipette, then put in a cuvette paired with an electrode. When the nanoparticle solution was analyzed using zeta sizer, the particles in the nanoparticle solution would migrate to electrodes with different charges, where the velocity is proportional with the zeta potential magnitude. The zeta potential results of the test sample can be seen in Table 5. Based on Table 5, the zeta potential value of the three formulas meets the requirements (above +30 mV) (Kumar and Dixit, 2017). This shows that the three formulas have excellent stability and tend not to flocculate. The zeta potential value of three formulas increases with the increasing concentration of hEGF. Besides, zeta potential shows a correlation with particle size and polydispersity index. Nanoparticle, which has the smallest size and low polydispersity index, shows a higher zeta potential value, which means it is more stable. While nanoparticle, which has a larger size and higher polydispersity index, has lower zeta potential values. This is because the large particle size causes repulsive force between particles to decrease so that the particle tends to flocculate. Among the three formulas tested, FIII has the best stability and tendency to experience flocculation only slightly.

**Morphology of chitosan-hEGF nanoparticle**

In the morphological observation of chitosan-hEGF nanoparticle by using SEM, it can be seen how the surface of chitosan-hEGF nanoparticle surface looks. The surface can be observed using SEM by first taking a powder-shaped sample coated with conductive gold. The function of the coating sample by gold is to protect the sample from large electrical voltages on SEM devices. The observation shows that the surface morphology of the nanoparticle encloses hEGF in it. However, because hEGF is encapsulated in chitosan nanoparticle and chitosan itself has a larger size than hEGF, electrons cannot penetrate.

In Figure 5 (A), SEM can show the size scale of one particle in the sample. From this figure, a sphere size of more than 1000 nm was obtained and stuck together to form an aggregation due to the lyophilization of chitosan-hEGF nanoparticle using a freeze dryer. The average size of nanoparticle measured using a PSA was 600.6 nm.

The internal morphology of chitosan-hEGF nanoparticle was observed using TEM. The result can be seen in Figure 5(B). Chitosan-hEGF nanoparticle was sonicated first to avoid flocculation. Then the sample was dropped on the TEM grid and dried for further analysis. From observations using
TEM, it can be seen in the picture that there are darker colored dots, and there are like small pores. The more shaded colored sphere is hEGF, which was absorbed inside the chitosan nanoparticle matrix. But the matrix formed is still connected, and no single spherical free matrix was found that incorporates hEGF. This can occur because there is still a tendency for the nanoparticle to locculate so that the particles stick together. In a further development, it is necessary to ensure that aggregation does not occur by sonication when forming an ionic chitosan-hEGF nanoparticle.

**pH measurement of chitosan-hEGF nanoparticle**

pH measurement of chitosan-hEGF nanoparticle was carried out to determine whether the formulation made had the appropriate pH for its intended use. The pH of chitosan-hEGF nanoparticle with a variation of hEGF concentration was measured using calibrated pH meter. The purpose of this calibration was to determine the measurement accuracy of the pH meter. In the pH meter calibration, three calibrator solutions were used, each of which had a pH of 4.01; 7.00 and 9.21.

The test results in Table 6 show the average pH of FI, FII, and FIII. The increase in hEGF concentration did not provide a significant difference in pH values. The pH of chitosan dissolved in 0.1% glacial acetic acid has a sufficiently acidic pH of 5.13. So that the pH of the chitosan-hEGF nanoparticle was still in acidic pH. However, the pH result is still within the normal skin pH range of 4-6.

When there are injuries, especially chronic wounds caused by diabetes mellitus, the skin will have a pH close to the blood pH of 7.4. So chitosan-hEGF nanoparticle prepared to need to be added with a sodium hydroxide base to increase the pH (Harris et al., 2009).

**The entrapment efficiency of chitosan-hEGF nanoparticle**

The entrapment efficiency analysis was intended to determine the effectiveness of chitosan nanoparticle in trapping hEGF through the ionic gelation process. The percentage of hEGF trapped in chitosan can be seen in Figure 6.

Based on Figure 6, the entrapment efficiency of each formula is excellent, reaching 99%. So it can be said that the ionic gelation process produces excellent entrapment efficiency in chitosan. There was no significant difference between the formula containing and not containing Na-TPP - PEG 400. Entrapment efficiency increases by increasing dose. The highest entrapment efficiency value was on FIII.

**In Vitro Evaluation (Cell Proliferation)**

WST-8 is one of the reagents of tetrazolium salt, which has a high sensitivity. In cells, WST-8 is converted into formazan products by NADPH, whose absorbance can be measured at wavelengths of 430-550 nm. The levels of formazan formed are proportional to the levels of NADPH as an indicator of cell viability. The metabolic scheme of WST-8 can be seen in Figure 7. (Chamchoy et al., 2019).

The results of NIH3T3 cell culture and inoculation of the chitosan-hEGF nanoparticle can be seen in Figure 8 and Figure 9. Cell culture is made of 24 well plates, which consist of 4 groups namely control, FI (hEGF 25 ng / mL), FII (hEGF 50 ng / mL), and FIII (hEGF 75 ng / mL). Each group was made in six replicas. Then WST-8 reagent was added to determine the number of living cells. Cell quantification can be expressed as cell viability in terms of the percentage of living cells compared to the control. The highest cell viability is in FII with a percentage of 192%. This value indicates that the cell proliferates by almost two times the original amount during 4 hours of incubation. hEGF 50 ng / mL is more optimal than a higher dose. The order of cell viability percentage from highest to low is FII> FIII> FI> control (can be seen in Figure 10).

**CONCLUSIONS**

The optimization results of chitosan nanoparticle-containing 0.15% Na-TPP and 2% PEG 400 produced the smallest particle size of 550 nm. Based on the characterization results of chitosan-hEGF nanoparticle, which contains variations in hEGF concentration, the most optimal formula is FIII with a hEGF concentration of 75 µg / mL. Where FIII has the smallest average particle size of 600.6 nm with a polydispersity index value of 0.259, zeta potential of +41.29 mV, and has an entrapment efficiency of more than 99.9%. The results of in vitro testing showed that hEGF 50 ng/mL had an optimal cell viability percentage with a value of 192%.

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