

Expression, Microencapsulation of Recombinant Human Epidermal Growth Factor, and Release Study in Gastric Ulcer Representing Condition

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ABSTRACT

Recombinant human epidermal growth factor (rhEGF) has been studied and expressed in various expression systems. It has been also commercialized and clinically used, yet limited to topical diseases. However, being naturally expressed in different tissues, the rhEGF is potential to be applied not only for external wound and skin disorders, but also to regenerates internal damaged epidermal cells such found in gastric ulcer. In the recent study, chitosan microparticles were developed to facilitate delivery of the rhEGF and to overcome gastric degradation that majorly interfere protein, particularly rhEGF oral administration. The rhEGF was expressed in *E. coli* BL21(DE3) and purified using Ni-NTA chromatography. The refolded rhEGF showed proliferation activity on MCF7 cells. rhEGF loaded chitosan microparticles were stable in the gastric and specifically released the loaded rhEGF in the high oxidative environment in acidic pH representing gastric ulcer condition.

Key words: epidermal growth factor, chitosan microparticle, gastric ulcer, targeted release

INTRODUCTION

Gastric ulcer is a manifestation in health condition caused by infection of *Helicobacter pylori*, non-steroidal anti-inflammatory drugs (NSAIDs), and hypoxia or stress condition in the gastric (Huang *et al*, 2002; Bhattacharyya, *et al*, 2014). The ulcer is formed by margin layer containing non-necrotizing mucosa such as epithelial cells, and base layer consisting of granulation tissue eg. fibroblast, macrophage, also endothelial cells (Syam, *et al*, 2009). Enhancing the proliferation of the epithelial cells on the margin layer is the priority strategy for the ulcer healing by re-epithelization of the mucosa surface and reconstruction of the gastric gland (Tarnawaski, 2000).

Epidermal growth factor (EGF) is the most important cytokines in healing mechanism in

gastric ulcer that induces proliferation and maturation of epithelial cells (Goodsell, 2003; Bodnar, 2011). However, at severe stage of the gastric ulcer, EGF which is naturally secreted in the body is inadequate to induce proliferation and maturation of the epithelial cells due to degradation by proteases from *H. pylori* and competition with these bacteria to bind to EGFR (Posselt, *et al*, 2017). Therefore, exogenous EGF is requested in the severe gastric ulcer therapy.

The exogenous EGF is provided by protein engineering to produce the recombinant protein using an appropriate expression system. *E. coli* is the most favorable expression system used to produce recombinant protein. In the previous study, we constructed our synthetic gene of hEGF with plasmid pET21b(+) and then transformed it into *E. coli* DH5 α subcloning host (Nurmalasari,

2010). In this study, the recombinant plasmid pET21b(+)_hEGF_6×His-tag was expressed in *E. coli* BL21(DE3).

The rhEGF was then formulated into microparticles to ensure its delivery to the damaged gastric. We intended to apply chitosan microparticles for the rhEGF oral delivery because the materials used are available in the nature and safe. In addition, the preparation is simple, organic solvent free, and applicable for large production.

The delivery system is designed to adsorb the rhEGF in a physiological environment, including in neutral and acid conditions and then it has the ability to release the rhEGF in damaged tissue in the gastric ulcer. In patients with gastric ulcer, their gastrointestinal pH is around 2.4 ± 1.9 (as comparison, gastric pH in healthy subject is 1.7 ± 0.2) (Machida, 1981). Moreover, the damaged condition has higher oxidative stress compared to healthy tissue (Suzuki, *et al*, 2012). This condition is used to trigger the rhEGF release from the microparticles.

The rhEGF has been encapsulated in several microcarrier systems for local or dermal administration (Sriwidodo, *et al*, 2020; Zhang, *et al*, 2020). Therefore, this is the first study of encapsulation of the rhEGF which is purposed for nondermal application, particularly for gastric ulcer. This study was aimed to produce purified and active rhEGF and then prepare a suitable formulation for oral delivery of the rhEGF, specifically for gastric ulcer therapy.

MATERIALS AND METHODS

Expression, purification, and characterization of recombinant human epidermal growth factor

The recombinant plasmid pET21b(+)_rhEGF_6×His-tag obtained from the previous study was isolated from its subcloning host *E. coli* DH5 α using alkaline lysis method and transformed into an expression host *E. coli* BL21(DE3) by heat-shock method (Ausuble, *et al*, 1999). The rhEGF was expressed in Luria Bertani media by IPTG induction which was optimized at concentration 0.1 mM and 4 h, 37°C incubation. The cells pellet was harvested by centrifugation at 6,000rpm; 15min; 4°C and solubilized with solubilization buffer (8 M urea, 80 μ M β -mercapto-ethanol, 30 mM glycine; 60 mL solubilization buffer for pellet from 300 mL cells culture) incubated at 4°C for 24–48 h). Solubilized protein was separated with the pellet residue by centrifugation 12,000 rpm, 4°C, 20 min.

The solubilized protein was then mixed with slurry of Ni-NTA agarose enriched with equilibrium buffer (0.05% Tween 20, 30mM imidazole in phosphate saline buffer) and incubated at 4°C for 18 h. Contaminant proteins were removed in washing steps using washing buffer (50mM in phosphate saline buffer). Subsequently, target protein (rhEGF_6×HisTag) was eluted with elution buffer (100 mM imidazole in phosphate saline buffer). The purified rhEGF_6×HisTag was refolded by two methods of dialysis against stepwise concentrations of arginine (0–0.05 M arginine and 0.5–0 M urea) and one concentration of GSH (0.03 M GSH and 0.5–0 M urea). Refolding was performed at 8–10°C, 6 h for each step with urea and overnight for the last dialysis without urea. Except, the first step with GSH and urea was performed in room temperature for 3 h.

The refolded rhEGF_6×HisTag was concentrated by filtration using 10 kDa membrane filter with low speed centrifugation (8,000 rpm; 5min; 10°C). Tricine-SDS PAGE with 15% acrylamide was used for analyzing protein profile. For protein quantification, image of tricine-SDS PAGE was converted into green colored band with black background for a better contrast with Image Studio Digit® and then the specific bands were measured using ImageJ®. Western blot was done by using anti-rhEGF antibody. The rhEGF stability in acidic solution was performed by incubating the refolded rhEGF in HCl 0.1 N at 37 °C for 0, 1, and 2 h, respectively. After incubation the protein was analyzed with tricine-SDS PAGE. Concentration ratio of the incubated and not incubated protein was semi-quantified using ImageJ software (Arya, *et al*, 2015).

In vitro bioassay of rh-EGF proliferation activity on MCF7 cell line

MCF7 cell line (confluency 80–90%) was trypsinized and resuspended in serum free DMEM (1.105 cells/mL). The cell suspension (100 μ L/well) was incubated in 96 well plate, 24 h, 37 °C, 5% CO₂. On the next day, the media was discharged and refreshed with new media supplemented with rhEGF. The cells were incubated with the rhEGF for 24 h, followed by MTT addition (20 μ L/well). The cells were then incubated with MTT for 4 h. The media was recharged with DMSO supplemented media. Prior to read absorbance at 570 nm, the cells were incubated with DMSO for 20 min at room temperature.

Formula optimization for chitosan microparticles with BSA as model protein and preparation of rh-EGF loaded microparticles. Bovine serum albumin (BSA) was used as protein model in formula optimization. BSA and chitosan were dissolved in 0.1 N acetic acid pH 5, respectively. STPP/carrageenan were dissolved in water for injection. Chitosan and STPP were prepared in various concentration (Table. 1). BSA, and chitosan, and STPP/carrageenan solutions (ratio 1:1:1) were mixed with vortex (Thermo Scientific) at maximum speed, approximately 3,200 rpm. First, BSA and chitosan solutions were mixed for 60 s, then added with STPP/carrageenan solution and mixed for 60 s as well.

One optimized formula was used to entrap the rhEGF. The refolded rhEGF was concentrated using membrane filter 10 kDa by centrifugation at low speed, 6,000 rpm, 5 min, 4 °C. This concentrated rhEGF (0.3 mg/mL) was directly mixed with chitosan and STPP solution, without any solvent addition. Standard rhEGF was dissolved in water for injection before being mixed with chitosan and STPP.

Entrapment efficiency measurement

Entrapment efficient of the protein was calculated from the ratio of bound and unbound protein. The bound protein was separated from unbound protein by centrifugation at 12,000 rpm for 15 min. The unbound protein in optimization study was analyzed with Nanodrop™ spectrophotometer and confirmed with SDS PAGE, but unbound protein in rhEGF loaded microparticles was analyzed by bicinchoninic acid (BCA) assay, for more sensitive detection.

$$\% LE = \frac{A}{B} \times 100\% \dots\dots\dots(1)$$

$$\% LC = \frac{A}{B + C} \times 100\% \dots\dots\dots(2)$$

A= Unbound protein (mg); B= Bound protein (mg); C= empty chitosan microparticle.

Physical characterization of chitosan microparticles

Hydrodynamic size was measured using Zetasizer Micro Range, Malvern Analytical. Morphology of microparticle was observed under scanning electron microscope JSM IT200, JEOL, Japan.

In vitro release study of rh-EGF loaded microparticles in condition representing gastric ulcer

Dispersion of rhEGF loaded microparticles (1 mL in each microtube) was centrifugated at 12,000 rpm; 15 min to obtain pellet of rhEGF loaded microparticles. The pellet was then added with HCl 0.1 N, pH 1 and incubated at 37 °C. Samples were collected at 0; 5; 10; 15; 30; 60; and 120 min after being incubated in HCl 0.1 N pH 1 by centrifugation at 12,000 rpm; 15 min. Other pellets from samples which had been incubated in HCl 0,1 N pH 1; 37 °C; 2 h were added with HCl 0.1 N pH 1 supplemented with GSH 10 mM and incubated again at 37 °C. The GSH incubated pellets were collected at 0; 15; 30; and 60 min by centrifugation at 12,000 rpm; 15 min. Supernatants obtained after centrifugation were analyzed with BCA assay. All samples were performed in duplicates

RESULT AND DISCUSSION

Expression, purification, characterization, and activity study of recombinant human epidermal growth factor

The rhEGF has been successfully expressed in *E. coli* BL21(DE3) under T7 promoter which was induced with IPTG. The protein was obtained as inclusion bodies, thus a solubilization process is required. Figure 1(a) lane 2 shows solubilized rhEGF before purification. A thick band was observed at approximately 6.5 kDa.

In order to remove contaminants or endogenous proteins, purification using Ni-NTA matrix was performed. The rhEGF was designed with 6×Histag fusion at C-terminal. Therefore, the 6×Histag was bound to nickel on the matrix. Proteins without any multiple histidine could not bind to the matrix and collected in the flow through fraction. Figure 1(a) lane 3 depicts that no rhGEF was released in the flow through which means that the protein target entrapment was sufficient. Contaminant proteins containing multiple histidine were also found in the first elution fraction (Figure 1(a) lane 7). However, in the next elution fractions, a single band of purified rhEGF was obtained. All elution fractions, except the first elution were collected and then refolded with arginine.

Prior to confirm whether the refolded rhEGF has bioactivity, the purified protein was characterized using western blot against anti-rhEGF monoclonal antibody. Positive blotted bands were characterized (Figure 1(b)).

Activity of the refolded rhEGF to induce cell proliferation was tested on MCF7 cell line. In this study, the MCF7 cell line was selected for the bioactivity assay of the rhEGF because it showed a significant sensitivity and mitogenic response among other cancer cells lines tested in a previous study (Fitzpatrick, *et al*, 1984). The EGFR specifically responded to the EGF during the S/G2 phase of the cell cycle (Walker, *et al*, 2007).

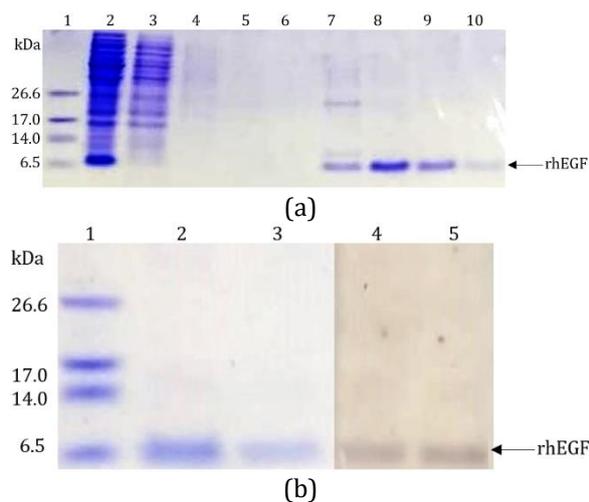


Figure 1. Expression, purification, and characterization of rhEGF. (a) The rhEGF profile before and after purification using Ni-NTA matrix. Lane 1: protein marker; lane 2: protein crude (before purification); lane 3: flow through fraction; lane 4 – 6: washing fractions; lane 7 – 10: elution fractions. (b) The rhEGF blotting profile against anti-rhEGF monoclonal antibody. Lane 1: protein marker; lane 2-3: purified rhEGF on SDS PAGE gel; lane 4-5: blotted rhEGF on nitrocellulose membrane.

Regarding to the bioassay for the refolded rhEGF, the MCF7 cell line was considered as an appropriate and sensitive method.

The MCF7 cell line was treated with rhEGF and incubated for 24h. Significant activity towards negative control or untreated cells was found in rhEGF 30ng/mL. This is the optimized concentration of the rhEGF to induce the MCF7 cell proliferation. The highest concentration of the rhEGF showed opposite result which caused cell death. Kim *et al* (2015) reported that at certain concentration, the rhEGF might shows paradox effect leading to cell death which is related to suppression of PI3K/AKT/mTOR signaling pathway. The MTT assay proved that the refolding process was appropriate (Figure 2).

Refolding of denaturated rhEGF is a complex process. This protein possesses three six cysteine residues which contributes to form three intrachain disulfide bonds. These disulfide bonds determine particular hEGF loops. Three loops of hEGF specifically interact with two among three domains of C-shaped hEGFR (Ogiso, *et al*, 2002). A molecule of hEGF binds with a molecule of hEGFR and triggers rhEGF dimerization by interaction with another complex of hEGF-hEGFR (Ogiso, *et al*, 2002). Once the hEGFR dimer is arranged, the following tyrosine kinase-related cascade is activated (Goodsell, 2003). Thus, inappropriate refolding or mismatched cysteine bounds generate no biological activity (Kozlova, *et al*, 2016). The refolded rhEGF was then encapsulated with chitosan after the optimized formula was obtained.

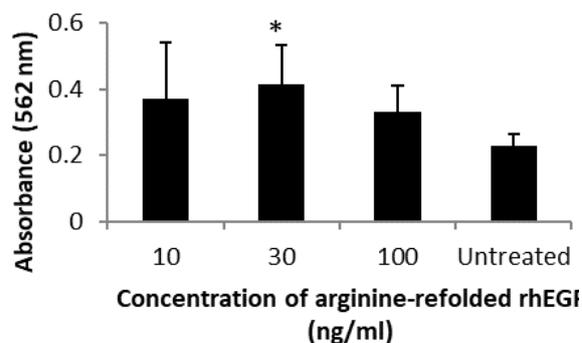


Figure 2. Bioactivity of arginine refolded-rhEGF to induce proliferation of MCF7 cell line detected with MTT assay

Formula optimization for chitosan-based particles

Formula optimization was carried out using a model protein, bovine serum albumin (BSA). The model protein was used instead of the rhEGF in the optimization step by considering the economic value and difficulty level of producing the purified protein. In addition, BSA was chosen because of similarity of substantial physical characterization with rhEGF, in terms of chitosan encapsulation. Protein pI is the most important parameter in the recent study in which protein loading was highly depended on ionic interaction of protein and chitosan, in other words it relies on protein pI and pH of chitosan solution. pI of BSA and EGF was quite similar, i.e 4.8 and 4.5, respectively (Zeng, *et al*, 1998; Deng, *et al*, 2006).

Combination formulas of chitosan and its crosslinker (STPP and carrageenan) are listed (Table I). Chitosan and carrageenan are natural polymers having opposite ionic charges in neutral

pH. Combination of those polyelectrolytes physically forms an ionotropic hydrogel. Concentrations of polyelectrolytes determine structure of the hydrogel which are micro/nanoparticles or beads. This physical interaction is reversible and disrupted by changes of pH, ionic strength, or temperature (Grenha, *et al*, 2009). Similar with carrageenan, STPP is also a compound having a negative charge, therefore can be used as a crosslinker for the chitosan-based particles (Yang, *et al*, 2009). Crosslinks between $-NH_3^+$ of chitosan and $P_3O_5^-$ or $HP_3O_4^-$ of STPP and $-R-SO_3^-$ of carrageenan are the main intra and intermolecular interaction to form the particles (Triwulandari, *et al*, 2018).

Table I. Formula optimization of chitosan microparticles using bovine serum albumin as protein model

Formula	Chitosan (%)	Carrageenan (%)	STPP (%)
A1	0.1	-	0.01
A2	0.4	-	0.01
A3	0.1	-	0.04
A4	0.4	-	0.04
B1	0.1	0.01	-
B2	0.3	0.01	-
B3	0.1	0.05	-
B4	0.3	0.05	-

Table II. Analysis of BSA loaded microparticles

Formula	Particle size (nm)	PDI	% EE	% LC
A1	400.7; 0.271	0.271	71.24	8.85
A2	308.3; 0.225	0.225	65.77	2.35
A3	426.8; 0.290	0.290	58.66	5.91
A4	516.6; 0.312	0.312	59.76	1.99
B1	514.4; 0.295	0.295	77.24	9.50
B2	860.7; 0.484	0.484	67.39	3.15
B3	1196.0; 0.524	0.524	59.76	5.63
B4	732.1; 0.380	0.380	63.01	2.62

The particle characterization in formula optimization study including particle size, polydispersity index (PDI), loading efficiency (LE), and loading capacity (LC) (Table II). In general, STPP crosslinked-chitosan microparticles were smaller in size and in PDI compared with the particles crosslinked with carrageenan. Chitosan based particles produce the smallest size using STPP as crosslinker, followed by carrageenan,

alginate, and sodium dodecyl sulphate (Wulandari, *et al*, 2018). Formula A2 which resulting in the best PDI and the smallest size was then chosen to encapsulate the rhEGF.

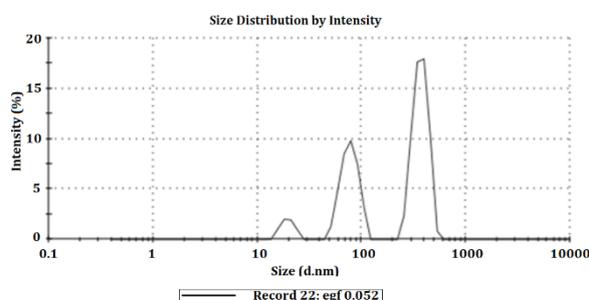


Figure 3. Size distribution by intensity of rhEGF loaded chitosan microparticles

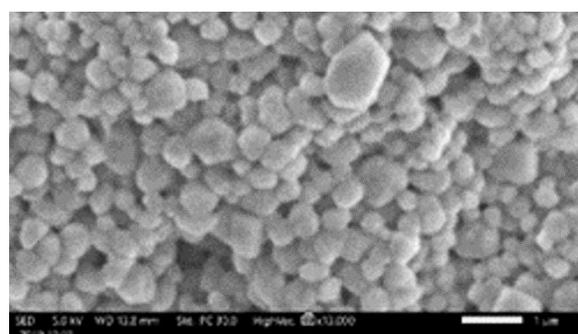


Figure 4. Morphology of rhEGF loaded chitosan microparticles ($\times 13,000$)

rh-EGF loaded chitosan microparticles, *in vitro* release study and physical characterizations thereof

Using the exact formula with BSA (Formula A2), rhEGF loaded chitosan microparticles have approximately twice bigger in size, which is 617 nm in average with PDI 0.619 compared than that of BSA loaded chitosan microparticles (Figure 3 and Figure 4). This result proves that protein molecular weight does not linearly correlate with final hydrodynamic size of a protein loaded particles. Hydrodynamic size or dimension of a protein not only depends on the molecular weight, but also its tertiary structure which is affected by a number of parameters such as folding/unfolding states and environmental pH. Hydrodynamic size/dimension also reflects oligomer or aggregate of protein (Zheng, *et al*, 2016). In hydrodynamic size, BSA and EGF are almost similar. BSA is reported having 3.3 – 4 nm or 3.7 nm hydrodynamic size and so is EGF (3.7 nm) (Jachimska, *et al*, 2008; Thone *et al*, 2004;

Brownsey, *et al*, 2003). In addition, unfolded state of rhEGF is also potential to contribute to the bigger rhEGF-loaded chitosan microparticles (Zheng, *et al*, 2016).

In order to assess capability of the chitosan microparticles to entrap the rhEGF in gastric ulcer representing environment, a release *in vitro* study was performed. The system mimicked pH and temperature of the gastric condition, i.e pH 1.2 37 °C and incubated for 2 h – minimal time needed for gastric digestion (Hoebler, *et al*, 2002). This is an extremely destructive environment that must be passed by the protein delivery system.

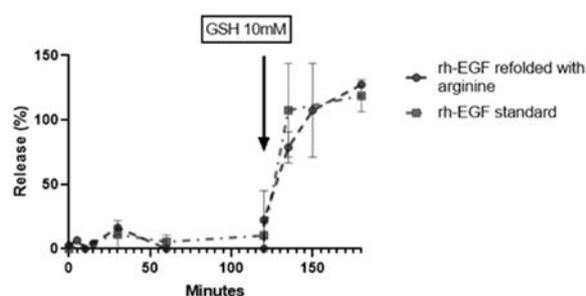


Figure 5. rhEGF release profile in gastric ulcer representing environment LE standard: 59.03%; LC standard: 5.3%, EE rhEGF: 51.33%, LC rhEGF: 7.15%

Release profile of the arginine folded rhEGF and rhEGF standard loaded chitosan microparticles was compared (Figure 5). Both of delivery system show alike release profile. Corresponding to stability, the chitosan microparticles exhibit capability to entrap the rhEGF in the extremely low pH of gastric environment in which approximately less than 20% of rhEGF was released (Figure 5) (Raza, *et al*, 2020).

Gastric ulcer is characterized with elevated level of oxidative stress which closely related to numerous gastric diseases such as *H. pylori* infection, ischemic injury, and lifestyle-related gastric diseases, for instance hypertension gastropathy and diabetes (Suzuki, *et al*, 2012). Therefore, the *in vitro* release testing system was added with GSH 10 mM to trigger the rhEGF release from chitosan microparticles as one of typical indicators for gastric ulcer (Bhattamisra, *et al*, 2019). Started in 30 min after incubated with GSH, the rhEGF was completely released (Figure 5).

EGF is known stable in gastric fluid containing pepsin and gastric acid (Britton, *et al*, 1987). In our study, the refolded rhEGF was

incubated in HCl 0.1 N at 37 °C, for 0, 1, and up to 2 h representing gastric transit condition. No other bands were found except a band of the rhEGF meaning that no major degradation caused by the related condition (Figure 6). Yet, comparing with the rhEGF without reaction with HCl 0.1 N, the protein concentrations were slightly decreased which were 90.61; 91.26; and 90.60%, respectively, quantified with ImageJ software. Playford *et al* (1995) reported that in an *in vivo* study, four amino acids (AA 49 – 53) at C-terminal of the EGF was degraded in gastric acid and cleavage between Asp 46 and Leu 47 was found due to pepsin degradation. However, as previously mentioned, bioactivity of the EGF was strongly determined by the EGF loops formed by Cys 6 – Cys 20; Cys 14 – Cys 31; and Cys 33 – Cys 42 (Ogiso, *et al*, 2002). In addition, the EGF preserves its bioactivity after gastric degradation up to 30 % (Playford, 1995). Combination with omeprazole was recommended in order to obtain 3-4 times higher concentration of EGF in gastric fluid (Dutta, *et al*, 1987).

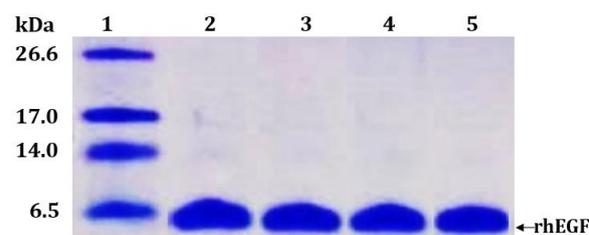


Figure 6. Stability of rhEGF in HCl at 37.5C. Lane 1: protein marker; lane 2: not incubated; lane 3: after 2 h incubation; lane 4: after 1 h incubation; lane 5: after 0 h incubation

Oral drug delivery is the most convenient and less invasive route administration. However, it is much challenging for macromolecule drugs, like protein because of gastric acidic condition and low permeability (Brown, *et al*, 2019).

Recombinant human epidermal growth factor (rhEGF) is one of top proteins used in therapies, but still limited in topical usages. Topical products containing rhEGF has been commercialized for diabetic foot, scald, and skin rejuvenation (Berlanga-Acosta, *et al*, 2017; Wong, *et al*, 2016; Aldag, *et al*, 2017). However, rhEGF is highly potential not only for topical cells renewal purposes, but also for damaged epithelial cells related-diseases, such gastric ulcer. In patient with gastric mucosal ulcer, the EGF level is lower than that of in patient with intact mucosa (Konturek, *et al*, 1997).

Itoh and Matsuo (1994) reported that combination of intravenous rhEGF and cetraxate HCl showed a significant effect to heal gastric ulcer in rats. However, individual administration of those drugs was not effective. Therefore, it was suggested that an oral application of rhEGF prospectively would show more benefits (Itoh and Matsuo, 1994).

Chitosan has been studied in pharmaceutical formulation since early 1990s (Bernkop-Schnürch and Dünnhaupt, 2012). As development of microcarriers for proteins become interesting, chitosan is also considered as one of polymers to encapsulate protein, particularly for oral delivery (Bowmand and Leong, 2006).

Chitosan is soluble in acidic pH, however in a large number of studies, drug-loaded chitosan microparticles did not show massive drug release in the gastric, yet floating and starting to perform drug controlled-release (Bowman and Leong, 2006; Pahwa *et al*, 2012). It is a mucoadhesive leading the chitosan-particles remain intact in the destructive gastric acidic environment. Moreover, chitosan is positively charged in acidic pH, thus it binds well to cell membranes which increases permeability towards gastric membrane (Deacon, *et al*, 2000).

In the recent application, the chitosan microparticles were aimed to entrap and protect the rhEGF towards the destructive gastric environment, and by being sized in micro/micro, the chitosan particles easily enter the opened damaged tissue of the gastric ulcer and then released the rhEGF triggered by high oxidative level. The free radicals contribute to degrade the chitosan polymer and reduce its molecular weight, thus the capability of chitosan microparticles to encapsulate the rhEGF is obliterated leading to its release in the therapeutic target (Jennings, 2017).

An appropriate drug carrier could overcome unfeasibility and difficulty in delivering drugs, such as protein/rhEGF for oral administration. In addition, a specific triggered-release mechanism is beneficial in avoiding toxicity at non-therapeutic target and increasing the drug accumulation at therapeutic target which eventually escalate drug effectivity (Kundu, *et al*, 2019).

In order to prove the efficacy of the rhEGF microparticle, *in vitro* or cell study alone is inadequate because it requires a condition in which biological fluids changes are available and allows oxidation triggered-release of the rhEGF from the microparticle prior to targeting the ulcer in the gastric. An *in vivo* study using gastric ulcer –

induced animal model is suggested for the further research.

CONCLUSION

Chitosan has been explored as a revolutionary delivery system for oral administration of rhEGF which is the most challenging route for protein. rhEGF has been expressed in *E. coli* BL21(DE3) using pET21 vector. In order to express the protein of interest, the expression system was induced with IPTG. The rhEGF was purified by Ni-NTA chromatography and then refolded with arginine. The refolded rhEGF showed a significant proliferation activity on MCF7 cells at 30 ng/mL compared with untreated cells. For oral delivery, the rhEGF was encapsulated with chitosan by adsorption mechanism forming microparticles. The chitosan microparticles successfully exhibited protection for rhEGF under extremely degradative gastric condition in an *in vitro* study and then specifically released the rhEGF in gastric ulcer representing environment.

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