Encapsulation of proteins with high/low pI in PLGA-particles for oral delivery

Riyona Desvy Pratiwi*, Dian Fitria Agustiyanti, Fitri Agustiani, Mega Ferdina Warsito and Apon Zaenal Mustopa

Research Center for Biotechnology, Indonesian Institute of Sciences, Cibinong Science Center, Jalan Raya Bogor Km. 46 16911, Indonesia

*Corresponding author email: riyona.desvy.pratiwi@lipi.go.id

Abstract. Polyactic co-glycolic acid (PLGA) is a biocompatible and biodegradable polymer used for controlled drug delivery of small molecules or macromolecules including protein. Proteins highly requires an advanced drug delivery system because of its sensitivity towards many factors such as temperature, hygroscopicity, and biological system. Particularly for oral dosage form, protein must be encapsulated in extremely low pH resistant particle. Protein’s intrinsic characterization determines effectivity of the encapsulation. The recent method of PLGA particle preparation was more suitable for proteins with low pI. PLGA showed higher effectivity to encapsulate protein with low pI such as BSA (pI = ~4.8) than that of high pI for instance lysozyme (pI = ~11). In the study, protein release and physico-chemical characterizations of BSA loaded PLGA-particle were performed.

1. Introduction

Polyactic co-glycolic acid (PLGA) is synthetic thermoplastic aliphatic polyester that has biodegradable and biocompatible characteristic. PLGA has been approved for medical applications by the US Food and Drug Administration (FDA) [1]. It has been reported as potential drug delivery agent for various therapeutic agents such as, chemotherapy, antibiotics, antiseptic, anti-inflammatory and antioxidant drugs, and also proteins [2,3].

The US FDA has been approved about 239 therapeutic proteins since 1982 [4]. The majority of commercially available therapeutic proteins are for parenteral administration [5, 6]. This type of administration had several downfall including not acceptable for self administration, painful, the need for sterile conditions, potential risks to health care practitioners from blood-borne pathogens, require specialized storage and cold chain transport for distribution. To date, oral administration is the most convenient and preferable administration route for most patient and it is usually the safest and least expensive option [7]. In addition, though, there are challenges to administer therapeutic protein orally because of multiple physicochemical and biological barriers in the digestive tract [8]. PLGA helps protein to pass through gastrointestinal tract and successfully deliver the active compound to be taken by M Cell of peyer patches [9].

This study used two proteins that have different isoelectric points, i.e. bovine serum albumin (BSA) and lysozyme, as the model proteins. BSA is reported to has isoelectric point (pI) of 4.8, while lysozymes 10.5-11 [10,11]. Physicochemical properties of the protein loaded particles were characterized using particle size analyzer, FTIR, and SEM, while its stability and release in the gastrointestinal tract were conducted in vitro and analyzed using SDS PAGE – semidensitometry.
2. Materials and Methods

2.1. Preparation of PLGA-particle
PLGA (lactide : glycolide 50 : 50; MW 30,000 – 60,000) was dissolved in chloroform and was dissolved in PBS pH 7.4. BSA solution was wisely dripped into PLGA solution, and then immediately sonicated using 40% A for 2 min in ice water bath. Subsequently, BSA – PLGA emulsion (emulsion 1) was dripped into 1% sodium cholate solution, then sonicated at 40% amplitude for 2 min. BSA-PLGA-sodium cholate emulsion (emulsion 2) was also wisely dripped into 0.3% sodium cholate solution and similarly sonicated with the previous condition. The last emulsion was added into 300 ml of 0.3% sodium cholate using 3 ml syringe and then stirred for overnight at 600 rpm, room temperature to evaporate organic solvent. On the following day, the PLGA-particles were separated by centrifugation at 12,000 rpm, 4°C. The particles were rinsed with dH2O three times. All materials were purchased from Sigma and Thermo.

2.2. Analysis of particles stability in acidic and neutral condition representing digestive system
Particles pellet was diluted in 0.1 N HCl pH 1.2 and 1× PBS pH 7.4 then incubated at 37°C for 4 h, respectively. Afterwards, the pellet was separated with the released protein by ultracentrifugation at 30,000 rpm, 10 min, 4°C. The supernate containing released protein and the pellet with the remained protein were analyzed using SDS PAGE with Coomasie blue staining. The concentration of protein was analyzed using semi-densitometer with a serial concentration of BSA standard and then calculated by using ImageJ software.

2.3. Loading capacity (LC) and entrapment efficiency (EE) of PLGA Particles
Total loaded protein was calculated from released and remained protein in the pellet. Loading capacity (LC) is calculated based on total of loaded protein to total particles produced. Entrapment efficiency (EE) is calculated based on quantification of successfully loaded protein to total protein used. Calculation following equation by Fernandez-urrusuno et.al. 1999 [12].

\[
\text{Entrapment Efficiency} = \frac{(\text{Total protein} - \text{unbound Protein})}{\text{Total protein}} \times 100
\]

\[
\text{Loading capacity} = \frac{(\text{Total protein} - \text{unbound protein})}{\text{Total nanoparticles}} \times 100
\]

2.4. Analysis of particles size, PDI and zeta potential
Measurement of particles size, PDI (polydispersity index), and zeta potential were determined using Particle Size Analyzer (Zetasizer Nano Range, Malvern Panalytical). Z-average size obtained by DLS (Dynamic Light Scattering), a mean value for size and width parameter known as PDI (zetasizer nano manual, Malvern 2004)

2.5. Analysis of Fourier Transform Infra-Red (FTIR)
FTIR was analysis of interfaces to investigate the surface adsorption of functional groups on particle. Samples were freeze-dried, then mixed with potassium bromide (KBr). IR spectra in ranged 4000-400 cm\(^{-1}\). IR spectra result by Spectrum Two FT-IR spectometer (Perkin Elmer, USA).

2.6. Analysis of Scanning Electron Microscopy (SEM)
SEM analysis was used to determine dispersion, size, shape and texture of particle surface morphology. A freeze-dried sampel was coated with gold, then observed under scanning electron microscope (JSM IT200, JEOL, Japan).
3. Result and Discussion

3.1. Amount of PLGA and solvent determined hydrodynamic size

Concentration of polymer (PLGA) and organic solvent significantly affect particle size. Table 1 shows large particles were obtained from high concentration or high amount of PLGA. Formula 1 and formula 2 resulted in higher particles than formula 3 and 4. Huang and Zhang reported that particles prepared from PLGA >40 mg/ml were found in large size (>500 nm) [13]. Polydispersity (PDI >0.7) was found in the particles with high concentration of PLGA (Table 1) [14]. High concentration of PLGA contributed to increase amount of polymers chains per unit volume of organic solvent leading to more polymer – polymer interaction and forming larger particles and aggregates [15].

PLGA particles are formed via precipitation polymer droplet (organic phase) in an aqueous phase [13]. Therefore, diffusion rate of the organic solvent into the aqueous solvent predominantly determine particle size [13]. The organic solvent was removed from the aqueous phase by evaporation using stirring method at room temperature for overnight[16]. This method is known simple and mild, howbeit, long stirring time is potential to cause particles coalescence and protein diffusion into the second water layer generating particle aggregation and low EE [17]. A faster organic solvent removal using rotary evaporator is then considered to improve the PLGA-particles, in terms of particle size and EE percentage [17].

Comparing formula 3 and formula 4 containing similar amount of PLGA, yet less organic solvent in formula 4 resulted in smaller particles in size (Table 1). In this study, chloroform was used. Chloroform is a water immiscible solvent which is the best solvent for PLGA, however it requires longer time to diffuse to the aqueous phase causing formation particles in large size. Similar with water immiscible solvent, fully water soluble solvent such as acetone also yield large particle. Song and colleagues reported that partially water soluble solvent, for instance ethyl acetate and propylene carbonate exhibited smaller particles [18].

Moreover, the formula 4 contained higher amount of surfactant, i.e sodium cholate. The surfactant facilitates organic droplet of polymer to diffuse into aqueous phase and stabilizes the system [15]. Surfactant is beneficial to avoid particles collide and coalesce among themselves. Therefore, the formula 4 also gave monodispersed particles indicated by PDI < 0.7 [18].

Size range in particles are varied depending on the characteristics of the particles themselves. The Nanomedicine European Technology Platform (ETPN) determines that particles (nanomedicine) is not more than 100 nm, but European Commission mentioned that particles, specifically nanomedicine has size up to 1000 nm because it is commonly provided with additional properties for better biological interaction [19,20,21]. Turning to particles obtained in this study, formula 4 resulted BSA – loaded particles in nanosize.

**Table 1. Formula and physical characterizations of PLGA-particles.**

| Component                  | Formula 1                                      | Formula 2                                      | Formula 3                                      | Formula 4                                      | Formula 4 (lysozyme)                   |
|----------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|-----------------------------------------------|----------------------------------------|
| PLGA                       | 200 mg (in 5 ml chloroform)                   | 400 mg (in 5 ml chloroform)                   | 50 mg (in 5 ml chloroform)                    | 50 mg (in 2 ml chloroform)                     | 1 ml (stock 1.8 mg/ml)                |
| Protein (BSA)              | 400 µl (stock 10 mg/ml)                       | 150 µl                                        |                                              |                                              |                                        |
| Trehalose 10%              |                                               |                                               |                                              |                                              |                                        |
| Sodium cholate 1%          | 2 ml                                          |                                               |                                              |                                              |                                        |
| Sodium cholate 0.3%        |                                               |                                               |                                              |                                              |                                        |
| Result                     | 134.5 mg                                      | 184.5 mg                                      | 70 mg                                        | 100 mg                                        | NA                                     |
| Hydrodynamic size          | 2666.5 nm ± 849.26                            | 2208.3 nm ± 117.78                           | NA (> 1000 nm)                               | 512.6 nm ± 130.35                            | NA                                     |
| Polydispersity index (PDI) | 1                                             | 0.85                                          | NA                                           | 0.659                                         | NA                                     |
| Zeta potential             | NA                                            | NA                                            | NA                                           | -26.63 mV                                     | NA                                     |
3.2. Protein loading and release study

Proteins are loaded into particles through several loading mechanisms depending on pH, ionic strength, denaturant, temperature or ‘breathing mechanism’ [22]. In this study, pre-load technique in which protein loading and particles preparation are performed at the same time was applied. The protein, BSA dissolved in PBS pH 7.4, was encapsulated into particles by mixing it with PLGA in organic solvent. Surprisingly, with a few modification in amount of organic solvent and surfactant which yielded significantly smaller size PLGA particles, the amount of encapsulated BSA was remarkably increased reaching loading efficiency up to 88.89 % (Figure 1c lane 9). In addition, encapsulation efficiency (EE) depends on type of PLGA polymer and ionic strength [23,24]. PLGA with low molecular weight (MW) is potential to give high EE. Increase in PLGA MW reduces percentage of EE. Among PLGA 15,000; 25,000; and 30,000 MW, the PLGA 15,000 MW resulted the highest EE, whereas PLGA 30,000 MW gave the lowest EE [23]. In this study, PLGA with 30,000 – 60,000 MW. The EE was quite high because the initial amount of protein was minimized to wasted un-encapsulated protein. However, in line with Fu and colleagues, percentage of LC was slightly low, about 3.2% which was caused by PLGA with high MW. Even so, particles made from PLGA with high MW is stable towards degradation, particularly hydrolysis [23].

The obtained PLGA particles showed capability to protect the encapsulated protein in neutral condition, and slightly released the protein in acidic solution. Figure 1 shows remained and released BSA/lysozyme in HCl pH 1.2 37°C and PBS pH 7.4 37°C representing acidic gastric and neutral esophagus condition, respectively [25]. Figure lane 1 12; 17; and 21 indicate slight release of BSA from PLGA-particles after 2 h incubation in acidic environment. Figure 1 lane 13; 16; and 20 no release of BSA after 2 h incubation in neutral environment. The results explain that the PLGA-particles protected BSA in neutral condition, but was slightly degraded in extremely acidic pH. Figure 1 lane 6 also shows slight release of BSA in the acidic and normal condition, yet not valuable compared with amount of loaded BSA.

Figure 1. Electroforegram of released and remained proteins in in vitro conditions representing gastric and esophagus environment. Lane 1 – 5: BSA standard (0.0625; 0.125; 0.25; 0.5; 1 mg/ml); y = 26520x + 6990.6, r² = 0.9966. Lane 6;12; 17; 21: BSA released in HCl 0.1N pH 1.2 37°C. Lane 7; 13; 16; 20: BSA released in PBS pH 7.4 37°C. Lane 8;11; 15; 19: BSA remained after incubation in HCl 0.1N pH 1.2 37°C. Lane 9; 10;14;18: BSA remained after incubation in PBS pH 7.4 37°C. Lane 22: Lysozyme released in HCl 0.1N pH 1.2 37°C. Lane 23: Lysozyme released in PBS pH 7.4 37°C. Lane 24: Lysozyme remained after incubation in HCl 0.1N pH 1.2 37°C. Lane 25: Lysozyme remained in HCl 0.1N pH 1.2 37°C. Lane 6 – 9: Formula 1. Lane 10 – 13: Formula 2. Lane 14 – 17: Formula 3. Lane 22 – 25: Formula 4.

Figure 1 lane 22 - 25 are remained and released profile of lysozyme from the PLGA-particles. No band were found in the lysozyme electroforegram meaning that the protein was not successfully encapsulated with PLGA using formula 4, the best formula for BSA. Lysozyme is commonly used as model protein for high pl [24]. In the recent study, proteins were dissolved in PBS pH 7.4 and the PLGA-
particles were prepared also in neutral pH aqueous solution. pH of the aqueous phase is the most important factor influencing EE. Proteins are known less ionized at pH near its isoelectric point, so the proteins are less soluble in the aqueous phase compared than that of in the organic phase. Therefore, the protein leakage into the aqueous phase is minimized leading to more effective encapsulation [26]. The applied pH of aqueous phase in this study was much lower towards lysozyme pI than BSA pI and it clearly contributed to no entrapment of lysozyme into the PLGA-particles. Besides, encapsulation of lysozyme in PLGA-particles was strongly dependant on the PLGA MW, in which lysozyme was only entrapped into the PLGA-particles made from low MW polymer. Otherwise, BSA entrapment into the PLGA-particles was not affected by the PLGA MW [24].

3.3. FTIR spectroscopy and SEM
BSA - loaded PLGA-particles formula 4 was characterized with FTIR spectroscopy and SEM. Interaction of each component after particles formation and washing was studied using FTIR spectroscopy. Spectra of BSA – loaded PLGA-particles was compared with all components thereof, which were PLGA polymer, sodium cholate, trehalose, and BSA. Major spectrum of sodium cholate and trehalose were not found in BSA – loaded PLGA-particles spectra, as expected. Those components were not incorporated with the particles and removed during the washing steps. The FTIR spectrum show that both components were succesfully cleared.

On the other hand, spectrum of PLGA polymer and BSA – loaded particles were almost similar, except there are two vibration peaks appear at wavenumber 1657 cm$^{-1}$ presenting asymetric stretching vibration of arginin for CNH$_3$H$^+$ and 1546 cm$^{-1}$ for asymetric stretching vibration of glutamin, specifically for COO$^-$.Those are specific vibration peak for amino acids [27]. It decribes that BSA has been bound with PLGA polymer forming BSA – loaded particles (Figure 2).

![Figure 2. FTIR spectrum of BSA – loaded PLGA particles and components thereof.](image)
The other vibration peaks depict functional groups of PLGA. A strong and narrow vibration peak at 1748 cm\(^{-1}\) points C=O of PLGA [28]. Fingerprint peaks of PLGA are observed at 1163, 1130, and 1085 cm\(^{-1}\) indicating C-O stretching vibration, whereas C-H stretching vibration peaks are shown at 1422 and 1382 cm\(^{-1}\) [28-30]. Two small peaks around 2950 cm\(^{-1}\) are stretching vibration of C-H from aliphatic CH\(_2\) and -C-H- of PLGA [28-30].

Figure 3 depicts BSA loaded particles under SEM with 13,000 \(\times\) magnification. As detected with particle size analyzer, the BSA loaded PLGA particles were found approximately 200 – 600 nm, yet some bigger particles which were around 1000 nm in size were also captured.

![SEM Image]

**Figure 3.** Visualization of BSA – loaded PLGA particles with SEM

### 4. Conclusion
Preparation of protein loaded PLGA particles is complicated and depends on a number of factors. The particle size was influenced by types of PLGA polymer, PLGA MW, organic solvent, and surfactant. The protein encapsulation into PLGA particles was determined by the pH system or aqueous phase and PLGA MW. The pH was required as near as the protein pI expecting high EE. Moreover, process of organic solvent removal also played significant role controlling particle size and EE percentage. The reported procedure was more suitable for BSA as protein with quite low pI than that of for lysozyme which has high pI. The recent protein delivery system was highly potential and suitable for oral delivery in which it showed capability to protect the protein in extremely acidic solution corresponding gastric environment.

### Acknowledgment
The authors would like to thank to Program of Prioritas Nasional (PN) Obat 2019, Research Center for Biotechnology, Indonesian Institute of Science, Research Center for Biology and Research Center for Biomaterial Indonesian Institute of Sciences for facilitating us in analysis with SEM and FTIR, and Ms. Nur Raisah Maddepungeng for her assistance on redrawing the FTIR spectrum using Origin\(^\circledR\) software.

### References
1. Pandey A, Jain D S and Chakraborty S 2015 *Handb. Polym. Pharm. Technol.* 2, ed V K Thakur and M K Thakur (Beverly: Scrivener Publishing LLC/Wiley) p 151–72
2. Danhier F, Ansorena E, Silva J M, Coco R, Le Breton A and Préat V 2012 *J. Control. Release* 161 505–22
3. Berthet M, Gauthier Y, Lacroix C, Verrier B and Monge C 2017 *Trends Biotechnol.* 35 770–84
[4] Usmani S S, Bedi G, Samuel J S, Singh S, Kalra S, Kuma P, Ahuja A A, Sharma M, Gautam A and Raghava G P S 2017. PLoS ONE 12 1–12
[5] Ramirez J E V, Sharpe L A and Peppas N A 2017 Adv Drug Deliv Rev. 114 116–31
[6] Ye C and Venkatraman S 2019 Ther. Deliv. 10 269–72
[7] Dinda A K, Bhat M, Srivastava S, Kottarath S K and Prashant C K 2016 Vaccine 34 3076–81
[8] Mamo B 2015 Asian J. Biomed. Pharm. Sci. 5 1–12
[9] Malathi S, Nandhakumar P, Pandiyavan V, Webster T J and Balasubramanian S 2015 Int. J. Nanomed. 10 2207
[10] Zeng X and Ruckenstein E 1998 J. Membrane Sci. 148 195–205
[11] Deng Q Y, Zhou C R and Luo B H 2006 Pharm. Biol. 44 336–42
[12] Fernandez-Urrusuno R, Calpo V, Remunan-Lopez C, Vila-Jato J L and Alonso M J 1999 Pharm. Res. 16(10) 1575 – 81
[13] Huang W and Zhang C 2018 Biotech. J. 13(1) 1 – 25
[14] Rahmawanty D, Anwar E and Bahtiar A 2015 Jurnal Ilmu Kefarmasian Indonesia 13(1) 1693 – 831
[15] Reddy P R V, Acharya S R and Acharya N S 2015 Asian J. Pharm. 9 152 – 61
[16] Kwon B S, Lee H E, Kim D H, Kang H K, Kang J S, Lee S and Choi S J 2007 I. Ind. Eng. Chem. 13(6) 1043 - 6
[17] Mishra N, Tiwati S, Vaidya B, Agrawal G P and Vyas S P 2011 J. Drug Target 19(1) : 67 - 8
[18] Song C, Lee H S, Choung Y, Cho K I, Ahn Y and Choi E J 2006 Physicochem. Eng. Aspects 276 162 - 7
[19] Tomellini R, Faure D and Panzer O 2005 European Technology Platform, Strategic Research Agenda for Nanomedicine, Nanomedicine Nanotechnology for Health
[20] Wagner V, Husing B and Bock A K 2008 JRC Scientific and Technical Reports – European Commission 1-13
[21] Lohcharoenkal W, Wang L, Chen Y C and Rojanasakul Y 2014 Biomed. Res. Int. 1-12
[22] Diaz A C and Sunna A 2018 Genes 9(7) 1 – 30
[23] Fu X, Ping Q and Gao Y 2005 J. Microencapsul. 22(7) 705 - 14
[24] Blanco D and Alonso M J 1998 European J. Pharm. Biopharm. 45(3) 285 - 94
[25] D’Souza S 2014 Adv. Pharm. 304757 1 - 12
[26] Swed A, Cordonnier T, Fleury F and Boury F J. Nanomed. Nanotechnol. 5(241) 1 - 8
[27] Barth A 2007 BBA – Bioenergetics 1767(9) 1073 – 101
[28] Arasoglu T, Derman S and Mansuroglu B 2016 Nanotechnology 27(025103) 1 – 12
[29] Dompeipen E J 2017 Majalah BIAM 13(01) 31-41
[30] Nandiyanto A B D, Oktiani R and Ragadhi R 2019 IJoST 4(1) 97 - 118