Characterization and Hemocompatibility Assay of Microencapsulation Chitosan-Alginate Single Garlic Extract-loaded (MCA-SGE)

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Abstract. Single garlic is a tropical plant with high bioactive compounds. Most bioactive compound found in single garlic is allicin but it has low bioavailability. Allicin was classified to reactive sulfur species (RSS) and has the ability to damage eukaryotic cells, for example is red blood cells (RBC). The damaged of RBC was minimized with a specific mechanism using the drug delivery system (DDS) with Microencapsulation Chitosan-Alginate (MCA). The aim of this study is to characterize and test the hemocompatibility of RBC. Single garlic extraction method using maceration with 70% ethanol. Optimal formulation of MCA-SGE determined using characterization with particle size analyzer (PSA) and hemocompatibility assay. PSA of MCA-SGE such as Z-Average (390.540± 11.460 nm), Polydispersity Index (PdI) (0.609 ± 0.011), and zeta potential (-23.067 ± 0.493 mV) shows that MCA-SGE categorized into optimal DDS. Hemocompatibility assay shows that MCA-SGE has low hemolysis percentage than SGE. The result of hemolysis percentage MCA-SGE does not cause the damage of RBC. Thus, it can be concluded that MCA-SGE was optimal increasing bioavailability of allicin compounds, hence MCA-SGE was compatible with RBC.

1 Introduction

Single garlic (*Allium sativum* L.) is one of the tropical plants in Indonesia. Single garlic has biological functions as an antioxidant, anti-cancer, anti-inflammatory, antibacterial, and immunomodulatory [1–5]. Most bioactive compounds were found in single garlic is organosulfur such as DAS, DADS DATS, ajoene, allicin, and alliin [6]. Allicin is a bioactive compound was formed when the alliin reacts to enzyme allinase while garlic is being cut. Allicin is claimed to be one of the compounds responsible for its pharmacological but is classified as an unstable compound. Factors that affect the instability of allicin compounds include pH, temperature, and chemical bonds [7]. Unstable compounds of allicin lead single

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garlic to has low bioavailability. Allicin was classified as RSS and has the ability to damage eukaryotic cells, for example, RBC [8]. Allicin should be protected with a specific mechanism using the drug delivery system (DDS) to reduce the damage of RBC.

Researchers developed several DDS to minimize hemolysis of RBC against its bioactive compound. Examples of DDS such as liposomes, dendrimers, micelles, self-nano emulsifying drug delivery systems (SNEDDS), microencapsulation, etc [9–12]. Microencapsulation is one of the DDS that was developed. Previous studies have proven that microencapsulation has succeeded in protecting bioactive compounds in turmeric with particle sizes < 400 nm [13]. Microencapsulation with Single Garlic Extract (SGE) using ionic gelation with the main component is a polymer [14]. Polymers have been used in microencapsulation were chitosan, alginate, and a crosslinker agent $CaCl_2$ [15]. Chitosan comes from the core shells of crustaceans while alginate comes from brown algae [16]. Polymers play an important role in Microencapsulation Chitosan-Alginate (MCA) formulation as biodegradable components, forming hydrogel beads and polyelectrostatic interaction when the components interact [17]. MCA also increases drug release and reduces the hemolysis percentage of RBC [18–20].

The optimal formulation of MCA-SGE is expected to be the next candidate for optimal DDS. Thus, MCA-SGE is well absorbed by oral administration, then to the gastrointestinal tract, circulated to the blood stream, and also not to cause damage of RBC. This study aims to characterize and asses the hemocompatibility of MCA-SGE against the hemolysis of RBCs.

2 Experimental Design

The materials used include single garlic from Oro-Oro Dowo Market Malang, 70% ethanol (Merck), aquabides (Ikapharmindo), chitosan (Sigma-Aldrich), alginate (Sigma-Aldrich), $CaCl_2$, Tween-80 (Sigma-Aldrich), 1% asetic acid, 1 M NaOH, Phosphate Buffer Saline (PBS), 1% Triton-X 100, Ethylenediamine Tetraacetic Acid (EDTA), *Mus musculus*, microfilter 0.45 µm, microtube 2 mL, syringe, gloves, aluminum foil, plastic wrap, blue tip (OneMed), and yellow tip (OneMed).

Preparation of Single Garlic Extract (SGE). The extraction of single garlic using maceration method with 70% ethanol as solvent. A total of 1 kg single garlic was peeled and mashed, then soaked in 3 L of ethanol 70% for 3 days [21]. The maceration results were continued with the evaporation process using a rotary evaporator, thus a single garlic liquid extract was obtained.

Preparation of MCA-SGE. Preparation of MCA-SGE components included production stocks of chitosan 0.6 mg/mL, alginate 0.6 mg/mL, and CaCl2 0.67 mg/mL. Chitosan dissolved in 1% acetic acid, alginate dissolved in 0.5% tween-80, and CaCl2 dissolved in aquabides. For the formulation of MCA-SGE, 1.5 mL SGE was added to 50 mL alginate 0.6 mg/mL then homogenized with a magnetic stirrer for 10 minutes and sonicated for 15 minutes. Then, continue with adding 10 mL dropwise of CaCl2 0.67 mg/mL and homogenized with ultra turrax for 5 minutes and homogenized with a magnetic stirrer for 30 minutes. 2.5 mL chitosan 0.6 mg/mL was homogenized with ultra turrax for 5 minutes and homogenized with a magnetic stirrer for 60 minutes. MCA-SGE was stored at 4°C [22].

Characterization of MCA-SGE. Characterization of MCA-SGE using Particle Size Analyzer (PSA) Nano-Zetasizer Ver. 7.01 (Malvern Instruments Ltd.) included Z-Average, Polydispersity Index (PdI), and Zeta potential. Data obtained from Z-Average is the particle size of MCA-SGE, while data of PdI includes the distribution of particle homogeneity. Zeta potential data includes the indicator of MCA-SGE dispersion stability. A sample of MCA-SGE was diluted with deionized water using a 2:1 ratio with three repetitions [22].

Hemocompatibility Assay. Hemocompatibility test using RBC of *Mus musculus*. Blood was obtained from heart organ, then put into a 2 mL microtube containing EDTA anti-coagulant and shaken for a while. Blood samples were then centrifuged for 10 minutes at 3000 rpm. The supernatant was discarded and 150 µL of RBC was transferred to a new microtube. Then, 0.9% NaCl was added to RBC for washing with a ratio of 1:10. Each RBC sample was added with PBS, 1% Triton-X 100, SGE, and MCA-SGE then homogenized with vortex [22]. Samples were incubated in an incubator shaker for 60 minutes at 37°C. After the incubation process, samples were centrifuged for 10 minutes at 4000 rpm. The supernatant was moved to a new microtube then measured the absorbance value at a wavelength of 540 nm [23]. The percentage value of hemolysis RBC is calculated by the following Equation 1.

$$\% \text{ Hemolysis} = \frac{(Sample - Negative\ control)}{(Negative\ control. - Positive\ control.)} \quad (1)$$

3 Results and Discussion

3.1 Particle Size Analyzer

The results of MCA-SGE Z-Average have been shown that the particle size are 390.540 ± 11.460 nm and the size is classified as microparticle size. According to [24], Z-Average using Dynamic Light Scattering (DLS), hence the particle size of the sample has a larger size than the measurement with a microscope because the MCA particles are swollen. The large particle size is also caused by the concentration of the MCA-SGE components that establish the microparticles. The graph of the MCA-SGE Z-Average value can be seen in Figure 1.

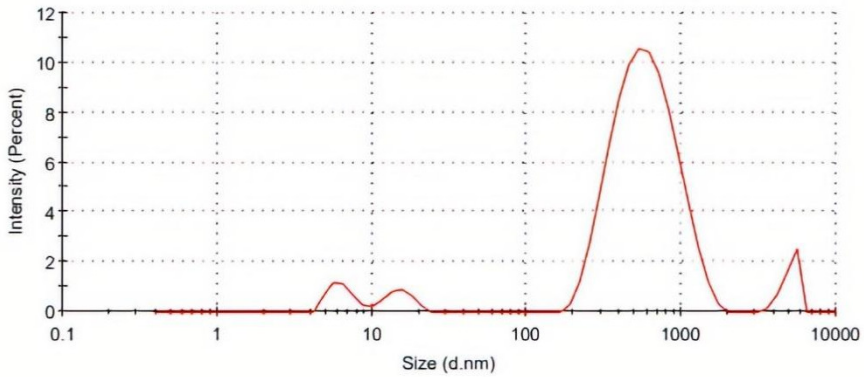

Fig. 1. Graph of MCA-SGE Z-Average

PdI aims to discover the distribution of particle homogeneity. The most optimal value of PdI is < 0.5 or close to 0 [25]. The results of the MCA-SGE PdI are 0.609 ± 0.011 (Table 1). A low PdI value indicates that the distribution of particle homogeneity is high (monodispersity), while a high PdI value indicates that the particles are widely distributed (polydispersity) [26]. The value of MCA-SGE PdI shows that it a slightly higher than 0.5, thus it can be categorized as heterogeneous because most of the MCA-SGE particles are not formed, resulting in various sizes. High PdI values also occur due to weak ionic interaction among the main component of MCA-SGE, hence aggregation occurs [27].

MCA-SGE particle stability is found with zeta potential. Criteria of optimal zeta potential is range between < -30 mV and > +30 mV [28]. The results of the MCA-SGE zeta potential are -23.067 ± 0.493 mV. The value of zeta potential is related to pH, ionic interactions, and the main component of the particles. Based on MCA-SGE zeta potential -23.067 > -30 mV,

accordingly, the formulation of MCA-SGE is classified as less stable due to particles forming an agglomeration [29]. The agglomeration of particles occurs of high Van der Waals forces [25]. The graph of the MCA-SGE Zeta Potential value can be seen in Figure 2.

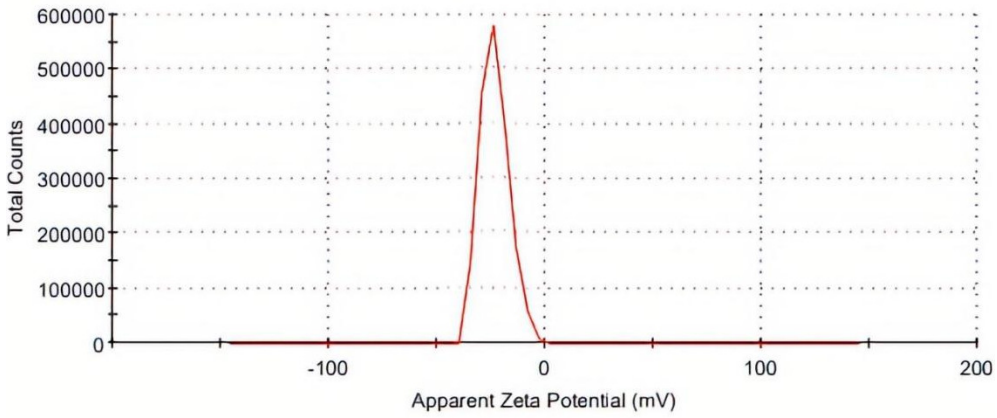

Fig. 2. Graph of MCA-SGE Zeta Potential

Table 1. Characterization of MCA-SGE using PSA

PSA Characterization of MCA-SGE	
Z-average (nm)	390.540 ± 11.460
Polydispersity Index	0.609 ± 0.011
Zeta Potential (mV)	23.067 ± 0.493

3.2 Hemocompatibility Assay

Hemocompatibility is one of the parameters used to test the biocompatibility of a DDS, therefore, the hemocompatibility test aims to determine the RBC response to unfamiliar objects in the blood circulation, such as bioactive compounds [30]. The MCA-SGE hemocompatibility test is to find out the hemolysis of RBC level against SGE and MCA-SGE. The graph of SGE and MCA-SGE Hemocompatibility Percentage can be seen in Figure 3.

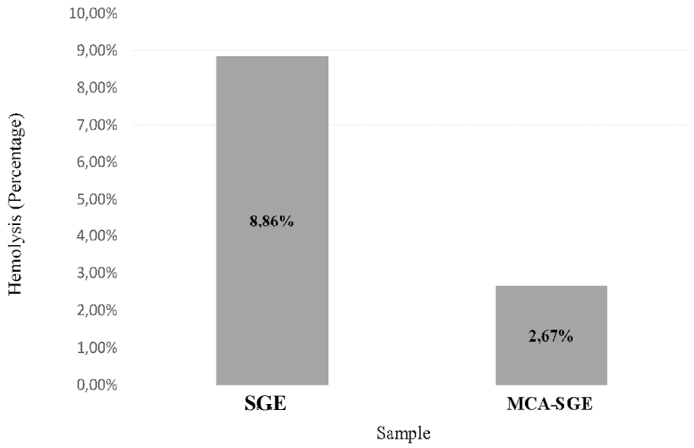

Fig.3. Graph of SGE and MCA-SGE Hemocompatibility Percentage

Hemolysis is the rupture of RBC caused by high osmotic pressure. In the hemolysis test, Triton-X 100 solution was used as a positive control, while PBS was used as a negative control [20]. From the SGE and MCA-SGE hemocompatibility result tests obtained there are 8.86 ± 0.61% and 6.04 ± 0.70% of hemolysis. The most optimal percentage of hemolysis is <5% or close to 0% [31]. However 10-25% hemolysis is considered a relative limit (10% are non-hemolytic, and 25% are hemolytic) [23]. The hemolysis SGE and MCA-SGE percentages are included as the non-hemolytic because <10%. MCA-SGE hemolysis percentage is lower than SGE which makes the MCA-SGE could be able to minimize the hemolysis in RBC.

The low percentage of MCA-SGE hemolysis indicates that allicin compounds are thought to be well absorbed by the gastrointestinal tract to blood circulation. With good MCA-SGE absorption, there is an optimal formation process. MCA components such as chitosan and alginate have been shown not to cause RBC to burst because they are biocompatible and biodegradable polysaccharides [32–33]. The amine group in chitosan will form an ionic interaction with the carboxylate group on the alginate to become a gel at an acidic pH as in gastrin (stomach) [17]. On the outer surface of the MCA, there is chitosan which when it enters the gastrin will burst because its pH tends to be higher than the gastrin pH [22–34]. Alginate tends to be stable hence it can protect and release the bioactive compounds in the intestines with neutral pH. The bioactive compounds that were released from MCA will be absorbed by the intestine and circulated into the blood circulation [35].

The complexation of chitosan-alginate ionic interactions was also strengthened by the crosslinker agent, $CaCl_2$ [36]. $CaCl_2$ will bind to the alginate and cause a polyelectrostatic interaction between Ca^{2+} and the carboxylate group of alginate, therefore a gel will be formed by Ca^{2+} ions. $CaCl_2$ is used as a crosslinker agent because the Ca^{2+} ion is a divalent ion that has a faster gel formation rate than other divalent ions [37]. DDS with multiple components and reinforced with crosslinkers have been shown to have better biocompatibility. It has also been shown not to cause toxicity and hemolysis in RBC [18]. MCA-SGE is expected to reduce hemolysis and increase allicin bioavailability.

4 Conclusion

The results showed that characterization of MCA-SGE using PSA was classified to microparticles size (390.540 ± 11.460 nm), heterogeneous distribution (0.609 ± 0.011), and had less stable particle (-23.067 ± 0.493 mV). Based on hemolysis criteria, SGE and MCA-SGE are included as the non-hemolytic with the results SGE (8.86 ± 0.61%) and MCA-SGE (6.04 ± 0.70%). MCA-SGE hemolysis percentage is lower than SGE, thus MCA-SGE is expected to reduce hemolysis and be compatible with RBC.

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