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Research Article

FORMULATION OF SELF-NANOEMULSIFYING DRUG DELIVERY SYSTEM OF BOVINE SERUM ALBUMIN USING HLB (HYDROPHILIC-LYPOPHILIC BALANCE) APPROACH

Lina winarti[1,2], Suwaldi [3], Ronny Martien[1,3]*, Lukman Hakim[4]

[1]Postgraduate Programme, Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta 55281, Indonesia;
[2]Dept of Pharmaceutics, Faculty of Pharmacy, University of Jember, Jember 68121, Indonesia;
[3]Dept of Pharmaceutics, Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta 55281, Indonesia;
[4]Dept of Pharmacology and Clinical Pharmacy, Faculty of Pharmacy, Universitas Gadjah Mada, Yogyakarta 55281, Indonesia.

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*Corresponding author
Ronny Martien

Email:
ronnymartien@ugm.ac.id

ABSTRACT

Self-Nanoemulsifying Drug Delivery System (SNEDDS) has potential to be developed for oral protein delivery because it is free from water, hence preserving the stability of protein, protecting protein from enzymatic degradation, and enhancing the protein permeability in the gastrointestinal tract (GIT). However, protein-based SNEDDS formulation is challenging due to low solubility property of protein in oil, which is towards zero. This present study aimed to obtain the most compatible SNEDDS for protein using HLB approach. Bovine serum albumin (BSA) was used as a protein model. A number of 78 formulas with HLB ranging between 11 and 15 were screened to acquire stable SNEDDS composition without the presence of phase separation. Of 13 stable formulas, two were selected (F30 and F45) with HLB 15, and then loaded with BSA. Physical characteristics of both formulas were then evaluated and these results suggested that SNEDDS with single hydrophilic surfactant (F45) and HLB 15 was the best formula for protein template as the stability testing showed that phase separation and precipitation did not appear. It was robust to pH and dilution with percentage of transmittance of 96.40±1.05% and the droplet size of 180.9nm. F45 had also uniform distribution of droplets size since the polydispersity index was less than 0.1. The zeta potential of F45 was -0.12mv with loading efficiency 83.57±1.77%. The emulsifying time of F45 was > 2min due to the formation of crystalline gel that was difficult to disperse.

Key words: SNEDDS, BSA, hydrophllic surfactant, lypophilic surfactant, protein delivery

INTRODUCTION

Oral delivery system is the most convenient route because it can improve patient adherence, especially among patients with chronic diseases (Noha *et al*., 2006). However, the use of protein in the development of oral delivery system remains a challenge due to enzymatic degradation, acidic environment of the stomach, less epitelial permeability, and rapid clearance from gastrointestinal tract (GIT) (Zhang *et al*., 2012; Saffran *et al*., 1986; Fix, 1996). This urges researchers to develop more on oral delivery system of protein. Some techniques have been tested to improve protein bioavailability, including the use of absorption enhancer (Takatsuka *et al*., 2006), protease inhibitor (Bernkop-Schnürch and Scerbe-Saiko, 1998), encapsulation on several delivery systems using nanoparticles (Sonaje *et al*., 2009), microemulsion (Sharma *et al*., 2010), self-nano-emulsifying drug delivery system (SNEDDS) (Li *et al*., 2014; Ma *et al*., 2006; Sakloetsakun *et al*., 2013), and liposom (Wu *et al*., 2011).

This present research employs Bovine Serum Albumin (BSA) as a protein model as it is sufficiently stable and widely used to evaluate a drug delivery system (Ravi Kumar *et al*., 2009). BSA is a very hydrophillic macro-molecule with molecular weight of 66,000 DA (Rachmawati *et al*., 2008). As a protein, the backbone of the protein chain is highly polar with the amino (-NH-) group as a strong donor of hidrogen bonds and the carbonyl (-CO-) group an acceptor of hydrogen bonds. Protein is made of lots of amino acids joined together by peptide bonds. In the presence of water the peptide bonds can be broken by hydrolisis. On the other hand, SNEDDS offers an advantage

for protein delivery since it has no water on it (Chime *et al.*, 2014; Sakloetsakun *et al.*, 2013; Patel *et al.*, 2013). Hence, protein physical and chemical stability can be assured.

SNEDDS is anhydrous form of nanoemulsion (preconcentrated nanoemulsion) consisting of oil, surfactant, co-surfactant/co-solvent and drug that forms fine oil in water (O/W) nanoemulsion when introduced into aqueous phase under mild agitation (Rao and Shao, 2008; Shakloetsakun, 2013). However, protein solubility in the oil phase is almost zero (Rao and Shao, 2008). Therefore, to formulate protein into SNEDDS, it is necessary to find suitable solvent for protein. Glycerine in the SNEDDS formula is used as co-solvent to increase the solubility of therapeutic agents (Parul *et al.*, 2013). According to Houen (1996) BSA solubility in glycerine was 20mg/mL (half dissolved). Because of its solubility in glycerine, BSA can be incorporated in SNEDDS by dissolving first in glycerine. Another problem may appear because protein has relatively large molecular weight and glycerine is water soluble compound, forming less homogenous SNEDDS due to phase separation during storage. Therefore, it is important to determine surfactants with suitable HLB to stabilize and facilitate glycerine and soluble protein in SNEDDS.

In this study, a mixture of surfactant with similar structure, Tween and Span, were used (Wang *et al.*, 2009; Weerapol, 2014) to obtain HLB range 11 to 15. As non-ionic surfactants, Tween and Span offer many advantages over ionic surfactant including increased stability, formulating flexibility, and wider compatibility. By combining Tween and Span at different ratios, a wide HLB range to emulsify most oils can be created (Croda, 2010). Combinations of both surfactants were designed to have mixed HLB > 10 in order to form O/W system which is easy to become emulsion spontaneously in aqueous media.

Mygliol 812 was selected as oil phase. Mygliol 812 is a medium chain triglyceride use for oral and commonly is used as penetration enhancer, drug carrier, and solvent (Warner Graham Company, 2016).

Previous work was done on SNEDDS formulation using BSA complexed with phospholipid to increase protein solubility in oil (Rachmawati *et al.*, 2008). Currently, there has not been a report exploring the influence of HLB value on SNEDDS as protein template. Hence, the present study developed SNEDDS containing BSA to establish a SNEDDS system for protein using HLB method. Afterward the formulation was subsequently subjected to the physicochemical characterization for oral delivery of protein.

MATERIALS AND METHODS

BSA, Span 20, and Span 80 were obtained from Sigma Aldrich, Germany. Mygliol® 812 was purchased from Cremer Oleo GmbH & Co.KG. Chremophor EL 40 was a gift from Shanghai Terppon China. Glycerine and trichloro acetic acid (TCA) were from Merck KgaA Germany. Tween 20, Tween 80 were from Bratachem Indonesia, and Bio-Rad Protein Assay was from Bio-Rad Laboratories, USA.

Oil phase selection

Selection of oil phase for SNEDDS preparation was based on our previous study (Winarti *et al.,* 2016). Four oil including Mygliol 812, oleic acid, Span 85 and Virgin coconut oil (VCO) were used. It was found that only Mygliol 812 in SNEDDS with three surfactants (Tween 20, Tween 80, cremophor EL 40) and two co-surfactants (propylene glycol and Span 20) gave the widest nanoemulsion area in terner diagram. This became the main consideration on choosing Mygliol 812.

Surfactants selection

In SNEDDS formulation, HLB method can be used as a starting point to acquire good emulsification characteristics. More than one surfactant can be blended to get desirable HLB. Selected surfactants should have good miscibility with other components in SNEDDS formula to produce stable and homogenous system. Another criterion is having relatively low toxicity for oral administration. HLB method was used to predict HLB value of emulsion and to design mixed ratio of two or more surfactants to yield a system with HLB of >10. Tween was selected due to structure similarity with Span (Wang *et al.*, 2009), while selection of Chremophore EL 40 was due to its

Table I. Mixed surfactant Ratio at various HLB

HLB	Surfactant mixing ratio (weight percent)					
	Tween 20/Span 20	Tween 20/Span 80	Tween 80/Span 20	Tween 80/Span 80	Chremophor® EL 40/Span 20	Chremophor® EL 40/Span 80
11	29.6/70.4	54.0/46.0	37.5/62.5	62.6/37.4	49.0/51.0	72.8/27.2
12	42.0/58.0	62.1/37.9	53.1/46.9	72.0/28.0	69.4/30.6	83.7/16.3
13	54.3/45.7	70.2/29.8	68.8/31.3	81.3/18.7	89.8/10.2	94.6/5.4
14	66.7/33.3	78.2/21.8	84.4/15.6	90.7/9.3	N/A	N/A
15	79.0/21.0	86.3/13.7	100.0/0.0	100.0/0.0	N/A	N/A

N/A: not applicable HLB

Table II. Characteristic of SNEDDS forming materials

Component	Name	Chemical Name	HLB value
Oil	Mygliol® 812	Capric triglyceride	15.36
Surfactants	Tween 20	Polyoxyethylene 20 sorbitan mono laurate	16.7
	Tween 80	Polyoxyethylene 20 sorbitan mono oleat	15
	Chremophor® EL 40	Ethoxylated Castor Oil	13.5
	Span 20	Sorbitan monolaurat	8.6
	Span 80	Sorbitan monooleat	4.3
Co-surfactant/Co-solvent	Glycerine	Propane-1,2,3-triol	

structure dissimilarity with Span. Chremophore EL 40 is polyoxyls. Therefore, in this research, the combination of surfactants with different structure in producing good self-nanoemulsion was also evaluated.

Co-surfactants selection

Glycerine was used as co-solvent for BSA and as co-surfactant. As a co-surfactant, altogether with surfactants, glycerine reduce interfacial tension to a very minimum, and even negative value. Therefore, glycerine was selected as co-solvent and co-surfactant in the present research.

Preparation of mixed surfactant system used the HLB method

The selection of surfactants was based on required HLB value to form O/W nanoemulsion, which should be greater than 10 (Kommuru *et al*., 2001). Three hydrophillic surfactants (Tween 20, Tween 80, and Chremopor EL 40) were mixed with two hydrophobic surfactants (Span 20 and Span 80) to formulate six binary surfactant combinations with HLB ranging from 11 to 15 (Table I). HLB_{mix} of each surfactant was calculated by using the following equation:

$$HLB_{mix} = f_A HLB_A + f_B HLB_B$$

where HLB_A and HLB_B are HLB value of surfactant A and surfactant B; and f_A and f_B are the fraction weight of surfactant A and surfactant B, respectively.

Construction of SNEDDS

SNEDDS was constructed from oil, surfactant, and co-surfactant in suitable composition to form a stable isotropic mixture. Mygliol 812 was used as oil phase, Tween 20, Tween 80, Chremophor EL 40, Span 20, and Span 80 were the surfactants, and glycerine was the co-solvent or co-surfactant (Table II). SNEDDS was prepared by stiring hydrophillic and hydrophobic surfactants at 300rpm for ten minutes. Glycerine was then added to the mixture and stirred for 10min. By the end of preparation process, oil component was added drop-by-drop and stirred for 10min.

Various surfactant-co-surfactant and oil ratios were used to obtain the most stable SNEDDS (Table III). SNEDDS with HLB between 11-15 were stored for 24h and they were observed for phase separation. The most stable SNEEDS formulation, which had the lowest surfactant composition, the highest oil component, and the highest HLB was selected as BSA template.

Preparation of BSA-loaded SNEDDS

BSA was dissolved in glycerine at a 10mg/mL concentration (Houen, 1996) and was stirred then. Glycerine with 1mg/mL concentration of BSA was added into the surfactant mixture and then was stirred for ten minutes. Mygliol 812 was added drop-by-drop and then was stirred again for 10min. BSA-loaded SNEDDS formulation was stored at 4°C for characterization on the next step.

Characterization of BSA-loaded SNEDDS

Thermodynamic Stability

Centrifugation study

To investigate the effect of centrifugation and temperature changes on the stability of nanoemulsion, the formulation of BSA-loaded SNEDDS was evaluated by using the thermodynamic stability tests, including the centrifugation study and freeze thaw cycle test. Deionized water was added into the formulation of BSA-loaded SNEDDS in a ratio of 1:20. The mixture was centrifuged at 3500rpm for 30min and then was observed whether phase separation and precipitation occured (Khedekar and Mittal, 2013; Reddy and Sowjanya, 2015).

Freeze Thaw Cycle

The SNEDDS formula remaining stable after centifugation was tested using freeze thaw cycle test. The formula was diluted with deionized water in a ratio of 1:20 and stored at temperatures of -20°C and 25°C for 48h for each temperature. This test was performed for two cycles. Phase separation and precipitation were observed if they occured (Khedekar and Mittal, 2013; Reddy and Sowjanya, 2015).

Phase separation and stability study of emulsions

Each 50μL of SNEDDS formula was added into a vial containing 5mL of doubles distilled water and SGF with pH of 1.2 at room temperature and was vortexed for one minute. Each emulsion was stored and observed for 24 hours for phase separation and precipitation (Reddy and Sowjanya, 2015).

Robustness to dilution

SNEDDS was diluted 100x and 1000x by using distilled water, 0.1N HCl, and phosphate buffer pH 6.8. Nanoemulsion was then stored and observed for 24h for phase separation and precipitation (Suresh and Sharma, 2011; Reddy and Sowjanya, 2015).

Visual observation of Self-emulsification

Self-emulsifying ability of the BSA-loaded SNEDDS formula was evaluated by using visual observation until homogenous nanoemulsion system was formed. A total of 250μL of BSA-loaded SNEDDS formula was quickly dropped in a beaker glass containing 250mL distilled water, simulated gastric fluid pH 1.2 and phosphate buffer pH 6.8 at 37±0.5°C. This medium was stirred at 100rpm. The duration of spontaneous nanoemulsion formation was recorded as self-emulsifying time (Weerapol *et al.*, 2014).

Percentage of transmittance

SNEDDS BSA formula was added into a vial containing 10mL of double distilled water at room temperature, vortexed for 1min (Reddy *et al.*, 2012), and measured for its transmittance using visible spectrophomo-meter at λ 650nm.

Droplet size and zeta potential determination

Distilled water was added into the SNEDDS BSA formula in a ratio of 1:1000 in a test tube. It was then vortexed for one minute. Particle size and polydispersity index (PDI) of nanoemulsion was analyzed at 25°C using dynamic light scattering (DLS) at an angle of 165° (Nagarsenkar, 2007). Zeta potential was measured with electrophoretic light scattering technique using Delsa™ Nano Beckman Coulter.

Calibration curve

Drug loading efficiency of BSA in SNEDDS formula was defined by using BSA calibration curve. Five serial concentrations of the formula that were 12.5, 25, 50, 75, and 100μg/mL yielded a regression equation of $y = 0.0063x + 0.0965$ with $r = 0.9986$.

Drug loading efficiency

To prepare the measurement of drug loading efficiency, 0.5g of BSA SNEDDS was precipitated by using 2mL of 2% TCA in an Eppendorf tube. It was centrifuged at 12,000rpm for 5min. Supernatant was removed and protein precipitate was washed three times using 100μL TCA and then diluted with 1mL of distilled water (Rachmawati and Haryadi, 2014). Eight hundread micro liter of BSA solution was added with 200μL of Bradford reagen and was incubated for five minutes. Absorbance was measured using visible

spectrophotometer at λ 595nm. Free BSA in supernatant was calculated using the regression equation from calibration curve which made previously. The equation of drug loading efficiency was as follow:

Drug loading efficiency =

$$\frac{Initial\ BSA – free\ BSA}{Initial\ BSA} \text{ X } 100\%$$

RESULTS AND DISCUSSION

Preparation of mixed surfactant

Despite the oil, surfactant is a vital component of SNEDDS (Shahba *et al*., 2012). The properties of surfactant, such as HLB value in oil, viscosity, and affinity for oil strongly affect the process of nano-emulsification and the size of nanoemulsion droplet (Makadia *et al*., 2013). The mixture of hydrophilic and hydrophobic surfactants can be used to form nanoemulsion with desired characteristics. HLB of Surfactant with < 10 is hydrophobic (Span) and can produce W/O emulsion, whereas over > 10 is hydrophilic and can form O/W emulsion. Surfactant concentration play a role in the formation of droplets in nanometric size (Singh, 2009). The bigger the ratio between hydrophilic and hydrophobic surfactants, the higher the HLB. The proper mixture of surfactants with lower and higher HLB may produce nanoemulsion that is stable even diluted with water. The proper mixture may also lower the interfacial tension to facilitate dispersion process by forming flexible film that is able to readily deform around droplet (Kommuru *et al*., 2001).

A number of 78 formulas of SNEDDS with HLB between 11-15 using different ratios of oil, surfactant, and co-surfactant (Table III) were prepared and evaluated for their stability after 24h of storage at room temperature. The most stable formula, which did not show phase separation, was selected as a template for BSA formulation.

Thirteen stable formulas were obtained after 24h of storage (Table IV). Higher HLB value showed higher hidrophylicity affected reduction of curvature on oil interface, thereby increasing the solubility and making the smaller droplets. Therefore, in this study the stable SNEDDS formula with the highest HLB value was selected. Moreover, the selection of the best formula was also based on higher oil concentration to obtain protective effect to protein drugs, but producing nanoemulsion with small droplets (Patel, 2011). The selection of surfactant with as low as possible concentration could reduce the risk of toxicity and irritation (Hauss, 2007). From 13 stable formulas, two formulas, F30 and F45, were selected as they had the highest HLB that make the emulsification process easier, the highest concentration of oil component, and the lowest concentration of surfactant. Further analysis was done for those two formulas.

Thermodynamic stability studies

Thermodynamic stability studies were performed to detect metastable formulas by using centrifugation and freeze thaw cycle methods. Together with zeta potential, thermodynamic stability was used to confirm the stability of prepared SNEDDS on the minimum possible time. The tests showed that the formula F45 remained stable after being centrifuged at 3,500rpm and stored at the temperatures of -20°C and +25°C. Creaming occured after the formula F30 was centrifuged, but it was able to redisperse forming homogenous emulsion. Reversible creaming also happened after the formula F30 underwent freeze thaw cycle.

Nanoemulsion is a system, that is thermodynamically stable and is produced on the presence of oil, surfactant, and co-surfactant without phase separation, creaming, or cracking. This differentiates nanoemulsion and macroemulsion, which is kinetically unstable and may result in phase separation (McClements, 2012).

The absence of precipitate after centrifugation showed that protein was already dissolved in oil phase aided by surfactants. Moreover, the amount of protein dissolved in oil can be calculated by using evaluation of drug loading efficiency.

Phase separation and stability study of emulsions

The solvent in which SNEDDS is formed, pH and ion affect the phase stability and the drug solubility (Morais *et al*., 2006). The stability of F30 and F45 formulas were studied using the medium of double distilled water and

Table III. Component ratio of SNEDDS (Rachmawati, 2008)

Component	Ratio		
	1	2	3
Surfactant-co-surfactant	8:1	7:2	7:1
Oil	1	1	2

Table IV. Stable SNEDDS template formulas after 24-hours of storage

Formula code	HLB	Mixed surfactant	Ratio oil:surfactant:co-surfactant/co-solvent
F10	14	Tween 20/Span 20	1:8:1
F13	15	Tween 20/Span 20	1:8:1
F24	13	Tween 20/Span 80	2:7:1
F25	14	Tween 20/Span 80	1:8:1
F28	15	Tween 20/Span 80	1:8:1
F30	15	Tween 20/Span 80	2:7:1
F34	12	Tween 80/Span 20	1:8:1
F40	14	Tween 80/Span 20	1:8:1
F43	15	Tween 80	1:8:1
F45	15	Tween 80	2:7:1
F58	15	Tween 80/Span 80	1:8:1
F64	12	Chremophor EL 40/Span 20	1:8:1
F66	12	Chremophor EL 40/Span 20	2:7:1

SGF pH 1.2 after being stored for 24h at room temperature. The results showed that the formula F45 was stable as phase separation and precipitation did not occur.

Robustness to dilution

F30 and F45 were subjected to dilution of 3 media which were distilled water, 0.1N HCl, and phosphate buffer with pH 6.8 in 100x and 1000x dilution. They were stored at room temperature for 24h. The volume of dilution varied because of the natural variation of gastrointestinal fluids between subjects particularly on the fed and fasted states. The successful formulation of SNEDDS depends on dilutability process to produce nanodroplets. The formula F45 did not show phase separation and precipitation. F45 was robust to dilutions with various media. No significant effect of pH on F45 was observed. It confirmed that preparation was robust to high dilution and variation in pH.

Visual assesment of emulsification time

Emulsification time was conducted to ascertain the rapidity of nanoemulsion formation of SNEDDS after oral administration (Jain *et al*., 2013). Self-emulsifying time of BSA SNEDDS was evaluated based on the rate of dispersion in aqueous media with mild agitation (Table 5). F30 was emulsified more faster than F45. The use of Tween 80 in the F45 possible to form crystalline gel that was difficult to disperse, resulting in the emulsifying time of > 2min. On the other hand, F30 was readily dispersed, but the dispersion was turbid due to lypophilic Span 80.

Percentage of transmittance

The percentage of transmittance showed the turbidity of emulsion made from SNEDDS in aqueous media that was evaluated by using visible spectrophotometer with λ 650nm (Table V).

Table V. Percentage of transmittance and visual assesment of self-emulsification (n=3)

Formulation Name	Percentage of transmittance	Self emulsification time (second)		
		Double distilled water	0.1N HCL	Phosphate Buffer pH 6.8
F30	0.37±2.88	29.30±0.25	16.93±0.30	16.32±0.09
F45	96.40±1.05	159.00±0.30	153.00±0.08	144.00±0.08

Table VI. Droplet size, zeta potential and Drug loading Efficiency

Formulation Name	Droplet Size (nm)	PDI	Zeta Potential (mV)	Drug Loading efficiency (%)
F45	180.9	0.255	-0.12	83.91±1.73

Percent transmittance of F45 indicated that the formulation was transparent. The transparency of the system is due to the fact that the droplets of the dispersed phase was not larger than 1/4th of the wavelength of visible light (Shintov and Shapiro, 2004). The nanoemulsion scatters light and therefore appears transparent or translucent. F30 was more turbid due to the present of lypophilic Span 80, although its HLB value was comparable to F45 that was produce using single hydrophilic surfactant.

Droplet size and zeta potential measurement

After being reconstituted in distilled water, the droplet size of emulsion from SNEDDS formula was evaluated. The determination of particle size and zeta potential were only performed on F45 using Particle Size Analyzer since the F30 was more turbid (Table VI).

The droplet size is a critical parameter of SNEDDS evaluation. The smaller the droplets, the larger the area of absorption and the faster the drug release. The small droplets may provide larger surface area that enables pancreatic lipase to hydrolize and promotes more drug release (Mueller *et al*., 1994). The mean droplet size of F45 was in the nanometer range. Globul size in the range of nano good transparency and increase surface area (Figure 1). The F45 also showed the narrow size distribution. The polydispersity value was < 1 in F45 formulation. Polydispersity index is the standard deviation of the average size of droplets that can be used as an indicator for the uniformity of droplet size in a formulation (Chudasama *et al*., 2011).

The size of nanoemulsion doplet is affected by the ratio of oil to surfactant (Fernandez *et al*., 2004) and the ratio of surfactant to co-surfactant. The larger the particle size, the more turbid the oil globule and the nanoemulsion system (Larsen *et al*., 2013).

Zeta potensial is measured to determine the surface charge of emulsion droplet. The charge depends on the surfactant used. Non ionic surfactant was selected for this study as it is less toxic than ionic surfactant (Patil *et al*., 2004). The zeta potential of BSA nanoemulsion droplet was -0.12mV (Table VI) due to the presence of free fatty acids (Figure 2).

Drug loading efficiency

F45 showed drug loading efficiency of BSA SNEDDS was 83.91±1.73%. Loading efficiency was analyzed using Bradford method with the principle of the coumassie blue dye bounds to primarily basis and aromatic amino acid residues especially arginin. The bound form of the dye has an absorption spectrum maximum at 595nm. The increase of absorbance at 595nm was proportional with the amount of bound dye and the protein concentration in the sample.

CONCLUSION

The present study showed the results of SNEDDS design for BSA based on HLB value approach. The problem found in preliminary study was glycerine separation from oil phase.

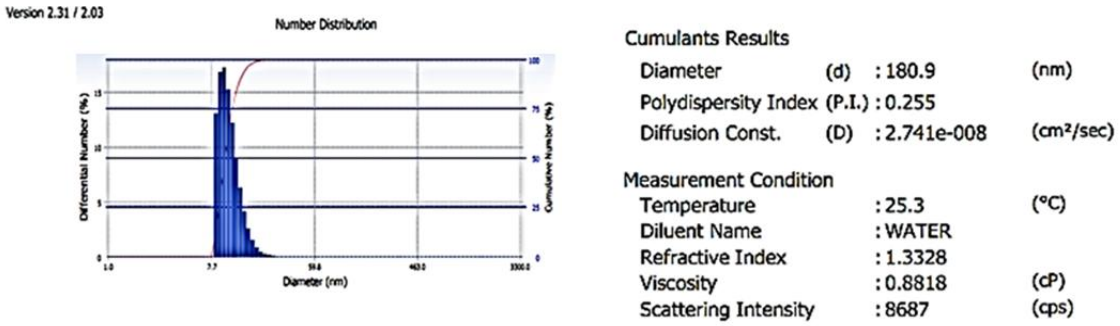

Figure 1. Globule size and size distribution of BSA SNEDDS

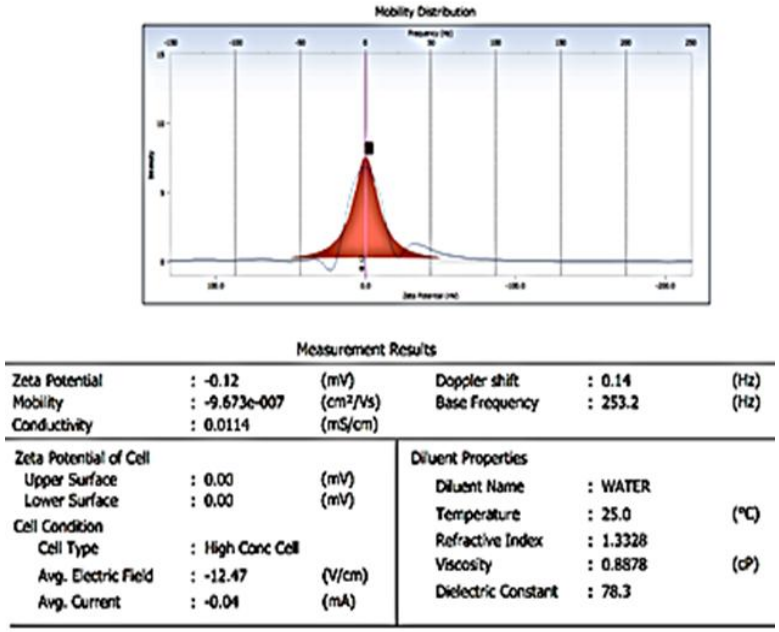

Figure 2. Zeta Potential of BSA SNEDDS

This should be tackled to make protein can be dissolved in oil. Therefore, single surfactant and combination of surfactant with HLB of 11-15 were used to aid water soluble glycerine entering into oil phase without the occurrence of phase separation was designed. The evaluation of 78 SNEDDS templates with HLB 11-15 showed that SNEDDS formula F45 containing single surfactant (Tween 80) was able to produce stable and clear SNEDDS preparation after being dispersed in aqueous media. It can be concluded that the F45 was the best SNEDDS formula in this study. The stability studies and the characterization of BSA SNEDDS suggested that it has potential as a protein carrier.

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