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Formulation and Characterization of Nanoparticle-Protein Sirtuin 1 (NPS1) by Nanoprecipitation Technique

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Atherosclerosis is a cardiovascular disease caused by endothelial dysfunction. This situation will trigger the bone marrow to immediately replace it with new endothelial progenitor cells (EPC) cells. However, some studies suggest that EPC can experience premature senescence. Sirtuin-1 (SIRT1) is a cellular post-translational protein that has the task of repairing dysfunctional EPC cells. Studies have tried to develop SIRT1 activation, but currently, there are no studies that have attempted to increase SIRT1 levels in cells. Nanoparticles (NPS) are one of the methods in nanomedicine, which has the advantage of being a drug carrier. So, further research is needed on adding exogenous SIRT1 levels, NPS, which can improve the quality of EPC cells and prevent premature senescence. This study aims to report the formulation and characterization stages of nanoparticles carrying SIRT1 (NPS1) with different solvents, such as ethanol and aquadest. The method used in this formulation uses nanoprecipitation. The characterization of nanoparticles at this stage included organoleptic tests, pH tests, and quantifying using Nanodrops in determining the presence of adsorbed proteins. The pH and organoleptic test showed that the NPS1 formulation was acidic (K1 = 5.412 ± 0.73; K2 = 3.624 ± 0.45; F1 = 5.418 ± 0.55; F2 = 4.182 ± 0.07), yellow in color, and had a characteristic odor. Thus, the formulation and characteristics of NPS1 can be used as a method in drug development for anti-senescence therapy in EPC cells in further research, both in vitro, in vivo, and evaluation of preparations that are still very possible to be developed.

Keywords: Chitosan; Endothelial Progenitor Cells; Nanoparticle; Nanoprecipitation; SIRT1; Senescence.

Atherosclerosis is a chronic inflammatory disease of the arteries that alters the structure and function of the three layers of the coronary artery wall[1,2]. According to current theories, endothelial cell dysfunction is one of the first steps in forming atherosclerosis[2]. The process of atherosclerosis is caused by several stages: the formation of fat lines, the construction of

atheroma, and the formation of atherosclerotic plaques. Various risk factors cause endothelial cell damage, one of which is inflammation. Acute inflammation has two impacts on EPC mobilisation and recruitment: the temporary restricted inflammatory response and the prolonged or exaggerated inflammatory stimulation. EPC mobilisation may be triggered by the transient restricted inflammatory response[3]. After vascular trauma, EPCs are immediately mobilised and help wounded arteries revascularize in response to increased proinflammatory cytokine levels[4]. IL-1â upregulates the expression of VEGF, VEGFR-2, and adhesion molecules in endothelial cells and is implicated in EPC recruitment and transit to ischemic tissue in a VEGF-dependent manner[5].

SIRT1 is a histone deacetylase that relies on nicotinamide adenine dinucleotide and helps regulate the cell cycle and apoptosis[6]. Sirtuin protein 1 (SIRT1) is the human sirtuin closely related to the yeast protein Sir2 sequence. SIRT1 is widely expressed in EPC cells and is a longevity gene that plays an essential role in its protective function[7,8]. An age-dependent decrease in SIRT1 levels was observed in arteries, indicating its role in the aging of the cardiovascular system. SIRT1 delays replicative senescence and premature senescence in stem cells and differentiated cells exposed to oxidative stress[9].

Nanoparticles are one of the technologies that develop the development of cardiovascular drugs. In nanotechnology, a particle is defined as an object that is small in size, has a role, and behaves as a unit concerning its properties and transport. Nanoparticles have many advantages so that they are considered capable of delivering drugs: easy to absorb, easy to dissolve, and bioavailability and safety in target tissues and organs[10]. One of the most popular nanoparticles used is chitosan. Chitosan is a valuable biomaterial generated from the deacetylation of chitin (poly(β-1,4-N-acetyl-d-glucose-2-amine)), which is one of the most prevalent natural polysaccharides found in the cell walls of microorganisms such as yeast, fungus, and the exoskeletons of crustaceans and insects. The molecular weight and degree of deacetylation primarily determine chitosan's physical and chemical characteristics. Chitosan is a biocompatible, biodegradable, bioadhesive, and nontoxic substance. Chitosan, a naturally occurring biocompatible polymer, has been widely studied in pharmaceutical and industrial research for drug delivery and biological applications.

Chitosan can control the rate of medication release, extend therapeutic efficacy, and deliver pharmaceuticals to the proper spot in the body. Chitosan may be dissolved in water under acidic circumstances to yield a high positive charge density. Chitosan's positive charge allows it to interact with polyanions and create complexes, then develop a gel and adhere to mucous membranes, increasing drug absorption across cellular barriers[11]. However, there are currently no studies that explain further the specific packaging of chitosan and SIRT1 protein. Therefore, this study aims to report the formulation and characterization stages of nanoparticles carrying SIRT1 (NPS1) with different solvents, to meet the optimal solvent used in drug design development.

MATERIALS AND METHODS

Nanoparticle formulation-SIRT1 (NPS1)

This study uses four groups of formulas: K1, K2, F1, and F2.

K1 is a control NPS1 with a solvent ratio of acetone:methanol (3:2) without protein SIRT-1 (Cat. Number ab54334, ABCAM, USA);

K2 is control of NPS1 with aquadest as a solvent without protein SIRT-1;

F1 is a formulation of NPS1 with a solvent ratio of acetone:methanol (3:2) with SIRT-1 protein 7.5 µL;

F2 is the formulation of NPS1 with aquadest as a solvent with SIRT-1 protein 7.5 µL.

Aquadest used in this research is water for injection (WFI).

Preparation of Nanoparticle-SIRT1 (NPS1) by the nanoprecipitation method

NPS1 with ethanol or aquadest solvent was prepared by the nanoprecipitation method. The manufacture is done by preparing an organic solvent (acetone methanol = 3:2). Then, the organic phase (FO) consists of chitosan and phosphatidylcholine mixed with 5 mL of organic solvent until dissolved. Dissolution can be assisted by using a sonicator or stirring on a hotplate stirrer for 1-2 hours. After everything was dissolved, 7.5 µL of SIRT-1 recombinant protein was added. Afterward, prepare an aqueous phase (FA) consisting of 1% Tween 80 (1 gram of tween 80 dissolved in 100 mL WFI).

Then perform FA quantification. Comparison FA:FO = 15:1 (FA 15 times of FO). If the FO is dissolved in 5 mL, prepare the FA 15 times (75 mL), then add the FO into the FA slowly while stirring, followed by ultraturax at a speed of 8000 rpm for 5 minutes. Stirring was carried out for 18 hours. In this stirring, overheating is not carried out to prevent the protein from being degraded. After 18 hours of stirring, the nanoparticles were ready to be harvested. The procedure was carried out twice for K1 and F1. The difference from this operation is that no protein is added to K1.

NPS1 with aquadest solvent was made by preparing the organic solvent (chitosan dissolved in WFI and HCl 0.2 N) and then melting phosphatidic choline at 80°C. Temperature lowered, and the organic solvent was added in the previous step. After the temperature is confirmed to drop and the FO is at room temperature, 7.5µL of SIRT-1 recombinant protein is added. Comparison FA: FO = 15:1, followed by ultraturax at a speed of 8000 rpm for 5 minutes. Stirring was carried out for 18 hours. In this stirring time, overheating is not carried out to prevent the protein from being degraded. After 18 hours of stirring, the nanoparticles were ready to be harvested. This procedure was repeated twice for K2 and F2. The difference from this operation is that no protein is added to K2.

Characterization of NPS1 by pH Test, Organoleptic Test, and Identification of Protein in NPS1 by Nanodrop and SDS-PAGE

The pH test was carried out using a pH meter, and in the organoleptic test, the color and odor of the preparation were observed. The formulation results were then observed based on pH 4. Protein levels contained in SIRT1 were identified using Nanodrops in 2 stages. Identification of protein in the supernatant was made by centrifuging the formulation that had been stirred for 18 hours at a speed of 5000 rpm for 5 minutes (LW Scientific EZ Swing 5K (C5) Centrifuge, Hettich Mikro 22R Refrigerated Centrifuge). Then, the pellets separated from the supernatant (containing the harvested nanoparticles).

Identification of proteins using Nanodrop at an absorbance of 280 nm, both in the pellet and in the supernatant. Protein is expected to be present in the supernatant

The concentration value indicates that protein is present in the solution. The blank used for both supernatant and pellet was WFI. For the step 2 procedure, 10 mL of each supernatant was centrifuged at 10,000 rpm for 15 minutes. All ten samples were divided into eight Eppendorf tubes and centrifuged for 15 minutes. The supernatant and pellets were collected according to each sample to obtain four pellet and four supernatant samples. Pellets were dissolved with Tris-HCl pH 8.0 100 µL per tube, pipetting was carried out slowly to mix everything and then collected into new tubes for each pellet sample in all formulations. The samples identified proteins using Nanodrop at an absorbance of 280 nm, both in the pellet and the supernatant. At this stage, it is expected that the protein will be present in the pellet. If the reading is positive, the concentration value indicates that protein is present in the solution. The blank used for pellets was Tris-HCl pH 8.0, while for the supernatant using WFI.

Table 1. Results of SIRT1 Protein Identification on NPS1

Nanodrop Identification	Formulation K1	K2	F1	F2
Step I				
Pellet	-0.14	0.02	-0.00	0.03
Supernatant	0.30	0.20	0.28	0.04
Step II				
Pellet	-0.01	0.36	0.09	0.19
Supernatant	0.07	-0.05	-0.16	0.05

Note: Stage I (5000 rpm centrifugation); Phase II (10,000 rpm centrifugation); negative, and a value of 0 indicates no undetectable protein in NPS1. A positive value indicates the presence of a detected protein. In this study, the protein is expected to be present in the supernatant in step I and the pellet at stage II.

Table 2. Observations of pH and organoleptic NPS1

Group	pH	Organoleptic
K1	5.412 ± 0.73	Clear yellow; smells like egg yolk (phosphatidylcholine-like)
K2	3.624 ± 0.45	Deep yellow; smells like egg yolk (phosphatidylcholine-like)
F1	5.418 ± 0.55	Deep yellow; smells like egg yolk(phosphatidylcholine-like)
F2	4.182 ± 0.07	Clear yellow; smells like egg yolk (phosphatidylcholine-like)

Note: Each formulation has an acidic pH range (each N=5). The organoleptic test showed that the formulation's color and odor were yellow, a mixture of chitosan and phosphatidylcholine.

RESULTS

NPS1 Formulation

The results showed that in the first confirmation stage (Stage I) there was a protein concentration of 0.28 mg/mL and in the second stage there was protein in the supernatant at 0.09 mg/mL. The results of the nanodrops are presented in Table 1.

NPS1 characterization (pH and organoleptic)

The pH observations were repeated to show that the pH of the SIRT1 nanoparticles was acidic in each formulation (Table 2).

DISCUSSION

SIRT1 modulates various molecular signaling pathways important for vascular function in various types of vascular cells[11]. SIRT1 inhibits the expression of endothelial adhesion molecules such as intracellular adhesion molecule-1 and vascular cell adhesion molecule-1 by suppressing nuclear factor NFKB, where SIRT1 deacetylates and inhibits the RelA/p65 subunit of NF-kB[12]. As a result, SIRT1 inhibits monocyte-to-endothelial cell binding, as well as monocyte transmigration to the arterial wall, indicating its anti-inflammatory effect on endothelial cells[13]. For this reason, in an *in vitro* study in this study, SIRT1 packaging used a drug carrier method with the nanoprecipitation method as a carrier for SIRT1 protein to penetrate the EPC cell membrane.

The nanoprecipitation method refers to the technique carried out by Luque-Alcaraz et al.[10] with several adjustments to protein conditions that do not require heating and normal room temperature while manufacturing nanoparticles[14]. The purpose of not heating is to keep the protein from being degraded during the stirring process for 18 hours. Normal room temperature refers to previous research which states that nanoparticles will be stable at room temperature[15]. Further confirmation was carried out using Nanodrop to determine whether there was a SIRT1 protein in the previously prepared nanoparticles. As in one of the formulations, F1, the results showed that in the confirmation of the first stage (Stage I), there was a protein concentration of 0.28 mg/mL, and in the second stage, there was protein in the supernatant at 0.09 mg/mL. The results of the nanodrops are presented in Fig. Table 1. Thus, the results show that there is the protein in F1.

The yellow color in NPS1 is the color of phosphatidylcholine mixed with chitosan as a polymer carrier. This study also found that the odor produced from NPS1 is a distinctive odor of phosphatidylcholine. The average pH value in each formulation is at an acidic pH. The size of the nanoparticles is highly dependent on pH, as shown in previous studies, which showed that the measure was due to the nucleation process in the slow formation of silver nanocrystals, so the particles formed were quite large[16-19]. On the other hand, at a high pH, it is easier to form small sizes[20-23].Thus it can be seen that NPS1 may have a relatively large particle size when viewed from the pH point of view. F1, a formulation made with acetone:methanol solvent, has a low pH, but in the process of checking nanoparticles with nanodrops, F1 tends to adsorbed proteins. Therefore, in this study, F1 has the potential to be further developed.

The nanoparticles in this study are in line for use in drug delivery, and have been published in several reports. The use of nanoparticles in delivering SIRT1, for example, is to influence transcription factors and mitochondrial biogenesis[24],

deliver siRNA to target cells[25], and reduce the side effects of using a drug[26]. In this study, SIRT1 packaging used a drug carrier method with the nanoprecipitation method as a carrier for SIRT1 protein to penetrate the EPC cell membrane. In the manufacture of nanoparticles, the chosen polymer is chitosan. Previous research revealed that chitosan could protect grape seed polyphenols from degradation and EPCs from oxidative stress caused by H_2O_2[27-29]. The protection of chitosan makes a valid reason for choosing the polymer as a nanoparticle drug carrier. In addition, chitosan is also a carrier with good biodegradability, low toxicity, hemostatic, and good biocompatibility. Therefore, efforts to package SIRT1 protein using chitosan as a polymer are significant to study.

CONCLUSION

This research is an early stage research that uses NPS, which is a nanoparticle, as an external carrier for the SIRT1 molecule. This research observes solvents that can be used so that NPS can work well in carrying SIRT1. Based on the pH and organoleptic test showed that the NPS1 formulation was acidic, yellow in color, and had a characteristic odor. The limitations of this study were that SEM, particle size, and nanoparticle validation were not performed according to NPS1. However, from this study, it is possible to be developed further because nanoparticles are considered a good alternative as a technology to add exogenous SIRT1 protein.

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Conflict of Interest

The authors have no conflicts of interest regarding this investigation.

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