RESEARCH ARTICLE
doi: 10.2306/scienceasia1513-1874.2025.022

ScienceAsia 51 (2): 2025: ID 2025022: **1–9**

Vasculo-protective effect of human serum albumin nanoparticles encapsulated recombinant human secretory leukocyte protease inhibitor (rhSLPI-HSA-NPs) against ischemia/reperfusion injury in vascular endothelial cells

Chayanisa Phutiyothin, Faprathan Pikwong, Wannapat Chouyratchakarn, Phornsawat Baipaywad, Sarawut Kumphune*

Biomedical Engineering Institute (BMEI), Chiang Mai University, Chiang Mai 50200 Thailand

*Corresponding author, e-mail: sarawut.kumphune@cmu.ac.th

Received 10 Oct 2023, Accepted 27 Dec 2024
Available online 22 Apr 2025

ABSTRACT: The reduction of vascular endothelial cell injury may potentially contribute to the preservation of cardiomyocytes from ischemia/reperfusion (I/R) injury. Previous investigations have shown that secretory leukocyte protease inhibitors (SLPI) have the potential to reduce I/R-induced cardiac cells and vascular endothelial cell injury. Nonetheless, the short half-life of SLPI and its susceptibility to degradation by circulating enzymes limit its potential for therapeutic applications. In this study, we employed human serum albumin (HSA) nanoparticles (NPs) to encapsulate recombinant human SLPI (rhSLPI) protein. Subsequently, we investigated the effect of these rhSLPI-encapsulated-HSA NPs (rhSLPI-HSA-NPs) on vascular endothelial cells that had been exposed to simulated ischemia/reperfusion (sI/R) injury in an *in vitro* experimental model. For the physical properties of HSA-NPs and rhSLPI-HSA-NPs, the average sizes, the polydispersity index (PDI) values, and the zeta potentials of the two NPs were 131.7 nm and 250.2 nm, 0.5 and 0.6, and −15.27 and −13.83 mV, respectively. Furthermore, the rhSLPI-HSA-NPs inhibited the trypsin activity without causing toxicity to human vascular endothelial cells (EA.hy926). The results showed that pre-treatment with 1 µg/ml and 10 µg/ml rhSLPI-HSA-NPs could significantly reduce sI/R and induce vascular endothelial cell death and injury. In summary, the current study demonstrated for the first time the vasculoprotective effect of rhSLPI-HSA-NPs in decreasing vascular endothelial cell damage generated by an *in vitro* simulated ischemia/reperfusion.

KEYWORDS: ischemia/reperfusion injury, secretory leukocyte protease inhibitor, vascular endothelial cells, nanoparticles, human serum albumin

INTRODUCTION

The involvement of vascular endothelial cells is crucial in the pathogenesis of ischemia/reperfusion (I/R) damage, a condition characterized by transient deprivation and subsequent restoration of blood supply to an organ or tissue [1]. The aforementioned phenomenon may manifest in several therapeutic interventions, including angioplasty, stent implantation, and surgical bypass grafting, among others, whereby the absence of an efficacious treatment modality is now observed [2]. Moreover, I/R injury aggravates vascular endothelial dysfunction through several mechanisms, such as cytotoxicity by pH changes, oxidative stress by the generation of reactive oxygen species (ROS), inhibition of endothelial nitric oxide synthase (eNOS), and subsequent reduction in nitric oxide (NO) production [3]. Therefore, limiting vascular injury is a significant therapeutic challenge for ischemic heart disease [4]. Injury in vascular endothelial cells (VECs) produces proteases that contribute to vascular pathology [5]. Protease inhibition, by using synthetic protease inhibitors, could lead to significant side effects [6]. One of the interesting alternative protease inhibitory strategies is using endogenous protease inhibitory peptides or proteins.

The secretory leukocyte protease inhibitor (SLPI) [7], belonging to the cationic-whey acidic protein family, has been demonstrated to protect host cells against the detrimental effects of proteolytic enzymes generated during inflammatory processes. SLPI selectively inhibits serine proteases, such as elastase and cathepsin G from neutrophils, chymotrypsin and trypsin from pancreatic acinar cells, and mast cell chymase [8]. Our previous studies reported that SLPI provided cardioprotection [9–12] and vasculoprotection [13–15] against I/R injury. However, the SLPI protein is unstable and inefficient in entering target cells, and there has been no work on delivering recombinant human SLPI (rhSLPI) to maximize intracellular efficiency [9, 13, 14].

To overcome such limitations, nanoparticles have been utilized to extend controlled release, target a specific bodily location, and safeguard carriers [16]. The recent decade has seen a rise in research into protein nanoparticles as a medication delivery option due to their unique properties [17], such as biocompatible, biodegradable, and non-toxic [18]. One previous research has conducted numerous studies on the use of human serum albumin (HSA) as nanoparticles. In addition, HSA has attracted significant attention in the field of protein nanoparticles for drug administra-

tion in recent years due to its high abundance as a plasma protein. The FDA's authorization of Abraxane, a nanoparticle formulation of paclitaxel incorporating HSA, for the treatment of various malignancies, serves as an indication of the favorable reception of HSA within the domain of drug delivery [18, 19]. Furthermore, there has been only one previous research utilizing HSA-NPs to encapsulate rhSLPI for anti-bacterial activity [19]. However, the cytoprotective effect of HSA-NPs encapsulated SLPI against I/R damage in vascular endothelial cells has not been investigated. Therefore, the current study hypothesized that the HSA-NPs could encapsulate rhSLPI and reduce vascular endothelial cell injury under the conditions of an *in vitro* model simulated I/R injury. The study results might be a potential therapeutic option for ischemic heart disease as well as other disorders caused by vascular diseases.

MATERIALS AND METHODS

Chemical and reagents

rhSLPI was purchased from Sino Biology Inc. (Beijing, China). Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), and penicillin/streptomycin were purchased from Gibco BRL, Life Technologies, Inc. (NY, USA). HSA, ethyl alcohol (ACS reagent, ⩾99.5%), and 25% glutaraldehyde solution were purchased from Sigma-Aldrich (St. Louis, USA). Pierce Lactate dehydrogenase (LDH) Cytotoxicity Assay Kit and 3-(4,5-dimethyl-2-thiazol)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Thermo Fisher Scientific (Rockford, USA) and Ameresco (Solon, Ohio, USA), respectively [20].

Cell type and cell culture

The vascular endothelial cell line (EA.hy926) was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle's Medium (ATCC 30-2002) supplemented with 5 ml of 10 mg/ml heparin, 10% (v/v) fetal bovine serum, 500 µl of 30 mg/ml Endothelial Cell Growth Supplement (ECGS), and 5000 units/ml of penicillin/streptomycin and maintained at 37 °C under a humidified atmosphere of 95% air and 5% CO_2 [20].

Preparation of rhSLPI-HSA-NPs

The preparation of HSA-NPs was conducted using the modified nanoprecipitation method as outlined by Mohamad Tarhini et al [19]. The rhSLPI-HSA-NPs sample was prepared by mixing 0.1 g of HSA in 5 ml of ultrapure water and 150 µl of 1 µg/µl of rhSLPI. Then, the rhSLPI-HSA mixture was added to a 1:2 solution of ethanol in water. The mixture was stirred until a milky appearance was observed; then, 10 µl of 25% (v/v) glutaraldehyde was added and left at 4 °C overnight to obtain the crosslinked rhSLPI-HAS-NPs. The solution was washed twice to eliminate any residues of non-reacted compounds. The final mixture was then subjected to freeze-drying at temperatures ranging between −40 and −44 °C and a pressure of less than 10 Pa until a powdered sample of rhSLPI-HSA-NPs was obtained. The final step of the preparation was to coat an additional layer of HSA on the surface of the rhSLPI-HSA-NPs sample by mixing 0.05 g of HSA dissolved in 4 ml of ultrapure water and 1 ml of 0.01 g/ml rhSLPI-HSA-NPs, then, following the aforementioned process. The final sample was stored at 4 °C until further use [19, 20].

Determination of physical characteristics of nanoparticles

The morphology of nanoparticles was determined by field emission scanning electron microscopy (FE-SEM, JSM-6700F, JEOL, Japan). Dried powders of the rhSLPI-nanoparticles were placed on a piece of gold coated (30 sec under vacuum) carbon tape and observed under an acceleration voltage of 15 kV. Sizes of the nanoparticles (at least 100 particles) were measured manually and calculated using ImageJ software (National Institute of Health) [20].

The homogeneity of the nanoparticle was indicated by the polydispersity index (PDI) value, and the electrical charge of the nanoparticle by zeta potential was measured by Dynamic light scattering (DLS) from a Malvern Zetasizer (UK) at the Faculty of Pharmacy, Chiang Mai University [21].

Determination of the encapsulated rhSLPI concentration

The encapsulation of rhSLPI was measured by dissolving 0.01 g rhSLPI-HSA-NPs in 1 ml of ultrapure water and then degraded by heating at 65 °C for 10 min. After centrifugation at 15,000 rpm for 10 min, the supernatant was collected to measure the encapsulated rhSLPI by enzyme-linked immunosorbent assay (ELISA) and calculate the percentage of encapsulation efficiency (%EE) using equation (1) [22].

$$\%\,\text{Encapsulation efficiency (\%EE)} = \frac{\text{The amount of rhSLPI encapsulation}}{\text{The total amount of rhSLPI}} \times 100 \quad (1)$$

Determination of anti-protease inhibition activity

To determine the anti-protease inhibitor of rhSLPI-HSA-NPs, 50 µl of 0.05 mg/ml rhSLPI-HSA-NPs was degraded by heating at 65 °C for 10 min and then preincubating with 50 µl of 0.25% trypsin at 37 °C for 1 h. After that, 100 µl of 1% (w/v) casein was added to the reaction mixture and incubated at 37 °C for 30 min. The reaction was stopped by adding 150 µl of 5% trichloroacetic acid (TCA) solution to the mixture, followed by centrifugation at 12,000 rpm for

15 min. The supernatant was collected for measuring the absorbance at λ280 nm. The protease activity was shown as a percentage of trypsin activity [20].

Determination of the cytotoxicity effect of rhSLPI-HSA-NPs on vascular endothelial cells

Aliquots of 200 µl of 1×10^5 cells/ml EA.hy926 were added into 96-well cell culture plates and cultured for 24 h. Then, 200 µl of various concentrations of rhSLPI-HSA-NPs (0.01, 1, 10, and 100 µg/ml) were individually mixed with 1,800 µl of the cultured medium, and 200 µl of the mixture was added into 96-well plates, containing cells, and incubated at 37 °C under 95% air and 5% CO_2 for 24 h. Cell viability was determined by the MTT (3-[4,5-dimethylthiazol-2-y1]-2,5-diphenyltetrazolium bromide) cell viability assay. For the assay, the culture medium was removed, replaced with 150 µl of 0.5 mg/ml MTT reagent, and incubated at 37 °C for 2 h. After that, the MTT reagent was removed, and 150 µl of dimethyl sulfoxide (DMSO) was added to solubilize the formazan crystal. The obtained solution was collected for absorbance measurement at 570 nm, using DMSO as a blank [14, 20].

Released-lactate dehydrogenase (LDH) activity assay

The LDH activity assay was done by mixing 50 µl of the sample with 50 µl of reaction mixture and incubated at room temperature for 30 min, protected from light. Then, 50 µl of stop solution was added. The solution was mixed, and the absorbance was measured at 490 and 680 nm. The LDH activity was obtained by subtracting the absorbance value at 680 nm (instrument's background signal) from the absorbance value at 490 nm [23].

Optimization of simulated ischemia/reperfusion (sI/R) injury duration

The EA.hy926 was cultured in 96-well plates by adding 200 µl of 1×10^5 cells/ml cell suspension to each well and incubating the plates for 24 h until cell growth was approximately 80% confluence. Simulated ischemia (sI) was induced by exposing EA.hy926 cells to ischemic buffer [(137 mM NaCl, 3.8 mM KCl, 0.49 mM $MgCl_2$, 0.9 mM $CaCl_2$, 4.0 mM HEPES) containing 20 mM 2-deoxyglucose, 20 mM sodium lactate, and 1 mM sodium dithionite at pH 6.5 [13]. Cells were subjected to sI at 37 °C for 0, 10, 20, 30, 40, 50, and 60 min before changing to a complete medium for reperfusion and incubating at 37 °C under 5% CO_2 for 24 h [11]. After that, cell viability and cell injury were respectively measured by the MTT cell viability assay and the released-LDH activity assay. The optimal duration of sI/R was the duration with 50% cell viability, and the value was used in all subsequent simulated ischemia experiments [20].

An *in vitro* vasculo-protective effect of rhSLPI-HSA-NPs

The EA.hy926 was cultured in 96 well plates by adding 200 µl of 1×10^5 cells/ml of cell suspension to each well and incubating the plates for 24 h until cell growth was approximately 80% confluence. Then, various concentrations of rhSLPI-HSA-NPs (1, 10, and 100 µg/ml) were added to pre-treat the cells and incubated for another 24 h. After that, cells were subjected to sI at 37 °C under 95% air and 5% CO_2 for a period of the aforementioned optimum duration of sI/R. At the end of the ischemic period, the sI buffer was discarded, replaced with a complete medium for reperfusion, and incubated at 37 °C under 95% air and 5% CO_2 for 24 h [11]. After that, cell viability and cell injury were measured by the MTT cell viability assay and the released LDH activity assay [20].

Statistical analysis

The statistical tests were performed using commercially available software (GraphPad Prism). All values were expressed as Mean ± SD. All comparisons were assessed for significance using ANOVA and followed by the Tukey-Kramer test when appropriate. A p-value of less than 0.05 was considered statistically significant [20].

RESULTS AND DISCUSSION

Physical characteristics of fabricated nanoparticles

Field emission scanning electron microscopy (FE-SEM) technique was used to determine the morphological attributes of the nanoparticles. The findings indicated that all types of HSA-NPs had a spherical morphology with apparent aggregations, as shown in Fig. 1 (b–e). Dynamic light scattering (DLS) determines the size of nanoparticles by measuring the Brownian motion of nanoparticles in suspension. However, this technique may not accurately give the actual size of nanoparticles due to their aggregation, as directed measured the size by TEM image. In contrast, using TEM to determine the morphology of isolated single nanoparticles could provide images that can be directly visualized and individually measured by Image J, resulting in an accurate diameter of a single nanoparticle. Therefore, Image J was shown to have more precise measurements than DLS in this study, due to less effects of aggregation and a clearer picture of the actual nanoparticle size. The manually measured size of nanoparticles by Image J in this study was also reported in the previous study by Zhang et al [24] The size of the nanoparticles was quantified using Image J software. The results showed that the sizes of HSA-NPs and rhSLPI-HSA-NPs were 131.7±6.3 nm and 250.2±73.5 nm, respectively. The size of rhSLPI-HSA-NPs was considerably greater than that of HSA-NPs (Fig. 2a). The increase in particle size observed following the loading of rhSLPI could be related to the size of the rhSLPI molecule.

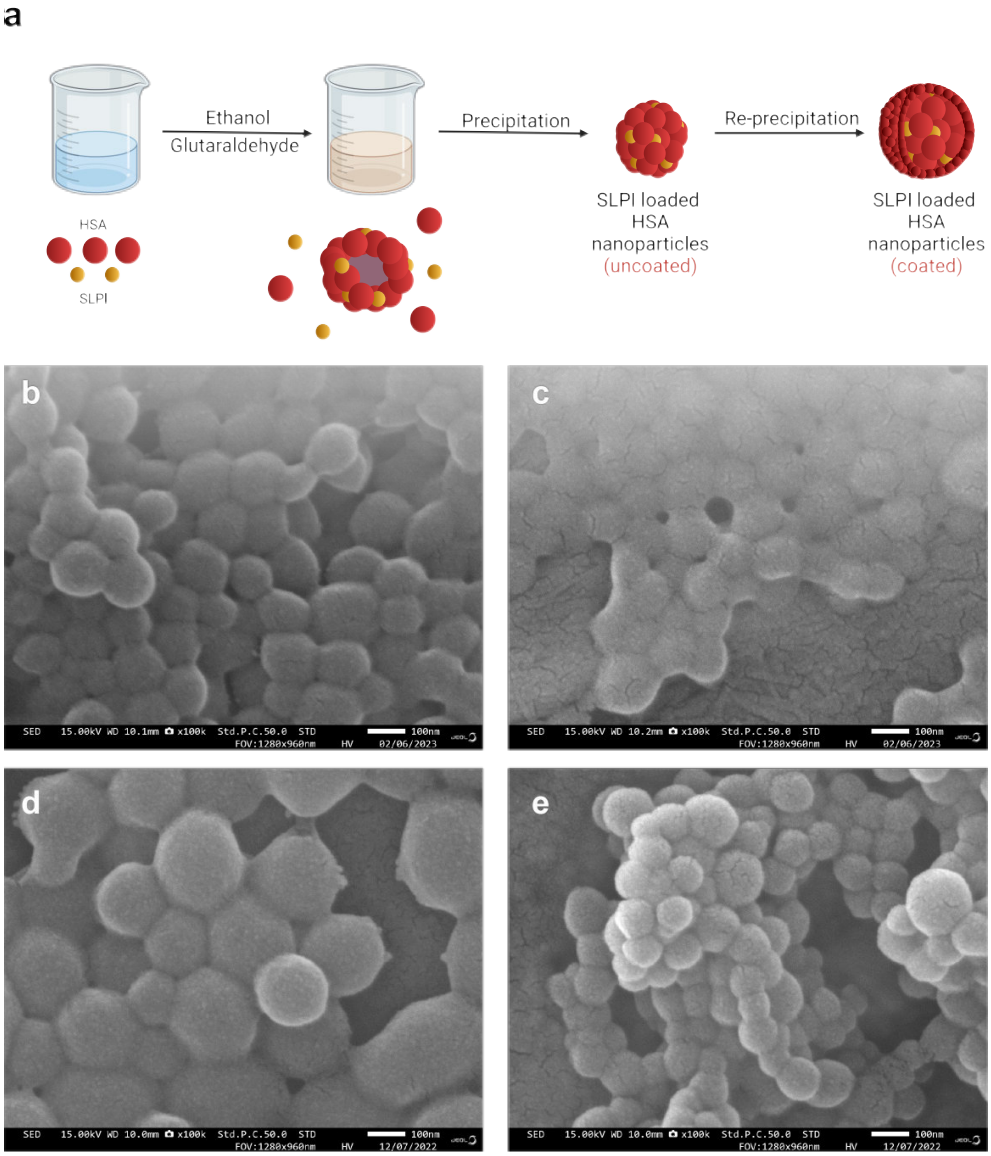

Fig. 1 (a), Schematic diagram of rhSLPI-HSA-NPs fabrication by nanoprecipitation method. Morphological characteristics of HAS-NPs by SEM: (b), uncoated-HSA-NPs; (c), coated-HSA-NPs; (d), uncoated-rhSLPI-HSA-NPs; and (e), coated-rhSLPI-HSA-NPs.

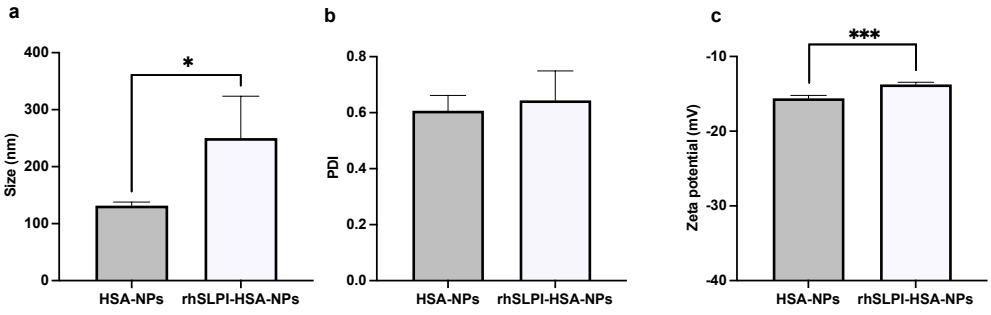

Fig. 2 Characterizations of nanoparticles: (a), size; (b), PDI; and (c), zeta potential. * $p < 0.05$ and *** $p < 0.001$ vs. each other group (unpaired t-test).

The PDI and zeta potential of nanoparticles were measured by Zetasizer, and the PDI values of HSA-NPs and rhSLPI-HSA-NPs were 0.4528±0.1762 and 0.5412±0.1309, respectively (Fig. 2b). The PDI indicates the uniformity of a nanoparticle in the solution [25]. From the results, PDI of rhSLPI-HSA-NPs was not significantly different from HSA-NPs. PDI value within the range of 0.05 to 0.7 is employed to assess the heterogeneity in the size distribution of nanoparticle. A PDI value below 0.05 indicates a high level of uniformity dispersity, whereas a PDI value above 0.7 indicates a low level of uniformity dispersity. Therefore, the PDI of all nanoparticle subtypes exhibited a value of approximately 0.7, indicating little uniformity in dispersity. This observation could be attributed to either the aggregation or the broad distribution of sizes of the nanoparticles.

Fig. 2c shows the zeta potential values of HSA-NPs and rhSLPI-HSA-NPs, which were −15.27±0.20 mV and −13.83±0.057 mV, respectively. The zeta potential value of rhSLPI-HSA-NPs was significantly higher than that of the HSA-NPs. The observed increase in the positive zeta potential value could be attributed to the electrostatic interaction between the positively charged rhSLPI protein [8] and the negatively charged HSA-NPs [26]. These results suggested that the HSA-NPs could encapsulate rhSLPI, and it was possible that the interaction between HSA and rhSLPI was mostly electrostatic in nature [19, 27]. On the other hand, the surface charge of HSA might cause protein attraction; and, hence, the aggregation of nanoparticles was accelerated [19, 27]. In addition, a previous study reported that a zeta potential value ranging from ±10 to ±30 mV indicated incipient instability, thereby indicating the propensity for nanoparticles to aggregate. The phenomenon of particle aggregation could be attributed to the impact caused by zeta potential, leading to the dispersibility of particles through electrostatic repulsion [28].

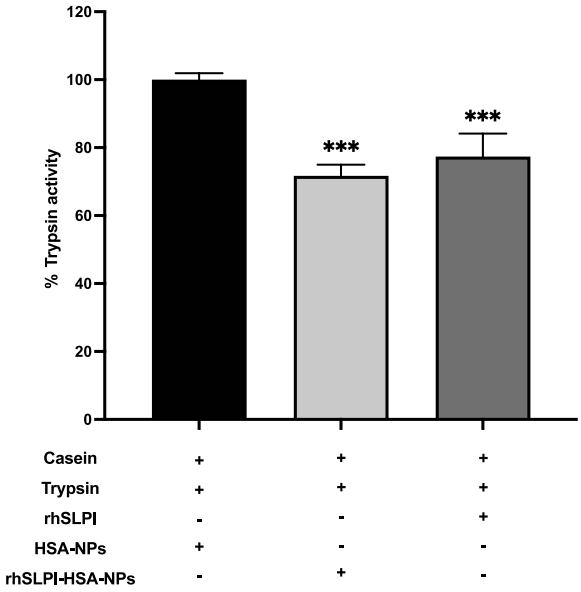

Fig. 3 Percentages of trypsin activity of HSA-NPs, rhSLPI-HSA-NPs, and rhSLPI were determined by anti-trypsin activity assay. *** $p < 0.001$ (ANOVA).

Biochemical characteristics of nanoparticles

The quantification of SLPI protein contained within rhSLPI-HSA-NPs was determined using the ELISA method. From a previous study, the concentration of SLPI protein in rhSLPI-HSA-NPs was significantly greater than that of HSA-NPs (519.4±3.964 pg/ml vs. 0.00±0.00 pg/ml, $p < 0.001$) [29]. The findings of the study demonstrated that the loading efficiency of rhSLPI-HSA-NPs, as measured by the percentage of rhSLPI loaded, was 0.00692%. However, the encapsulation efficiency of rhSLPI-HSA-NPs was not investigated [19], and there has been little information regarding the encapsulation of SLPI protein within HSA-NPs using the nanoprecipitation method [30]. The previous study from Tarhini et al [19], presented the existence of SLPI in HSA-NPs by Western blotting, without investigation the encapsulation efficiency, and the results of non-detectable SLPI, possibly due to the sensitivity of the method. However, in our study, we determined the existence of encapsulated SLPI in HSA-NPs by ELISA, a method having higher sensitivity than the Western blot. In general, ELISA immunological base technique, and it sensitivity depends on the affinity of the antibody against the protein of interest. Therefore, measuring the loading efficiency of protein is more challenging, compared with the encapsulation efficiency measurement of chemical drugs by HPLC or mass spectrometry, which could provide more accurate results. Although our current study showed very low encapsulation efficiency, we could still be able to see the effect of rhSLPI-HSA-NPs in both anti-protease activity assays as well as a cytoprotective effect against sI/R.

Furthermore, the observed low encapsulation efficiency (EE%) of rhSLPI-HSA-NPs could also potentially be attributed to the physicochemical characteristics of rhSLPI, including its charge [8] and hydrophilicity [31], which hinder its interaction with the nanoparticles' matrix. Another factor that might impact the loading process was the ratio of SLPI to HSA-NPs. In the case that the amount of SLPI surpasses the binding capacity of HSA-NPs, there is a possibility of a decrease in the encapsulation efficiency. Furthermore, the rhSLPI-HSA-NPs were gone through thermal fracturing before being tested. Hence, the process might potentially result in a decrease in encapsulation efficacy, due to the presence of HSA molecules blocking the SLPI binding sites [29].

The serine protease inhibitor, known as an SLPIs,

has the ability to block a variety of serine proteases, such as elastase, cathepsin G, chymotrypsin, and trypsin [32]. Trypsin was used as the target enzyme for rhSLPI-HSA-NPs so that the effect of a protease inhibitor could be measured using anti-trypsin activity assay. The findings indicated a significant reduction in trypsin activity when incubated with rhSLPI-HSA-NPs and rhSLPI compared with HSA-NPs (71.7±3.27% and 77.4±6.80% vs. 100±1.89%, $p < 0.001$), as shown in Fig. 3. In the present study, HSA-NPs, rhSLPI-HSA-NPs, and rhSLPI were preincubated with trypsin at 37 °C for 1 h, letting the NPs interact with the enzyme and inhibit its protease activity. Later, when casein was added to the reaction mixture, intact trypsin could digest the casein resulting in a reduction in optical density. The results showed that rhSLPI-HSA-NPs could inhibit the activity of trypsin less than the HSA-NPs, but similar to free rhSLPI (Fig. 3). Since dead cells secrete protease enzymes that can also damage nearby cells, the presence of anti-protease activity is advantageous for preventing the advancement of cell injury and cell death to the nearby cells. Hence, the presence of protease inhibitors could reduce cellular injury and mortality. However, previous research demonstrated that SLPI could independently decrease cell damage and mortality, irrespective of its anti-protease activity [33]. The finding has prompted researchers to hypothesize that SLPI not only has an ability to decrease protease activity, but also performs a "direct effect" in reducing cellular damage and death [33–35]. Therefore, under the scope of our study, we proposed that inhibition of protease activity could potentially assist in vasculoprotection, and the SLPI itself could partly provide these protective effects.

The cytotoxicity of rhSLPI-HSA-NPs on vascular endothelial cells

In order to evaluate the safety of utilizing synthetic nanoparticles, a cytotoxicity assessment was conducted on EA.hy926 for both HSA-NPs and rhSLPI-HSA-NPs. In a previous study [29], a cellular toxicity assay was conducted by treating EA.hy926 cells with various concentrations of nanoparticles (0, 0.1, 1, 10, and 100 µg/ml). The cell viability percentages of HSA-NPs treated cells (103.0±22.7%, 106.8±3%, 102.7±10.4%, and 102.9±10.2%, respectively) and rhSLPI-HSA-NPs (101.6±12, 110.3±7.3, 107.9±13.5, and 100.8±4.6, respectively) were not significantly different from the control group (100.0±0.3%), indicating that the exposure to both HSA-NPs and rhSLPI-HSA-NPs did not result in a significant reduction in cell viability. The results demonstrated that all of the human vascular endothelial cells exposed to different concentrations of HSA-NPs and rhSLPI-HSA-NPs exhibited cell viability percentages of more than 95% (Lethal Dose 5, LD5). This finding suggested that rhSLPI-HSA-NPs did not display any toxicity towards the cells, as it is commonly observed that about 5% of cells undergo cell death under normal circumstances [29].

Optimization of simulated ischemia/reperfusion (sI/R) condition

The current study aimed to optimize the sI/R condition on EA.hy926. This was achieved by adjusting the duration of ischemia, specifically at different time intervals of 0, 10, 20, 30, 40, 50, and 60 min; and cell viability of the individual time intervals were recorded as 94.3±12%, 62.9±3.36%, 47.2±4.48%, 37.3±5.05%, 23±5.48%, 20.6±0.26%, and 17.7±0.309%, respectively (Fig. 4a). The findings indicated that exposure durations ranging from 10 to 60 min resulted in a statistically significant decrease in cell viability compared with the control group. An ischemia period of 20 min resulted in an approximate 50% (47.2±4.48%) cell viability. Hence, the 20 min duration was selected as the best condition for inducing I/R injury, and this specific duration was employed in the other tests as follows. In order to simulate clinical circumstances, it is recommended to employ an sI/R duration that results in about 50% cell mortality, known as LD50. This LD50 value represents the degree of reperfusion harm following rapid reperfusion, as observed in experimental settings. Previous studies have established that a duration of 40 min is the ideal timeframe for inducing LD50 in EA.hy926 by the process of sI/R. The observed variations could potentially be attributed to the differences in cell passage number, the ingredients of cell culture media, the quality of employed reagents and chemicals, as well as variations in cell characteristics, including cell viability and growth rates [13].

The measurement of mitochondrial activity serves as an indicator for live cells; hence, determining the MTT [36]. The measurement of LDH activity indicates the quantification of cytoplasmic enzymes that have been released from damaged cells. It was reported that when plasma membrane was damaged, LDH was quickly released into the culture median [37]. Therefore, the extent of cellular injury could be assessed by measuring the activity of LDH released by cells subjected to different durations of ischemia. In our study, the activities of released-LDH at different duration of ischemia, time intervals of 0, 10, 20, 30, 40, 50, and 60 min were measured, and the values obtained were 0.5854±0.03, 0.8116±0.05, 0.9244±0.05, 1.028±0.098, 1.105±0.089, 1.140±0.09, and 1.095±0.07, respectively (Fig. 4b). The findings indicated that there was a statistically significant rise in LDH activity at time intervals ranging from 10 to 60 min as compared with the control group ($p < 0.001$). The LDH activity exhibited a considerable increase in comparison to the control group, which was consistent with the outcomes obtained from the MTT cell viability experiments.

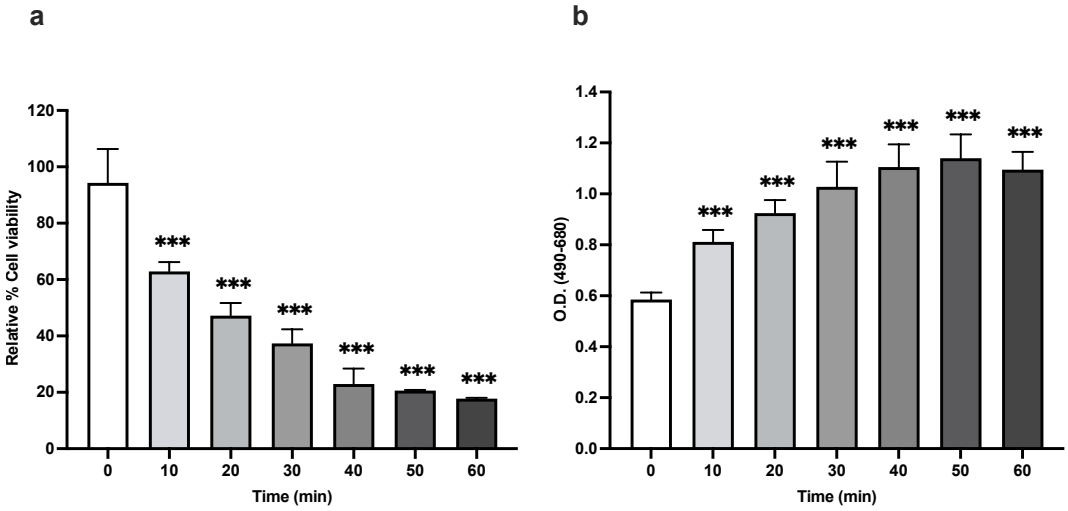

Fig. 4 The optimization of sI/R for EA.hy926 on: (a), cell viability; and (b), cell injury. *** $p < 0.001$ vs. control (ANOVA).

An *in vitro* vasculo-protective effect of rhSLPI-HSA-NPs against simulated ischemia/reperfusion (sI/R) condition

The vasculo-protective effect of rhSLPI-HSA-NPs was assessed using an *in vitro* sI/R model. Different doses of rhSLPI-HSA-NPs (0, 1, 10, and 100 µg/ml) were applied during the sI/R exposure. The cell viability percentages following pre-treatment with rhSLPI-HSA-NPs at concentrations of 0, 1, 10, and 100 µg/ml were determined to be 61.2±2.34%, 74.5±4.68%, 69.6±2.51%, and 65.8±5.22%, respectively, as shown in Fig. 5a. Therefore, the application of sI/R led to a significant decrease in cell viability compared with the control group (non-injured, without sI/R, cells) (61.2±2.34% vs. 93.9±10.7%, $p < 0.05$). The administration of rhSLPI-HSA-NPs at concentrations of 1 µg/ml and 10 µg/ml prior to treatment showed a significant increase in cell viability of the EA.hy926 cells compared with the sI/R group, with the values of 74.5±4.68%, 69.6±2.51%, and 61.2±2.34%, respectively (Fig. 5a). A prior investigation showed that the administration of rhSLPI at a concentration of 1,000 ng/ml resulted in a significant decrease in vascular endothelial cell injury and mortality caused by I/R [15]. The results of our study indicated that the administration of rhSLPI-HSA-NPs at concentrations of 1 µg/ml and 10 µg/ml, containing approximately 0.05–0.5 pg of rhSLPI, could enhance the reduction efficacy of vascular endothelial cell injury caused by I/R. Nevertheless, it was possible that the increased amount of the SLPI could result in a reduced cell survival rate.

In the present study, LDH activities in EA.hy926 cells with sI/R and cells pre-treated with rhSLPI-HSA-NPs at different concentrations were investigated. The results indicated that sI/R could significantly increase cellular injury compared with the control group (1.636±0.08398% vs. 0.6706±0.01878% control, $p < 0.001$). Pre-treatment with rhSLPI-HSA-NPs at concentrations of 1, 10, and 100 µg/ml showed a significant decrease in cell injury of EA.hy926 cells compared with the control group subjected to sI/R (1.167±0.2271%, 0.9287±0.1863%, and 1.089±0.2049%, respectively, compared with 1.636±0.08398% in the sI/R group, $p < 0.05$) (Fig. 5b). Although treatment with rhSLPI-HSA-NPs at the concentration of 100 µg/ml slightly increased the released-LDH activity, the increase was not significantly higher than the treatments with lower doses of 1 or 10 µg/ml (Fig. 5b) and similar to the percentage of cell viability (Fig. 5a). The current investigation involved the synthesis of HSA-NPs for the purpose of delivering rhSLPI. Subsequently, the vasculo-protective effect of the nanoparticles was evaluated on EA.hy926 cells under conditions of *in vitro* sI/R injury.

A previous study provided useful information speculating the mechanisms of cellular uptake and internalization of protein nanoparticles via caveolae and clathrin-mediated endocytosis pathways [37]. Intracellular trafficking of HSA-NP by co-incubating FITC was significantly reduced when treated with the transport inhibitor of caveolae or clathrin. For further study, we could investigate the cellular uptake of rhSLPI-HSA-NPs. The uptake of rhSLPI-HSA-NPs by vascular endothelial cells could be performed by labeling rhSLPI with fluorescent and then observing under the fluorescent or confocal microscope [38].

The rhSLPI-HSA-NPs could be potentially beneficial for treatments of ischemic heart disease and other vascular diseases, which might need a medication-releasing stent. Additionally, rhSLPI-HSA-NPs could be used as a supplement in preservation solutions to prolong the storage of preserved vascular grafts and

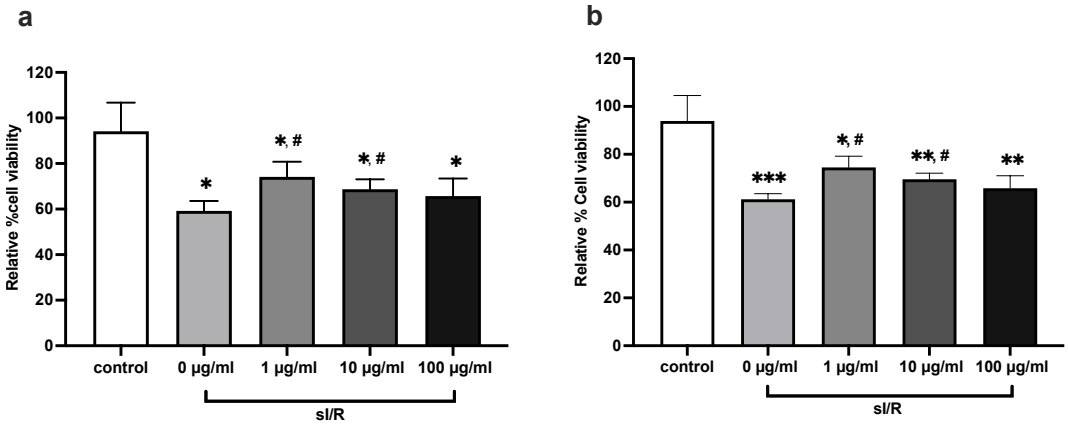

Fig. 5 Vasculo-protective effects of rhSLPI-HSA-NPs against an *in vitro* sI/R on: (a), cell viability; and (b), cell injury. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ vs. control group (ANOVA), # $p < 0.05$ vs. sI/R groups (ANOVA).

possibly improve the prevention of vascular injury. For HAS, it could be a novel candidate for drugs or biomolecule carriers. It has been reported that some miRNAs were found being attached to HSA, and HSA-miRNAs complex was crucial in regulating significant age-related changes in muscle pathophysiology, encompassing proliferation, differentiation, apoptosis, and senescence of skeletal cells. A previous study showed that silencing circRNA-TLK1 could reduce endothelial cell injury [39]; therefore, it could be delivered to vascular endothelial cells to reduce I/R injury. Moreover, the speculated potential of HSA-miRNAs complex and cardio-vasculo protection against myocardial I/R injury is a really interesting research question and should be considered for further investigation.

CONCLUSION

This study reported the protective properties of recombinant human SLPI (rhSLPI)-encapsulated human serum albumin nanoparticles (HSA-NPs), named rhSLPI-HSA-NPs, in mitigating vascular endothelial cell damage caused by ischemia/reperfusion (I/R). Administration of rhSLPI-HSA-NPs at a dosage of 1 µg/ml and 10 µg/ml prior to I/R considerably reduced the damage inflicted upon vascular endothelial cells because of I/R injury.

Acknowledgements: The present study was supported by Chiang Mai University through the Biomedical Engineering and Innovation Research Centre. The research and innovation activity were funded by the National Research Council of Thailand (NRCT) for CP and WC. This project is funded by the National Research Council of Thailand (NRCT): High-Potential Research Team Grant Program Contract no. N42A650870.

REFERENCES

1. Chen L, Luo W, Zhang W, Chu H, Wang J, Dai X, Cheng Y, Zhu T, et al (2020) circDLPAG4/HECTD1 mediates ischaemia/reperfusion injury in endothelial cells via ER stress. *RNA Biol* **17**, 240–253.
2. Yang Q, He GW, Underwood MJ, Yu CM (2016) Cellular and molecular mechanisms of endothelial ischemia/reperfusion injury: perspectives and implications for postischemic myocardial protection. *Am J Transl Res* **8**, 765–777.
3. Merz SF, Korste S, Bornemann L, Michel L, Stock P, Squire A, Soun C, Engel DR, et al (2019) Contemporaneous 3D characterization of acute and chronic myocardial I/R injury and response. *Nat Commun* **10**, 2312–2323.
4. Shi H, Gao Y, Dong Z, Yang J, Gao R, Li X, Zhang S, Ma L, et al (2021) GSDMD-mediated cardiomyocyte pyroptosis promotes myocardial I/R injury. *Circ Res* **129**, 383–396.
5. Slack MA, Gordon SM (2019) Protease activity in vascular disease. *Arterioscler Thromb Vasc Biol* **39**, e210–e218.
6. Lv Z, Chu Y, Wang Y (2015) HIV protease inhibitors: a review of molecular selectivity and toxicity. *HIV AIDS (Auckl)* **7**, 95–104.
7. Bouchard D, Morisset D, Bourbonnais Y, Tremblay GM (2006) Proteins with whey-acidic-protein motifs and cancer. *Lancet Oncol* **7**, 167–174.
8. Majchrzak-Gorecka M, Majewski P, Grygier B, Murzyn K, Cichy J (2016) Secretory leukocyte protease inhibitor (SLPI), a multifunctional protein in the host defense response. *Cytokine Growth Factor Rev* **28**, 79–93.
9. Mongkolpathumrat P, Kijtawornrat A, Prompunt E, Panya A, Chattipakorn N, Barrere-Lemaire S, Kumphune S (2021) Post-ischemic treatment of recombinant human secretory leukocyte protease inhibitor (rhSLPI) reduced myocardial ischemia/reperfusion injury. *Biomedicines* **9**, 422–439.
10. Prompunt E, Sanit J, Barrere-Lemaire S, Nargeot J, Noordali H, Madhani M, Kumphune S (2018) The cardioprotective effects of secretory leukocyte protease inhibitor against myocardial ischemia/reperfusion injury. *Exp Ther Med* **15**, 5231–5242.
11. Prompunt E, Nernpermpisooth N, Sanit J, Kumphune S (2018) Overexpression and pre-treatment of recombinant human secretory leukocyte protease inhibitor (rhSLPI) reduces an *in vitro* ischemia/reperfusion injury in rat cardiac myoblast (H9c2) cell. *Biomol Concepts* **9**,

17–32.
12. Paiyabhroma1 N, Nernpermpisooth1 N, Kumphune S (2018) The recombinant human secretory leukocyte protease inhibitor (SLPI) protects cardiac fibroblasts injury against an *in vitro* ischemia/reperfusion injury. *J Appl Pharm Sci* **8**, 156–162.
13. Kongpol K, Nernpermpisooth N, Prompunt E, Kumphune S (2019) Endothelial-cell-derived human secretory leukocyte protease inhibitor (SLPI) protects cardiomyocytes against ischemia/reperfusion injury. *Biomolecules* **9**, 678–695.
14. Kantapich K, Rungrueang Y, Nitirut N, Sarawut K (2020) Recombinant human secretory leukocyte protease inhibitor ameliorated vessel preservation in experimentally isolated rat arteries. *J Appl Pharm Sci* **10**, 107–114.
15. Nernpermpisooth N, Prompunt E, Kumphune S (2017) An *in vitro* endothelial cell protective effect of secretory leukocyte protease inhibitor against simulated ischaemia/reperfusion injury. *Exp Ther Med* **14**, 5793–5800.
16. Patra JK, Das G, Fraceto LF, Campos EVR, Rodriguez-Torres MDP, Acosta-Torres LS, Diaz-Torres LA, Grillo R, et al (2018) Nano based drug delivery systems: recent developments and future prospects. *J Nanobiotechnol* **16**, 71–88.
17. Lohcharoenkal W, Wang L, Chen YC, Rojanasakul Y (2014) Protein nanoparticles as drug delivery carriers for cancer therapy. *Biomed Res Int* **2014**, 180549–180562.
18. Kianfar E (2021) Protein nanoparticles in drug delivery: animal protein, plant proteins and protein cages, albumin nanoparticles. *J Nanobiotechnol* **19**, 159–176.
19. Tarhini M, Pizzoccaro A, Benlyamani I, Rebaud C, Greige-Gerges H, Fessi H, Elaissari A, Bentaher A (2020) Human serum albumin nanoparticles as nanovector carriers for proteins: application to the antibacterial proteins "neutrophil elastase" and "secretory leukocyte protease inhibitor". *Int J Pharm* **579**, 119150–119158.
20. Phutiyothin C (2023) Development of human serum albumin nanoparticles encapsulating recombinant human secretory leukocyte protease inhibitor against ischaemia/reperfusion-induced vascular endothelial cell injury. MSc Thesis, Chiang Mai University, Thailand.
21. Gordillo-Galeano A , Mora-Huertas CE (2021) Hydrodynamic diameter and zeta potential of nanostructured lipid carriers: Emphasizing some parameters for correct measurements. *Colloids Surf A* **620**, 126610–126621.
22. Judy E, Lopus M, Kishore N (2021) Mechanistic insights into encapsulation and release of drugs in colloidal niosomal systems: biophysical aspects. *RSC Adv* **11**, 35110–35126.
23. Chahine N, Nader M, Duca L, Martiny L, Chahine R (2016) Saffron extracts alleviate cardiomyocytes injury induced by doxorubicin and ischemia-reperfusion *in vitro*. *Drug Chem Toxicol* **39**, 87–96.
24. Zhang S, Wang C (2023) Precise analysis of nanoparticle size distribution in TEM image. *Methods Protoc* **6**, 63–69.
25. Danaei M, Dehghankhold M, Ataei S, Hasanzadeh Davarani F, Javanmard R, Dokhani A, Khorasani S, Mozafari MR (2018) Impact of particle size and polydispersity index on the clinical applications of lipidic nanocarrier systems. *Pharmaceutics* **10**, 57–74.
26. Fanali G, di Masi A, Trezza V, Marino M, Fasano M, Ascenzi P (2012) Human serum albumin: from bench to bedside. *Mol Aspects Med* **33**, 209–290.
27. Davidov-Pardo G, Joye IJ, McClements DJ (2015) Food-grade protein-based nanoparticles and microparticles for bioactive delivery: fabrication, characterization, and utilization. *Adv Protein Chem Struct Biol* **98**, 293–325.
28. Alsharef JMA, Taha MR, Ahmed Khan T (2017) Physical dispersion of nanocarbons in composites: a review. *J Teknol* **79**, 69–81.
29. Phutiyothin C, Chouyratchakarn W, Pikwong F, Jantrawut P, Baipaywad P, Kumphune S (2023) Development of nanoparticles for delivering recombinant human secretory leukocytes protease inhibitor (rhSLPI) for reducing vascular endothelial cell injury. In: *7th International STEM Education Conference 2022 (iSTEM-Ed 2022)*.
30. Herrera Estrada LP, Champion JA (2015) Protein nanoparticles for therapeutic protein delivery. *Biomater Sci* **3**, 787–799.
31. Hirano M, Kamada M, Maegawa M, Gima H, Aono T (1999) Binding of human secretory leukocyte protease inhibitor in uterine cervical mucus to immunoglobulins: pathophysiology in immunologic infertility and local immune defense. *Fertil Steril* **71**, 1108–1114.
32. Eisenberg SP, Hale KK, Heimdal P, Thompson RC (1990) Location of the protease-inhibitory region of secretory leukocyte protease inhibitor. *J Biol Chem* **265**, 7976–7981.
33. Mongkolpathumrat P, Kijtawornrat A, Suwan E, Unajak S, Panya A, Pusadee T, Kumphune S (2022) Anti-protease activity deficient secretory leukocyte protease inhibitor (SLPI) exerts cardioprotective effect against myocardial Ischaemia/Reperfusion. *Biomedicines* **10**, 988–1003.
34. McGarry N, Greene CM, McElvaney NG, Weldon S, Taggart CC (2015) The ability of secretory leukocyte protease inhibitor to inhibit apoptosis in monocytes Is independent of its antiprotease activity. *J Immunol Res* **2015**, 507315–507322.
35. Yang J, Zhu J, Sun D, Ding A (2005) Suppression of macrophage responses to bacterial lipopolysaccharide (LPS) by secretory leukocyte protease inhibitor (SLPI) is independent of its anti-protease function. *Biochim Biophys Acta* **1745**, 310–317.
36. Buranaamnuay K (2021) The MTT assay application to measure the viability of spermatozoa: A variety of the assay protocols. *Open Vet J* **11**, 251–269.
37. Decker T, Lohmann-Matthes M-L (1988) A quick and simple method for the quantitation of lactate dehydrogenase release in measurements of cellular cytotoxicity and tumor necrosis factor (TNF) activity. *J Immunol Methods* **115**, 61–69.
38. Chaiwaree S, Prapan A, Suwannasom N, Laporte T, Neumann T, Pruss A, Georgieva R, Baumler H (2020) Doxorubicin-loaded human serum albumin submicron particles: preparation, characterization and *in vitro* cellular uptake. *Pharmaceutics* **12**, 224–239.
39. Li J, Zhuo Z, Ren Z, Lid G (2023) Silencing circRNA TLK1 reduces endothelial cell injury in coronary heart disease. *ScienceAsia* **49**, 899–909.