Gelatin-coated silicon oxide nanoparticles encapsulated recombinant human secretory leukocyte protease inhibitor (rhSLPI) reduced cardiac cell death against an *in vitro* simulated ischaemia/reperfusion injury

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ABSTRACT

Ischemic Heart Disease (IHD) is the main global cause of death. Previous studies indicated that recombinant human secretory leukocyte protease inhibitor (rhSLPI) exhibits a cardioprotective effect against myocardial ischaemia/reperfusion (I/R) injury. However, SLPI has a short half-life *in vivo* due to digestion by protease enzymes in circulation. The application of nanoparticle encapsulation could be beneficial for SLPI delivery. Several types of nanoparticles have been developed to encapsulate SLPI and applied in some disease models. However, silica nanoparticles for rhSLPI delivery, particularly on myocardial I/R injury, have never been studied. In this study, we aimed to fabricate gelatin-covered silica nanoparticles (GSNPs) to encapsulate rhSLPI and cardioprotective effect of GSNP-SLPI against an *in vitro* simulated ischaemia/reperfusion (sI/R). Silica dioxide nanoparticles (SNPs) were fabricated followed by incubation with 0.33 mg/mL of rhSLPI. Then, SNPs containing rhSLPI were coated with gelatin (GSNPs). The GSNPs and rhSLPI-GSNPs were characterized by particle size, zeta potential, and morphology scanning electron microscope (SEM). The concentration of rhSLPI in rhSLPI-GSNPs and drug release was determined by ELISA. Then, cytotoxicity and cardioprotective effect were determined by incubation of GSNPs or rhSLPI-GSNPs with rat cardiac myoblast cell line (H9c2) subjected to simulated ischaemia/reperfusion (sI/R). The results showed the particle size of SNPs, GSNPs, and rhSLPI-GSNPs was 273, 300, and 301 nm, with a zeta potential of −57.21, −22.40, and −24.50 mV, respectively. One milligram of rhSLPI-GSNPs contains 235 ng of rhSLPI. The rhSLPI-GSNPs showed no cytotoxicity on cardiac cells. Treatment with 10 μg/ml of rhSLPI-GSNPs could significantly reduce sI/R induced cardiac cell injury and death. In conclusion, this is *the first* study to show successful of fabricating novel rhSLPI-encapsulating gelatin-covered silica nanoparticles (rhSLPI-GSNPs) and the cardioprotective effects of rhSLPI-GSNPs against cardiac cell injury and death from myocardial ischaemia/reperfusion.

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1. Introduction

It is anticipated that by the year 2030, Ischaemic Heart Disease (IHD) will be the primary cause of death worldwide [1]. Patients with myocardial infarction may eventually develop heart failure (HF), which is associated with an increased risk of morbidity and mortality. It becomes a challenging research problem to develop novel therapeutic approaches to lower the severity of myocardial damage, which will effectively save patients' lives.

Protease enzymes cause widespread destruction and have relatively long half-lives in tissue during myocardial ischaemia/reperfusion (I/R) injury [2]. Increasing in activity of proteolytic enzymes, such as caspases, calpains, cathepsins, matrix metalloproteinases, chymase, and matrix metalloproteinases, could aggravate cardiac cell injury and death [3]. Therefore, inhibition of protease activity can therefore be considered as powerful strategies for prevention of ischaemia/reperfusion (I/R) induced tissue injury. Our previous reports demonstrated that treatment with secretory leukocyte protease inhibitor (SLPI) via overexpression of the SLPI gene [4] or treatment with recombinant protein of human SLPI (rhSLPI) provided cytoprotection against I/R injury in an *in vitro* [4,5], *ex vivo* [5], and *in vivo* [6] study models. Moreover, SLPI can reduce infarct size, inflammation, oxidative stress, and apoptosis, as well as improve cardiac function [6]. It's interesting to note that while SLPI's positive benefits are assumed to be a result of its anti-protease action, a few studies have suggested they could exist independently on its anti-protease activity [7,8]. Our most recent report showed that the recombinant protein of the mutant SLPI (L72K, M73G, L74G), which is anti-protease defective (mt-SLPI), offered cardioprotection against I/R damage [9]. As a result, it is believed that SLPI's direct action contributes to some of its therapeutic potentials. One of the clinical drawbacks of employing recombinant human SLPI as a treatment is its brief half-life in circulation. The half-life of recombinant human SLPI in circulation was 10–60 min according to preclinical research in animal models, but it was 10–120 min in humans due to cleavage by other protease enzymes in circulation [10,11]. Hence, increasing the stability and half-life of SLPI in circulation by employing nanoparticles encapsulating SLPI might have a potent therapeutic benefit [12].

Several advantages of nanoparticles for peptide drug carriers have been listed, for example, reducing the enzymatic digestion, aggregation of peptide drugs, and increasing the transmembrane absorption [13]. Gelatin-covered silica nanoparticles (GSNPs) are thought to be one of the most efficient carriers for delivering and managing the release of medicines and biomolecules with reduced cytotoxicity because of their biocompatibility and biodegradability [14,15]. Gelatin-coated silica nanoparticles have never been investigated for their ability to deliver SLPI as a peptide medication, especially in cardiac cells subjected to I/R injury. In this work, we
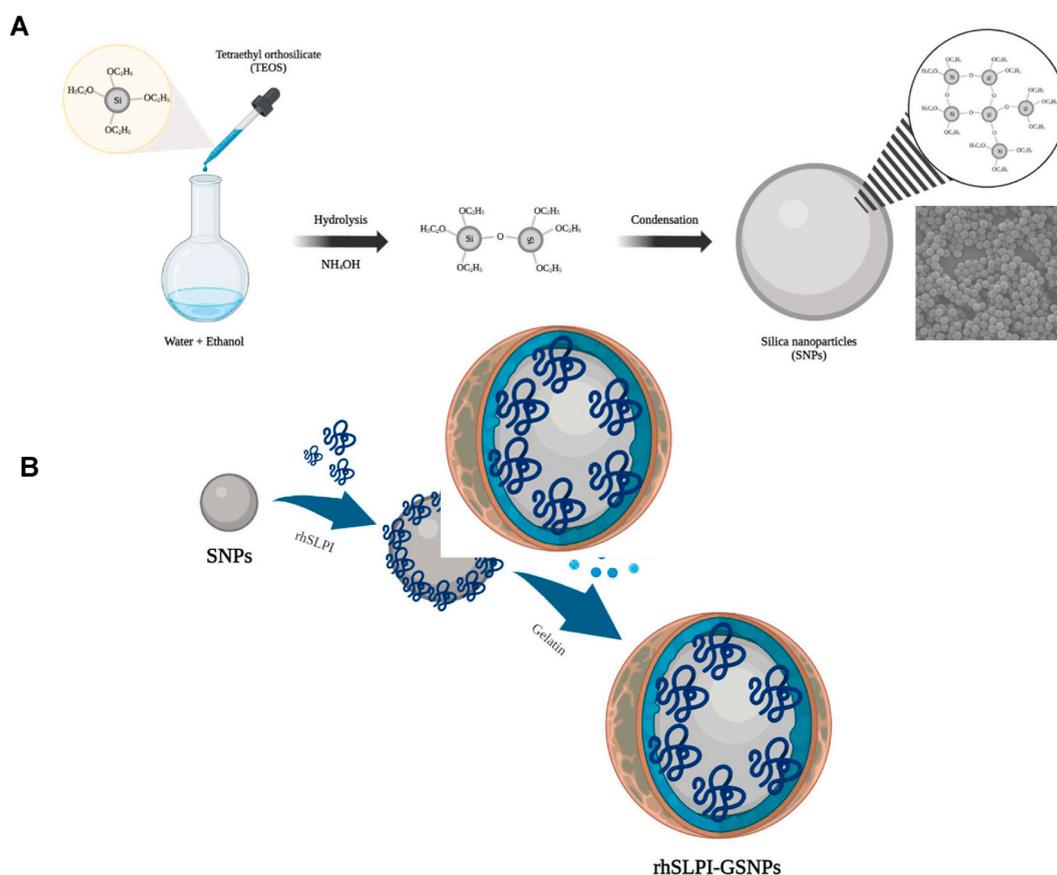
Fig. 1. Schematic diagram of gelatin covered silica nanoparticles-encapsulated recombinant human secretory leukocyte protease inhibitor (rhSLPI-GSNPs) fabrication by a modified Stöber process(A) and the purpose structure of rhSLPI-GSNPs (B).

demonstrated for the first time that recombinant human SLPI (rhSLPI) delivered by GSNPs could be fabricated without cardiotoxicity to cardiac cells and could provide cardioprotection against an *in vitro* I/R injury.

2. Material and methods

2.1. Chemical and reagent

The chemicals were purchased from Sigma–Aldrich including TEOS (98.0%), gelatin type A, Glutaraldehyde, 30% ammonia solution (NH4OH), and ethyl alcohol. Recombinant human SLPI (rhSLPI) was purchased from Sino Biology Inc. (Beijing, China), and Dulbecco's modified Eagle's medium (DMEM), foetal bovine serum and trypsin-EDTA (Gibco BRL; Life Technologies Inc. New York, USA). Other reagents CyQUANT™ LDH Cytotoxicity Assay Kit (Cat No: C20301) was purchased from Thermo Fisher, 3-(4,5-dimethyl-2-thiazol)-2,5-diphenyl-2Htetrazolium bromide (MTT) was obtained from Ameresco (Solon, Ohio, USA).

2.2. Cell type and cell culture

The rat cardiomyocyte cell line (H9c2) was obtained as ATCC-CRL1446 from the American Type Culture Collection (Manassas, VA, USA). Cells were cultured in DMEM supplemented with 10% (v/v) foetal bovine serum (FBS) and 5000 units/mL penicillin/streptomycin. Cells were kept at 37 °C in a humidified atmosphere of 95% air and 5% carbon dioxide.

2.3. Preparation of gelatin-covered silica nanoparticles-encapsulated recombinant human secretory leukocyte protease inhibitor (rhSLPI-GSNPs)

Silica nanoparticles (SNPs) were fabricated by a modified Stöber process as described in a previous study [16] (Fig. 1A). Briefly, a co-solvent containing distilled water and ethanol was added to a round flask. Then, TEOS (1 mL) was added to the solution. The solution was stirred and slowly dropped NH$_4$OH for the chemical reaction in 8 h. Then, the nanoparticles were centrifuged and washed three times with distilled water and ethanol. The rhSLPI-loaded gelatin-covered silica nanoparticles (rhSLPI-GSNPs) were prepared by using glutaraldehyde crosslinking [17] (Fig. 1B). SNPs were added in 0.33 mg/mL of rhSLPI solution and mixed overnight. Then, the solution was centrifuged to remove excess rhSLPI. Pellets were stirred in gelatin solution (1%, 1 mL) at 40 °C for 6 h. After that, cold deionized water (4 °C) was added to the mixture. Then, 1% glutaraldehyde solution was added and used for cross-linking the gelatin for 8 h. The solution was centrifuged to collect the particles and washed 3 times with distilled water. Finally, the rhSLPI-GSNPs were dried by freeze-drying and keep in a desiccator for the next use.

2.4. Physical characterizations of rhSLPI-GSNPs

Field emission scanning electron microscopy (FE-SEM) (CLARA, TESCAN) was used to examine the morphology of SNPs and rhSLPI-GSNPs after a nanoparticle solution was dropped on a copper tape and sputtered with platinum for 3 nm thickness. The images obtained from the FE-SEM were used to measure the particle sizes using ImageJ software (National Institute of Health).

The size and polydispersity index and surface charge of SNPs and rhSLPI-GSNPs were determined by Dynamic Light Scattering - Zetasizer (Malvern).

2.5. Determination of cardiac cytotoxicity of rhSLPI-GSNPs

MTT viability assay was used for cytotoxicity testing. First, 2×10^4 cells of H9c2 cells were seeded in a 96-well plate in complete DMEM medium supplemented with 5000 units/mL penicillin, 5000 g/mL streptomycin, and 10% Foetal Bovine Serum (FBS). The cell was then incubated for 24 h at 37 °C with GSNPs and rhSLPI-GSNPs concentrations of 0, 0.1, 1, 10, and 100 g/mL. Following that, the culture medium was discarded, and cell viability was determined using the MTT cell viability assay.

2.6. Simulated I/R (sI/R) injury

Simulated ischaemia (sI) was performed as described in previous publications [4,18–21]. Cells were incubated with specified modified Krebs–Henseleit buffer (137 mM NaCl, 3.8 mM KCl, 0.49 mM MgCl$_2$, 0.9 mM CaCl$_2$, and 4.0 mM HEPES) supplemented with 20 mM 2-deoxyglucose, 20 mM sodium lactate, and 1 mM sodium dithionite at pH 6.5. The reactions were incubated at 37 °C, 5% CO$_2$ for several incubation times before changing to complete medium for reperfusion and incubated at 37 °C, 5% CO$_2$ for 24 h. The incubation time that provides approximately 50% cell death is considered an optimal sI/R condition and was used for the whole experiment on I/R injury.

2.7. Determination of the cardioprotective effect of rhSLPI-GSNPs in sI/R injury

The MTT viability assay was used to determine the cardioprotective effect of rhSLPI-GSNPs. The H9c2 cell was seeded in 96-well plates at 1105 cells/well in complete DMEM medium containing 5000 units/mL penicillin, 5000 g/mL streptomycin, and 10% Foetal Bovine Serum (FBS). The cell was then exposed to rhSLPI-GSNPs at concentrations of 1, 10, and 100 g/mL for 24 h at 37 °C. The entire

DMEM medium was then removed, and sI/R injury was performed. Furthermore, the cellular injury was determined by the released-LDH activity assay.

2.8. Measurement of cell viability

H9c2 cells were cultured or exposed to experimental procedures. At the end of the experimental procedures, the culture media was removed, followed by adding 0.5 mg/ml MTT reagent in PBS and incubating for 2 h at 37 °C. After incubation, the MTT reagent was discarded and DMSO was added for solubilizing the formazan dye. The optical density (OD) was determined by a spectrophotometer at λ 570 nm using DMSO as a blank. The relative percentage of cell viability was compared to the control group.

2.9. Measurements of cellular injury

The released-lactate dehydrogenase (LDH) activity was determined to indicate the loss of permeability of the cell membrane. The LDH activity, in the cell culture medium, was measured by The CyQUANT LDH Cytotoxicity Assay Kit. Fifty microliters of culture medium were mixed with 50 μL of Reaction Mixture and incubated at room temperature for 30 min. Then, 50 μl of Stop Solution was added and mixed. Then, the absorbance at 490 nm and 680 nm was measured by spectrophotometric assay. To determine LDH activity,
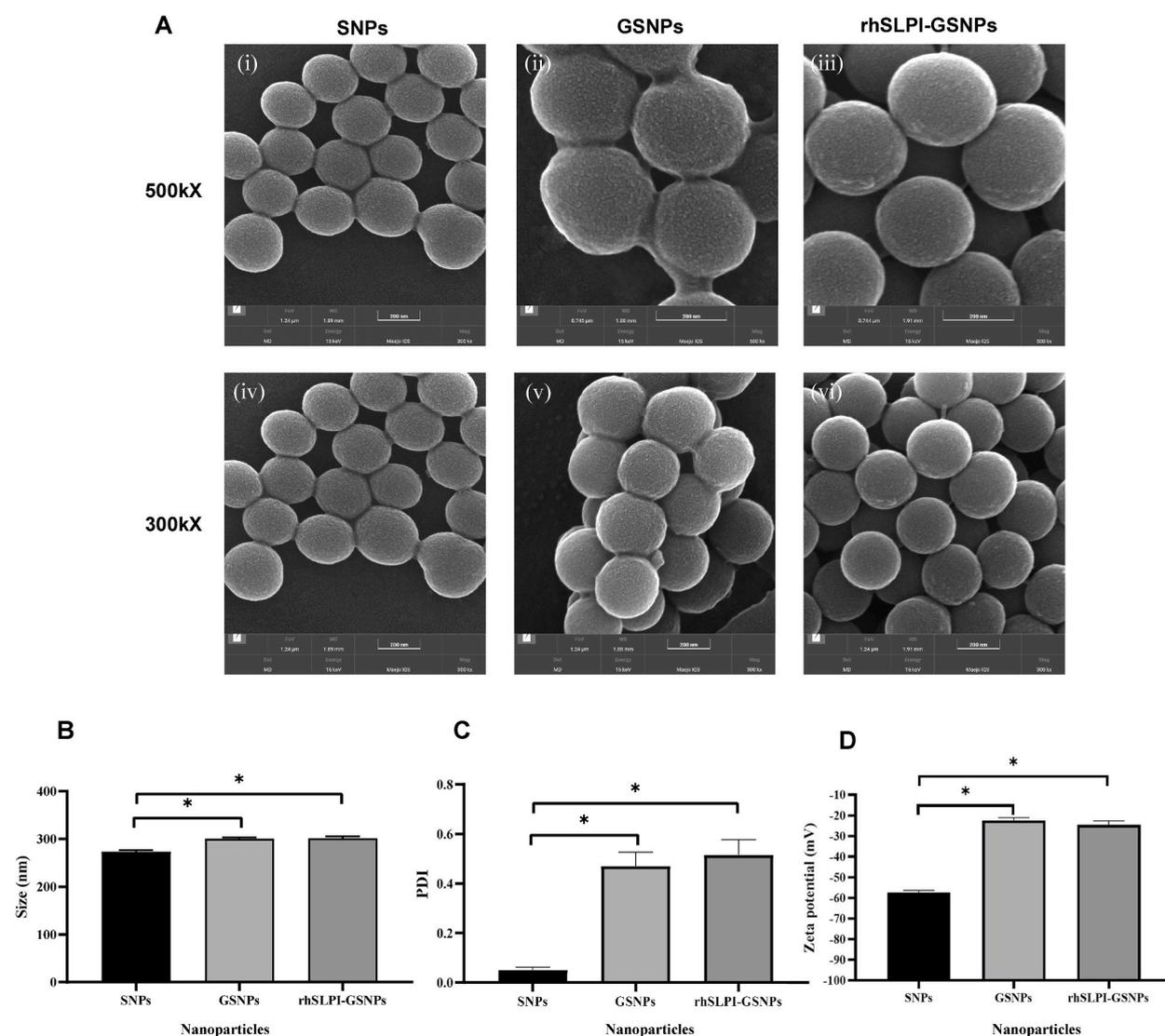
Fig. 2. Morphology of nanoparticles (A). Figure A (i), (iii), (iii) represented the SNPs, GSNPs, and rhSLPI-GSNPs with 500K magnification, respectively. Figure A (iv), (v), (vi) represented the SNPs, GSNPs, and rhSLPI-GSNPs with 300K magnification, respectively. The characteristics of nanoparticles including size (B); PDI (C), and zeta potential (D). *$p < 0.05$ versus each other group (ANOVA).

the 490-nm absorbance value was subtracted by the 680-nm absorbance value (background signal from the instrument).

2.10. Statistical analysis

The statistical tests were carried out using commercially available software (GraphPad Prism). All values were expressed as Mean ± S.E.M. The SLPI level was compared between GSNPs and rhSLPI-GSNPs using an unpaired t-test. The Tukey-Kramer test was used to determine the significance of all group comparisons. A p-value of less than 0.05 was considered statistically significant.

3. Results

3.1. Characterization of gelatin-covered silica nanoparticles-encapsulated recombinant human secretory leukocyte protease inhibitor (rhSLPI-GSNPs)

The FE-SEM was used to identify and determine the morphology of nanoparticles. The results showed that SNPs had a round shape with a smooth surface (Fig. 2A (i),(iv) with 500K and 300K magnification, respectively), while GSNPs (Fig. 2A (ii),(v) with 500K and 300K magnification, respectively)and rhSLPI (Fig. 2A (iii),(vi) with 500K and 300K magnification, respectively) showed a round shape with a rough surface. Each type of nanoparticle has similar diameters, which were averaged and measured by ImageJ. Sizes of SNPs, GSNPs, and rhSLPI-GSNPs are 273.3 ± 6.33 nm, 300.3 ± 6.55 nm, and 301.3 ± 12.73 nm, respectively (Fig. 2B). The size of GSNPs and rhSLPI-GSNPs was larger than the size of SNPs ($p < 0.05$). PDI and zeta potential values of nanoparticles that were obtained using Zetasizer. PDI of SNPs, GSNPs, and rhSLPI-GSNPs are 0.05 ± 0.02, 0.47 ± 0.10, and 0.51 ± 0.10, respectively (Fig. 2C). The PDI of GSNPs and rhSLPI-GSNPs was significantly higher than the PDI of SNPs ($p < 0.05$). The surface of nanoparticles has an impact on the zeta potential values. SNPs, GSNPs, and rhSLPI-GSNPs have respective zeta potentials of −57.27 ± 1.00 mV, −22.40 ± 1.37 mV, and −24.50 ± 1.92 mV, respectively (Fig. 2D). The zeta potential of GSNPs and rhSLPI-GSNPs was significantly higher than the zeta potential of SNPs ($p < 0.05$).

In addition, the results showed that there was no SLPI found in GSNPs compared to 1 mg of rhSLPI-GSNPs gave SLPI of 235.3 ± 1.041 ng (Fig. 3A). rhSLPI-GSNPs had a significantly SLPI concentration higher than GSNPs ($p < 0.05$). As a result, the calculated mass of SLPI from 50 mg of synthesized rhSLPI-GSNPs, which is the mass used for fabrication, was approximately 11.75 ng. The mass of rhSLPI, which loaded in nanoparticles was calculated by comparing the default value of rhSLPI for calculating encapsulation efficiency (EE %) as 3.56%.

Furthermore, the drug release study was separated into 2 protocols including performing the releasing profile in PBS solution for up to 72 h and performing the releasing profile in 0.1% collagenase solution for up to 240 min. Fig. 3B shows the releasing profile of rhSLPI from rhSLPI-GSNPs in PBS solution at 0 h, 24 h, 48 h, and 72 h. The accumulative percentage of release was 0%, 0.26 ± 0.3569%, 0.97 ± 0.6877%, and 1.05 ± 0.7593%, respectively. The results showed that rhSLPI could be released less than 2% in 72 h. A different study protocol was performed the releasing profile in 0.1% collagenase solution for up to 240 min. The result showed the accumulative release of rhSLPI from rhSLPI-GSNPs, in 0.1% collagenase solution for 0 min, 15 min, 30 min, 60 min, 120 min, and 240 min, was 0%, 13.97 ± 1.973%, 30.96 ± 1.032%, 52.28 ± 1.858%, 74.73 ± 3.902%, and 100 ± 3.270%, respectively. The results showed that rhSLPI could be released 100% within 240 min (Fig. 3C).

3.2. Cytotoxicity of rhSLPI-GSNPs on cardiac cell

Concentrations of GSNPs and rhSLPI-GSNPs were varied by 10-fold dilution from 0 to 100 μg/mL and incubated in cardiomyoblast (H9c2) cells for 24 h (Fig. 4). The results showed no significant difference in cell viability of cells treated with GSNPs or rhSLPI-GSNPs in every concentration ($p < 0.05$). There are two concentrations of rhSLPI-GSNPs including 1 μg/mL, and 100 μg/mL, which had a
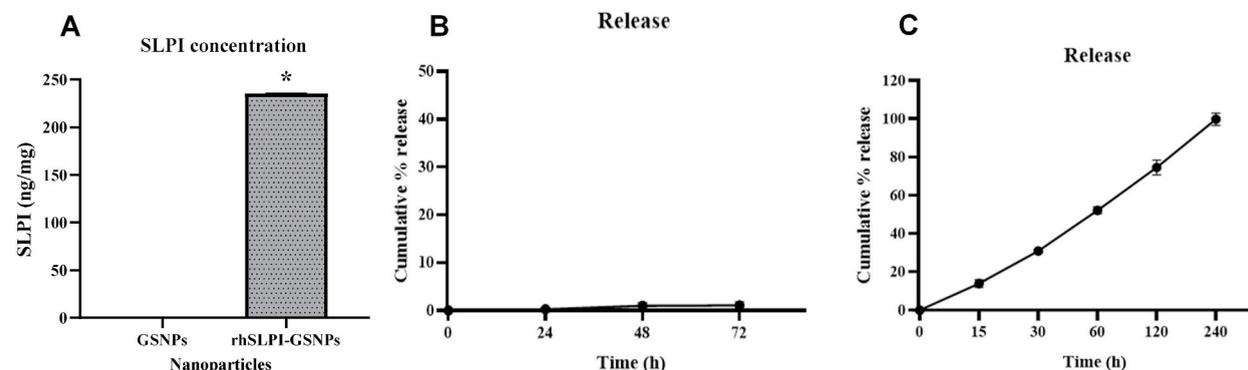
Fig. 3. SLPI concentration from synthesized nanoparticles GSLPI and rhSLPI-GSNPs by ELISA (A), release profiles of rhSLPI from rhSLPI-GSNPs in PBS solution (B) or in 0.1% collagenase solution (C). *$p < 0.05$ (Unpaired t-test).

percentage of cell viability below 95% (92.11 ± 10.27, and 84.80 ± 2.47, respectively).

3.3. Optimization of an in vitro simulated ischaemia/reperfusion (sI/R) injury

Optimization of sI/R time was separated from 0 to 60 min and measured by MTT assay and released LDH assay. the result showed the percentage of cell viability of time 0 min, 10 min, 20 min, 30 min, 40 min, 50 min, and 60 min was 100.00 ± 4.856, 101.8 ± 3.082, 99.63 ± 4.301, 92.64 ± 2.692, 82.14 ± 6.837, 71.69 ± 7.061, and 56.30 ± 8.955, respectively (Fig. 5A). In a time of 40 min, 50 min, and 60 min cell viability was significantly lower than the control group (0 min) ($p < 0.05$). In addition, the released LDH activity was 0.4519 ± 0.03, 0.47 ± 0.06, 0.52 ± 0.04, 0.58 ± 0.05, 0.71 ± 0.05, 0.74 ± 0.07, and 1.02 ± 0.12, respectively (Fig. 5B). In a time of 40 min, 50 min, and 60 min cell viability was significantly higher than the control group (0 min) ($p < 0.05$).

3.4. An in vitro cardioprotective effect of rhSLPI-GSNPs against simulated ischaemia/reperfusion (sI/R) injury

The result showed that sI/R could significantly reduce the percentage of cell viability when compared to the control group ($55.55 \pm 13.04\%$ vs $100.7 \pm 13.34\%$, $p < 0.05$). Treatment of rhSLPI-GSNPs at 1, 10, and 100 μg/mL significantly improved the percentage of cell viability ($p < 0.05$) when compared to the sI/R group ($54.43 \pm 13.40\%$, $74.48 \pm 8.23\%$, and $56.21 \pm 11.38\%$, respectively vs $55.55 \pm 13.04\%$, $p < 0.05$) (Fig. 6A). The sI/R increased the released LDH activity when compared to the control group (0.7727 ± 0.06 vs 0.3388 ± 0.03). Treatment with rhSLPI-GSNPs at concentrations 1, 10, and 100 μg/mL could significantly reduce released LDH activity (0.677 ± 0.06, 0.6886 ± 0.06, and 0.6787 ± 0.04, $p < 0.05$ respectively) (Fig. 6B).

4. Discussion

Previous studies reported a cardioprotective effect of SLPI in decreased cell injury *in vitro* studies [4], and decreased infarct size in the heart of rats in an *ex vivo* study [22]. However, SLPI has a short half-life *in vivo* due to protease enzymes in circulation [12]. Therefore, a delivery system is necessary. Gelatin-covered silica nanoparticles (GSNPs) are thought to be one of the most efficient carriers for delivering and managing the release of medicines and biomolecules with reduced cytotoxicity because of their biocompatibility and biodegradability [14,15]. However, there are only few studies showing fabrication of GSNPs for drug delivery (Table 1), which use for chemical drug delivery, and only our current study demonstrated for the first time that fabricating GSNPs could be used to deliver recombinant protein to cardiac cells and could provide a cardioprotective effect against an *in vitro* simulated ischaemia/reperfusion injury in the cardiac cell.

In the study, the surface of synthesized SNPs initially showed a smooth surface (Fig. 2A), and both surfaces of synthesized GSNPs and rhSLPI-GSNPs were later changed to a rough surface after coating with a thin layer of gelatin (Fig. 2A). Typically, the surface of SNPs is quite smooth or litter rough surface, however when covered with gelatin that has made an increased rough surface due to the formation of gelatin on the surface of SNPs. The previous studies showed gelatin could be adsorbed on the surface of SNPs, therefore gelatin was cross-linked by glutaraldehyde, which carboxaldehyde (CHO) of glutaraldehyde interacts with the amine (NH_2) of gelatin for preventing the adsorbed gelatin dissolution [17,23].

The size of GSNPs and rhSLPI is larger than SNPs showing that covered gelatin increased the diameter by approximately 30 nm indicating that the layer of covered gelatin is 15 nm. The result of SNPs nanoparticles showed that a similar size that made a PDI 0.05 ± 0.02 is monodispersed particles. The electric charge on the nanoparticle's surface was measured by zeta potential in which the charge of SNPs became more positive after coating with gelatin (GSNPs) and loading rhSLPI (rhSLPI-GSNPs). The surface charge of SNPs showed negatively charged from the silanol groups [24], while GSNPs and rhSLPI-GSNPs were slightly increased due to positively charged gelatin coating followed a previous study [25], which were covered by gelatin indicating a successful coating process. The stability of particles depends on the zeta potential value at a specific surface potential, usually ± 35 mV, particles tend to prevent coagulation via electrostatic repulsion [26]. As explained following Derjaguin–Landau–Verwey–Overbeek (DLVO) theory, when electrostatic repulses, driven by similarly charged electrical layers surrounding the particles, triumph over the attraction of the Van Der Waals interactions, particles become dispersible [26,27]. The zeta potential of GSNPs and rhSLPI-GSNPs is lower than +35 mV and
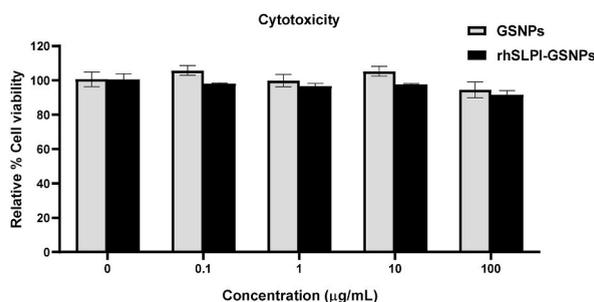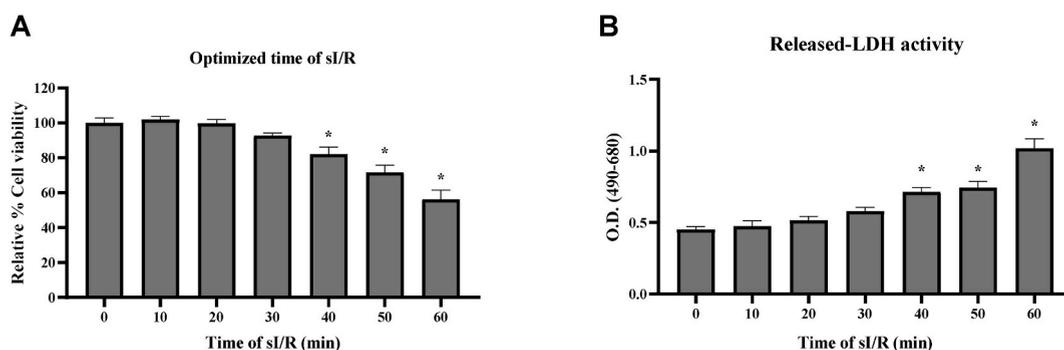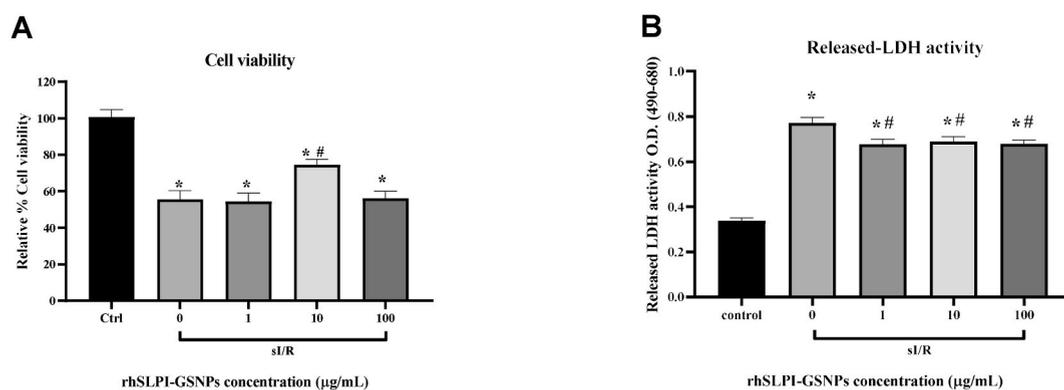

Fig. 4. The cytotoxicity test by determining the relative percentage of cell viability among nanoparticles treated groups (0.1, 1, 10, 100 μg/ml) vs. untreated control group. *$p < 0.05$ (ANOVA).

Fig. 5. Optimization of ischemic time for sI/R injury model. Determined cell viability by MTT viability assay (A), and cell injury by released LDH activity (B). *$p < 0.05$ vs untreated control (ANOVA).

Fig. 6. An *in vitro* cardiac protective effect of rhSLPI-GSNPs against sI/R in cardiac cells. Determined cell viability by MTT viability assay (A), and cell injury by released LDH activity (B). *$p < 0.05$ versus control group (ANOVA), #$p < 0.05$ versus sI/R groups (ANOVA).

Table 1
Comparison of gelatin-covered silica nanoparticles.

Nanoparticles	Drug loading	Size	PDI	Zeta potential	EE %	Releasing solution	Ref.
G-MSNs	doxorubicin	150 nm	–	−0.226 mV	–	pH	[17]
G-MSNs	pilocarpine	50 nm	–	−9.5 mV	–	MMP-2	[28]
GSNPs	rhSLPI	300 nm	0.47	−22.4 mV	3.56	collagenase	This study

higher than −35 mV, resulting, in particles occurring the aggregation, which had made increased the PDI value. Further study on stability of rhSLPI-GSNPs during storage, in different types of physiological buffer and different duration of storage, need to be performed by determining both of physical characteristics, qualification of rhSLPI as well as the physiological effects. SLPI, which is coated on the surface of NPs without a gelatin coating, is not effectively protected when delivered in circulation since protease enzymes are likely to destroy. Another layer can be shielded from destruction by being covered in gelatin.

In this study, we successfully encapsulated the rhSLPI, showing that rhSLPI-loaded nanoparticles (GSNPs) contain SLPI protein. Considering the concentration of rhSLPI used, the amount of rhSLPI loaded into the NPs was quite minimal, potentially because the volume of rhSLPI depends on the surface of the nanoparticle because rhSLPI can only bind to the surface of the core SNP that may be less loaded than the coreless nanoparticles. Another perspective point is the competitive between gelatin and rhSLPI c to bind the surface of SNPs, which may limit the binding capacity of rhSLPI.

The releases of rhSLPI in PBS solution are 1% from the beginning to 72 h due to the thick layer of gelatin, which covered the SNPs, could not be degraded in PBS due to the cross-linked process by glutaraldehyde. The result of rhSLPI release in PBS is similar to previous studies, which determined gelatin cover mesoporous silica nanoparticles (G-MSNs). The G-MSNs can release drugs with different pH concentrations (pH 7.4 is rarely released, and low pH is more released), indicating that covered gelatin is pH-dependent degrading [17]. However, low pH decreased the activity of SLPI. Thus, this releasing protocol seems unpractical in this study. We perform the alternative strategy for drug-releasing assay by using enzymatic digestion to assist degradation of gelatin, which could easily release the rhSLPI to the solution. In a previous study, breaking or digesting gelatin could facilitate drug or protein released. The rate of releasing is influenced by the concentration of enzyme. Gelatinase A was employed in earlier investigations to degrade the

gelatin layer [28], which is similar to our findings. The principle of glutaraldehyde property is generation of crosslinking at C-terminal region of protein. Therefore, in this study, it is possible that gelatin could be cross-linked with rhSLPI. Therefore, enzymatic digestion of gelatin, for drug releasing purpose, could possibly pulled rhSLPI together with the gelatin. Since the detection of released-rhSLPI was performed by ELISA, crosslinking form of rhSLPI and gelatin might not allow the antibody to detect the SLPI binding region, and therefore, reduce the amount of detected rhSLPI and subsequently results in low percentage of calculated encapsulation efficiency.

The nanoparticles generated from this study could provide a therapeutic effect without harming the cells, which reflect primary safety when implement in real clinical treatment. However, toxicity in an *in vivo* model still need to be performed to confirm the physiological toxicity.

The cardioprotective effect of rhSLPI has been reported for several years by our group. Our previous study demonstrated that giving recombinant human secretory leukocyte protease inhibitor (rhSLPI) by mean of overexpression of rhSLPI gene [4] or treatment with recombinant protein of human SLPI provided cytoprotective effect against I/R injury both in an *in vitro* [4,5], *ex vivo* [5], and *in vivo* [6] study model. Interestingly, although the beneficial effects of SLPI is believed to be due to the anti-protease activity, few studies showed that those effect of SLPI might be independent on its anti-protease activity [7,8]. Our recent unpublished data showed that recombinant protein of anti-protease deficient mutant SLPI (L72K, M73G, L74G) (mt-SLPI) also provided cardioprotection against I/R injury [21]. Recently, ectopic cardiac specific expression of slpi via Adeno-associated virus (AAV) serotype 9 delivery could also protect cardiac cell from I/R injury *in vivo* [9]. Therefore, the therapeutic potential of SLPI is believed to be in part by direct effect of SLPI.

An *in vitro* finding on therapeutic effect of rhSLPI showed that the effective concentration of rhSLPI that could provide an *in vitro* cardioprotection to cardiac cell (H9c2) under sI/R in both pre-treatment or during ischaemia with 1000 ng/ml of rhSLPI prior to sI/R significantly reduced the ischaemia-induced cell death [18]. However, in this study, treatment of 1, 10, and 100 μg/mL of nanoparticles suggest treatment of rhSLPI at concentrations of 0.23, 2.35, and 235 ng/mL, respectively (Fig. 6A). This amount of rhSLPI from NPs was found to be very different from the concentration of rhSLPI used in previous studies. The current study showed that 10 μg/mL of rhSLPI-GSNPs, which contain a rhSLPI concentration of 2.35 ng/mL (Fig. 6A), could sufficiently protect against cardiac cell injury and death in an *in vitro* sI/R injury. This concentration is much lower than the concentration of rhSLPI used in previous studies of 1000 ng/mL [18].

The rhSLPI-GSNPs of this study could be potentially used in several applications for improving strategic treatment. Percutaneous coronary intervention (PCI) is one of the revascularization techniques used to open clogged coronary arteries by using a stent, which helps keep the artery from narrowing or closing blood. Coating rhSLPI-GSNPs on the surface of a stent could be slightly released rhSLPI from nanoparticles to reduce disease severity of cardiovascular diseases and improve cardiac function. In addition, rhSLPI-GSNPs could be used as preservative agent of isolated vessel grafts, to prevent damage during transportation. We hope that this nanoparticle could be an alternative therapeutic agent for the treatment of IHD and other cardiovascular diseases.

Several points could be addressed as the limitations of the current study. Using scanning electron microscope (SEM) could not be able to identify between the surfaces of each nanoparticle. In order to conduct further research, I recommend the determination of the gelatin layer by using a transmission electron microscope (TEM) to identify the surfaces between each nanoparticle. In this study, intracellular uptake was not performed due to a lack of fluorescein-labeled peptides. In a previous report on intracellular uptake of SLPI, the SLPI protein is required to be conjugated with fluorescein, and subsequently purified the labeled-SLPI, which is then used for determining intracellular uptake.

The treatment of rhSLPI-GSNPs in the H9c2 cell is not directly connected to actual physiological conditions in the intact heart due to it does not exhibit the electrophysiological property. Therefore, treatment of rhSLPI-GSNPs in real models such as in an intact heart may provide more useful information similar to real physiological events in the heart. An experiment in a real model such as *ex vivo* and *in vivo* models might show the efficiency of the cardioprotective effect of rhSLPI-GSNPs against I/R injury such as reduction of infarct size, decreasing of ROS expression, reduction of inflammatory cytokines, as well as cardiac biomarkers such as cardiac troponin I, CK-MB, and myoglobin and improve cardiac function which is providing the therapeutic potential of rhSLPI. Thus, the more physiological cardiac models in an *ex vivo* and *in vivo* study of I/R injury, need to be intensively investigated, which could provide functional data for further clinical trials for therapeutic treatment.

5. Conclusion

This is the first study to demonstrate the successful of fabricating gelatin-covered silica nanoparticles (GSNPs) to encapsulate rhSLPI (rhSLPI-GSNPs), which could release rhSLPI in enzymatic digestion induce drug release manner. Moreover, this is also the first study on the cardioprotective effect of rhSLPI-GSNPs to protect cardiac cell from ischaemia/reperfusion injury, by reducing cardiac cells injury and death. This study provides useful information for rhSLPI-GSNPs in the cardioprotective effect. However, this method is required more investigation into the ischaemic heart disease treatment in terms of pre-clinical animal models.

Author contribution statement

Faprathan Pikwong: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper. </p>

Chayanisa Phutiyothin: Wannapat Chouyratchakarn: Performed the experiments; Analyzed and interpreted the data; Wrote the paper. </p>

Phornsawat Baipaywad: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper. </p>

Podsawee Mongkolpathumrat: Contributed reagents, materials, analysis tools or data; Wrote the paper. </p>

Sarawut Kumphune: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or

data; Wrote the paper. </p>

Data availability statement

Data will be made available on request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

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