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Research Article

Synthesis and Characterization of Chitosan-Saponin Nanoparticle for Application in Plasmid DNA Delivery

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Nonviral delivery system receives attention over the last decade. Chitosan (CS) is a cationic polymer whereas saponin (SP) is classified as glycoside. In this study, a spherically-shaped CS-SP nanoparticle was synthesized and characterized. The ability of the nanoparticle to protect DNA from enzymatic degradation, its thermostability and cytotoxicity were evaluated. The particle size was found below 100 nm as determined by Zetasizer, transmission electron microscopy (TEM), and field scanning electron microscopy (FSEM) results. The surface charge ranges from 43.7 mV to 38.5 mV before and after encapsulation with DNA plasmid, respectively. In terms of thermostability, Thermal Gravimetric Analysis (TGA) and Differential Scanning Calorimetry (DSC) revealed that CS-SP nanoparticle had a melting temperature of 110°C, with rapid decomposition occurring at 120°C. Encapsulation of DNA with the synthesized nanoparticle was evidenced by changes in the FTIR spectra including characteristic peaks at 3267.39 and 1635.58 cm^{-1}, wavenumbers. Additional peak was also observed at 1169.7 cm^{-1} following encapsulation. Electrophoretic mobility showed that CS-SP nanoparticle protected plasmid DNA from enzymatic degradation, while cell viability assays confirmed that the synthesized nanoparticle exhibited low cytotoxicity at different concentrations in avian cells. Taken together these, CS-SP nanoparticle showed potentials for applications as a DNA delivery system.

1. Introduction

DNA vaccines are getting considerable attention for use as alternatives in human and veterinary medicines. DNA vaccines are relatively safe and fast to produce and have the ability to stimulate the desired immune responses [1–3]. However, potential limitations, including degradation by endonuclease enzymes *in vivo*, effect of pH, and low immune response, have hindered their applications in field situation [1, 4, 5]. Thus, there is a growing need to improve their effectiveness in terms of delivery and sustained antigen release [6]. Recent advances in material sciences and nanotechnology allowed significant progress in the use of nonviral vectors for effective gene delivery [7]. Apart from their ability to carry large genes, nonviral vectors are less toxic, making them safe as compared to viral-vector systems [8, 9]. Several cationic polymers such as chitosan have been used as biodegradable nanomaterials for gene delivery [7]. Chitosan is a polycationic polysaccharide derived from crustacean as well as insects [10, 11]. On the other hand, saponin is a naturally occurring glycoside, with the ability to form stable soap-like foams in aqueous solutions. The amphiphilic nature of saponin compounds allows them to carry different antigens or protein for delivery into the cells and also serves as immunomodulators [12]. The aim of this study is to synthesize and characterize a chitosan-saponin (CS-SP) nanoparticle for application in plasmid DNA delivery.

2. Materials and Methods

2.1. Synthesis of Chitosan-Saponin Nanoparticles. A 1% chitosan (75%–85% deacetylated) solution (Sigma-Aldrich,

USA) was prepared by dissolving 0.1 g of chitosan in 10 mL distilled water containing 100 μL acetic acid (Fisher Scientific, Malaysia). The dissolved mixture was vigorously vortexed for 5 min, sonicated for 30 min, and centrifuged at low speed to remove air bubbles. The pH of the solution was adjusted to 5.5 by adding 0.1 M sodium hydroxide (Sigma-Aldrich, USA) while continuously stirring as described by Mao et al. [7]. Similarly, about 0.1 g of saponin (Sigma-Aldrich, USA) was dissolved in 10 mL of miliQ water (Merk Millipore, Germany) and filtered in 0.22 μm filter. To prepare a CS-SP nanoparticle, chitosan (0.03%) and saponin (0.001%) were mixed and stirred for 5 min at 500 rpm. The pH of the CS-SP mixture was further adjusted to 5.5 and then filtered through 0.22 μm syringe filter (Sartorius, Germany).

2.2. Preparation of Chitosan-Saponin Encapsulated DNA Plasmid. A bivalent IB DNA plasmid designated pBudCR88-S1/M41-S1 was encapsulated with a CS-SP nanoparticle to obtain pBudCR88-S1/M41-nanoparticle. Encapsulation was achieved by separate heating of the DNA plasmid as well as CS-SP mixtures at 55°C followed by immediate mixing of both plasmid DNA and CS-SP in a single tube and brief vortexing. Final mixture of CS-SP-DNA was obtained at a ratio of 3 : 1 : 1 respectively.

2.3. Characterization of Chitosan-Saponin Nanoparticle

2.3.1. Transmission Electron Microscopy. The morphology of the synthesized nanoparticle was evaluated by transmission electron microscopy (TEM) using Hitachi H-7100 (Hitachi, Japan). Briefly, a drop of nanoparticle suspension was placed on a parafilm and a carbon coated grid (Agar Scientific, UK) was floated on the nanoparticle drop and kept for 5 min, then, and fixed in 2% phosphotungstic acid (PTA) (Sigma, USA) for another 5 min. The grid was removed to blot excess liquid and dropped over a Whatman paper (GE Healthcare, UK) in a Petri dish. The grid was further dried in a desiccator and viewed under electron microscope (Hitachi, Japan) [13].

2.3.2. Field Scanning Electron Microscopy. Field scanning electron microscopy (FSEM) was used to determine the surface morphology of CS-SP nanoparticle. A drop of CS-SP nanoparticle suspension was placed on a curved aluminum foil and allowed to dry under a beam of light before coating with gold and subsequent viewing under FSEM (JEOL-7600F, USA).

2.3.3. Analysis of Zeta Size and Potentials. The prepared nanoparticle was subjected to zeta size and zeta potential measurements using a dynamic laser light scattering method in a Malvern zeta instrument 3000 (Malvern Instrument, UK) as described previously [7]. Briefly, about 100 μL of the sample was resuspended in 900 μL miliQ water and added to the cuvette. Measurement was carried out in a temperature of 25°C and a scattering angle of 90°C. Material dispersion and refractive indexes were set at 1.365 and 1.330 (viscosity (CP) 0.8872) while dielectric constant was 78.5, respectively [14].

2.3.4. Fourier Transform Infrared Spectroscopy. Fourier transform infrared spectroscopy (FTIR) spectra of the CS-SP encapsulated DNA were evaluated on Nicolet iS 50 FT-IR Spectrometer FTIR-Nexus (Thermo Fisher Scientific Inc., Waltham, MA, USA).

2.3.5. Thermal Gravimetric Analysis. Changes in the thermal properties of nanoparticle were determined using thermogravimetric analyzer, model TGA/SDTA85^e (Mettler Toledo, Switzerland). Briefly, about 200 μL of sample was placed in a previously tarred stainless-steel pan and heated from 25°C to 250°C at the rate of 10°C/min under nitrogen supply of 10 mL/min.

2.3.6. Differential Scanning Calorimetry. Differential scanning calorimetric analysis was carried out using DSC 823^e (Mettler Toledo, Switzerland). About 200 μL of the sample was heated from 25°C to 300°C at the rate of 10°C per minute under nitrogen gas carrier supplied at 10 mL/min.

2.3.7. Protection against Enzymatic Degradation. To determine whether CS-SP nanoparticle is able to protect plasmid DNA against enzymatic degradation, the CS-SP encapsulated DNA plasmids as well as naked DNA plasmids were digested with DNASE1 enzymes at 1 U/μg DNA. The CS-SP encapsulated DNA sample was also treated with chitosanase enzymes to serve as control. For DNASE1 digestion, samples were incubated at 37°C for 30 min following digestion and the reaction was terminated with a stop solution. All mixtures were analyzed for electrophoretic mobility in 0.8% agarose gel (FirstBase, Selangor, Malaysia) (BioRad, Hercules, CA, USA).

2.3.8. Cytotoxicity by MTT Assay. Cytotoxicity assay was carried out using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide solution (MTT) dye reduction reagent (Sigma-Aldrich, USA) as described previously [15]. A 96-well plate was seeded with DF1 cells at 10^5 cells/mL. The cells were grown in 50 μL, DMEM media supplemented with 10% fetal bovine serum. Cells were incubated at 37°C for 24 hrs under humidified 5% CO_2. When the cells reached 70–80% confluence, media were discarded and washed twice with 1X phosphate buffer saline (PBS) (pH 7.4). The cells were then treated with various concentrations of nanoparticle. Negative control cells were kept untreated; however, Dulbecco's Modified Eagle's Medium (DMEM) was added as mock-treatment. Both treated and control cells were further incubated overnight for 24 hrs under similar condition described in this section. After 24 hours of incubation, cells were washed twice with 1X PBS followed by addition of MTT reagent at a concentration of 0.2–0.5 mg/mL to each well. Further incubation was carried out for 3 hrs under 5% CO_2 condition. In order to stop the reaction, about 100 μL of dimethyl sulfoxide (DMSO) (Sigma-Aldrich) was added to each well for solubilization and the plates were incubated for 30 min before checking for absorbance at 570 nm in TECAN sunrise microplate reader (TECAN, Switzerland). Optical densities of treated cells were compared with that of negative control cells and percentage viability was determined

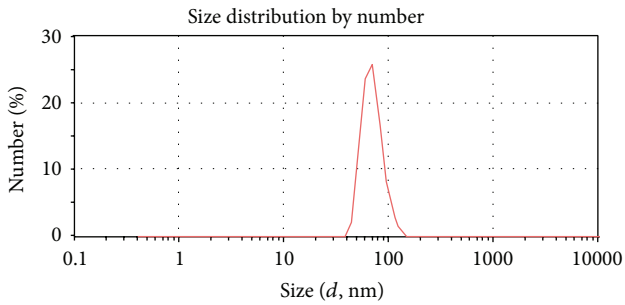


Figure 1: Size distribution of CS-SP nanoparticle as revealed by Malvern Zetasizer.

as absorbance of treated/absorbance of control × 100 and expressed in percentage.

3. Results

3.1. Synthesis of Chitosan-Saponin Nanoparticles. To obtain a nanoparticle suspension, various approaches such as vortexing, sonication, pH adjustment, and dilutions were carried out. Optimum size was achieved by premixing for 15–20 sec, followed by sonication for 15 min. The pH of the mixture was adjusted to 5.5 for maximum chitosan effects. A final mixture of the complexes nanoparticle was achieved by adding 300 μg (0.03%) chitosan, 100 μg saponin, and 100 μg plasmid DNA at a ratio of 3 : 1 : 1.

3.2. Analysis of Zeta Size. Since particle size is known to affect the interaction and uptake of a nanomaterial at the mucosa level [16], the size distribution of synthesized nanoparticle was evaluated using a Malvern Zetasizer. Mean nanoparticle size for CS-SP was found below 100 nm with a distribution of 70.67 nm (Figure 1).

3.3. Analysis of Zeta Potential. Determination of zeta potential was carried out to understand the charges as well as particle stability. Zeta potential for CS-SP nanoparticle was found to be 43.7 mV (Figure 2(a)). However, the particle surface charge decreases to 38.5 mV when negatively charged DNA plasmid was added into the mixture (Figure 2(b)).

3.4. Analysis of Nanoparticle Size by TEM. Results from TEM analysis revealed spherically shaped CS-SP nanoparticle (Figures 3(a) and 3(b)) with sizes ranging from 33.72 nm to 54 nm before complexion (a) and up to 79.37 nm after complexion (b), thus supporting the Zetasizer measurement result. The prepared nanoparticle was relatively homogenous in nature though some particles tend to agglomerate after encapsulation with DNA plasmid (Figures 3(a) and 3(b)).

3.5. Morphological Analysis by SEM. As in TEM, analysis of surface morphology by FSEM revealed that the synthesised CS-SP nanoparticle was spherical in nature. This is similar to that observed under TEM (Figure 4).

3.6. Fourier Transform Infrared Spectroscopy. Fourier transform infrared spectroscopy showed presence of characteristic peaks at 3239.27, 1635.31, and 1279.03 cm^{-1} chitosan molecule (Figure 5(a)). In the case of saponin, major peaks appeared at 3250.97 and 1635.18 cm^{-1} (Figure 5(b)). There was a shift in the chitosan peaks after interaction with saponin molecule as evident by characteristic peaks at 3249.09 and 1279.95 cm^{-1} (Figure 5(c)). On the other hand, following complexion of CS-SP nanoparticle with plasmid DNA, peaks at 3249.04 and 1635.09 cm^{-1} shifted from lower frequency wavenumbers to a high frequency wavenumbers as 3267.39 and 1635.58 cm^{-1}, respectively. Additional peak was observed at position 1169.7 following encapsulation with DNA plasmid (Figure 5(d)).

3.7. Thermal Gravimetric Analysis. Chitosan-nanosaponin recorded a sharp degradation at a temperature of about 110°C, thus indicating its melting temperature. There was an observable weight loss occurring from a temperature of 135°C to 280°C leaving only about 2.3% remnant of the total product (Figures 6(a) and 6(b)).

3.8. Differential Scanning Calorimetry. The glass transition temperature of the CS-SP nanoparticle was found to be 55.78°C and its stability reached 120°C, thus suggesting the optimum thermal stability of the product as shown in its endothermic peak (Figure 6(b)).

3.9. Protection against Enzymatic Degradation. The protective efficacy of CS-SP against enzymatic degradation was evaluated following treatment of encapsulated DNA plasmids with DNASE1 enzyme. The result indicates complete protection of the CS-SP encapsulated DNA as evident by the absence of DNA mobility in lanes 3 and 5, respectively. In contrast, DNA mobility was evident in CS-SP encapsulated DNA treated with chitosanase enzymes which removes chitosan (lane 6). Naked DNA group treated with DNASE1 did not show evidence of DNA band since the enzymes already degraded the unprotected DNA plasmid (Figure 7).

3.10. Cytotoxicity Assay. Percentage cell viability was used to determine the toxicity of CS, SP and CS-SP nanoparticle in chicken secondary fibroblast cells (DF1). Various concentrations ranging from 2500 mg/mL to 0.008 mg/mL were used. Chitosan was shown to be less toxic having cell viability of more than 50% even at 500 mg/mL concentration. On the other hand saponin was only nontoxic at 4 mg/mL concentration. Combination of chitosan and saponin in the nanoparticle led to a drastic reduction in the toxicity of saponin; thus, treatment with chitosan-nanosaponin resulted in more than 50% cell viability even at a concentration of 100 mg/mL (Figure 8).

4. Discussion

There is a growing interest in the use of nonviral vector as alternatives in gene delivery [17]. In this study, a CS-SP nanoparticle was synthesized and characterized for possible use in plasmid DNA vaccine delivery. Preparation of the

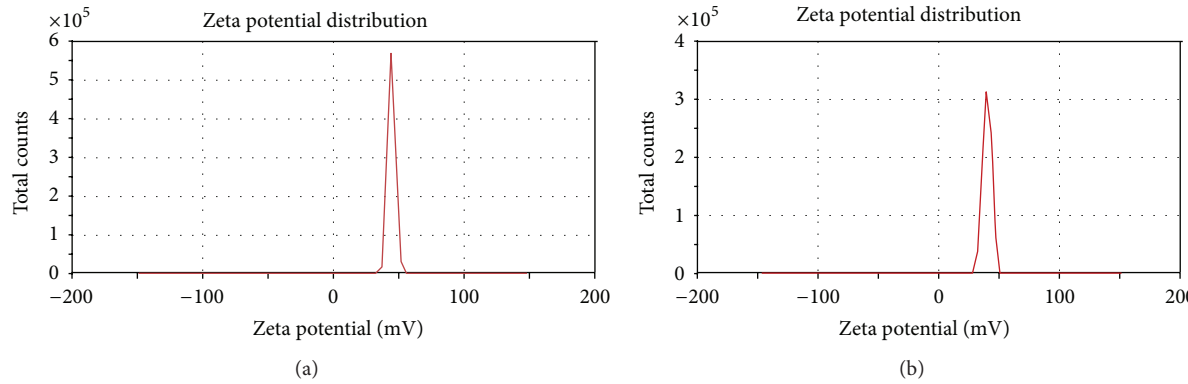


Figure 2: (a) Surface charges of chitosan-saponin nanoparticle before complexion with DNA. (b) Surface charges of CS-SP nanoparticle as determined by zeta potentials.

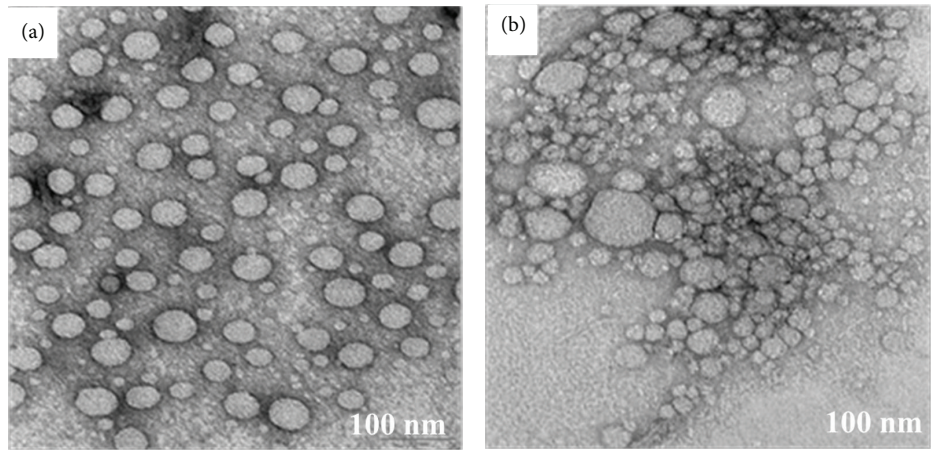


Figure 3: Transmission electron microscopy (TEM) showing a spherically shaped CS-SP nanoparticle before (a) and after (b) encapsulation with DNA plasmid. Bar = 100 nm.

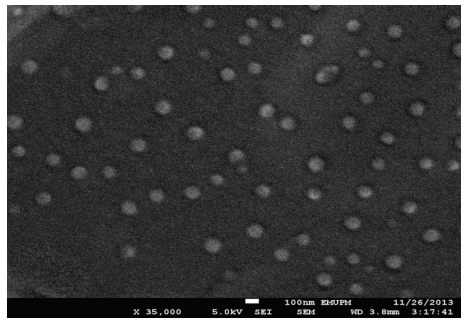


Figure 4: Surface morphology of CS-SP nanoparticle as revealed by field scanning electron microscopy (FSEM). Bar = 100 nm.

nanomaterial was achieved through adjustment of parameters such as pH as reported in previous studies [7, 15]. Apart from the ability of the chitosan to deliver vaccine antigen, the incorporated saponin has the advantage of acting as adjuvant or an immunomodulatory molecule to enhance immune response. This property was reported in saponin or ISCOM-based antigens [18–20]. Thus incorporation of saponin in this study might boost immune response in an *in vivo* system.

The synthesized nanoparticle was found within the nano range as evident by the zeta sizer result result and corroborated by TEM and SEM analyses. Interestingly, the size and particle morphology observed in this study are consistent with previous reports [15]. However, some studies reported large sizes of particle ranging from 170 to 580 nm. This variation could be as a result of the preparation methods, pH, and nature of starting materials used to synthesize the particle [21, 22]. Studies have also shown than a smaller particle of less than 10 μm is usually phagocytized by antigen presenting cells such as macrophages and dendritic cells, thus favoring the presentation of vaccine antigen [23].

The overall surface charges for CS-SP nanoparticle remained high, despite addition of a negatively charged DNA containing phosphate group. The strong positive charges are likely associated with chitosan, which is known to possess high positive charges at a pH of about 5-6 due to of protonation of amino group NH_3^+ in the presence of acetic acid medium. Other studies reported strong positive surface

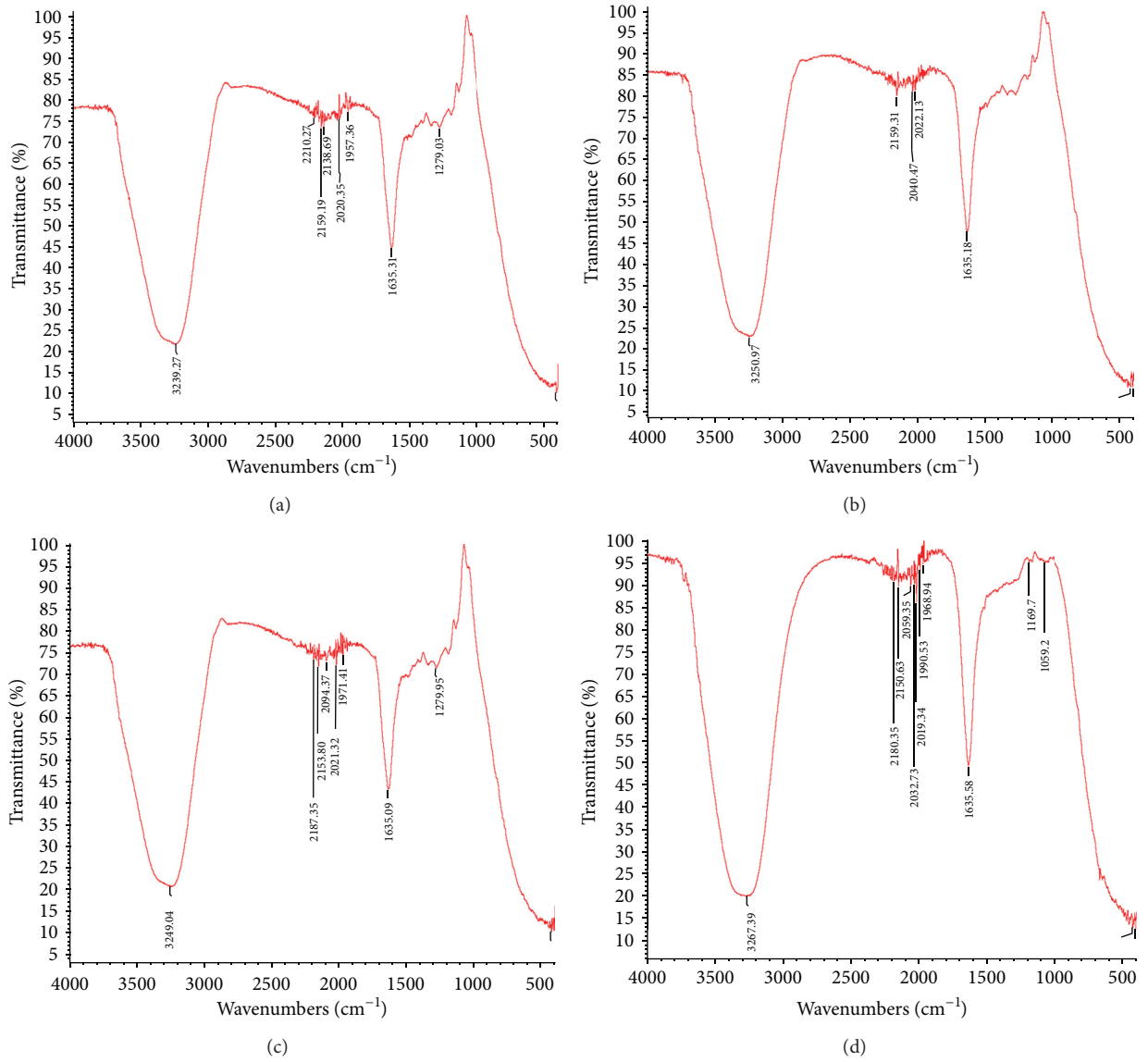


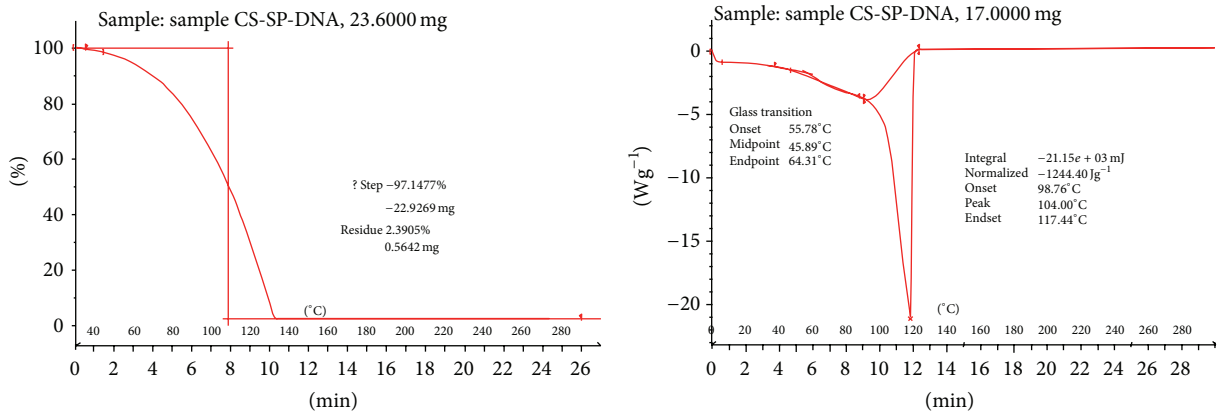
Figure 5: Fourier transform infrared spectra of chitosan (a), saponin (b), chitosan-saponin nanoparticle (c), and chitosan-saponin-DNA complexion (d).

charges in chitosan as well [24]. Adjustment of pH was similarly reported to affect the charges since NaOH may form the source of a hydroxyl group which may interact with protons (H+) of the amine group in chitosan, thus rendering it deprotonated [25].

Changes in the FTIR peaks observed at 3239.27, 1635.31, and 1279.03 cm^{-1} positions in the chitosan compound are likely associated with primary amine (-NH2), amide (-CONH2), and aromatic amine (C-N), respectively, present in chitosan. Similar result has been observed in other studies [15, 22]. On the other hand, major peaks occurring at 3250.97 and 1635.18 cm^{-1} in saponin compound might indicate a broad and strong stretching vibration resulting from hydrogen bonding (O-H) as well as a medium stretching due to secondary carbon (C=C) bonding, respectively [15].

Interaction of chitosan with saponin might have caused some peaks located at 3239.27 and 1279.03 cm^{-1} to shift from lower frequency wavenumbers to high frequency wavenumbers as indicated in 3249.09 and 1279.95 cm^{-1}, respectively. Similarly, peak at 1635.31 cm^{-1} was observed to shift from high to a lower frequency wavenumber as 1635.09 cm^{-1} in CS-SP nanoparticle. This may suggest hydrogen but not ionic interaction between the two compounds [15].

Increase in frequency of CS-SP peaks at 3267.39 and 1635.58 cm^{-1} in addition to the absence of signature peak at 1279.95 cm as well as appearance of new peak at position 1169.7 cm^{-1} also indicates interaction between CS-SP nanoparticle and the DNA compound. Evidently, the later peak indicates a stretching vibration probably due to phosphate group (P=O) and ester bonding in the DNA molecules.



(a) (b)

Figure 6: (a) Thermogravimetric analysis, curve showing melting temperature and % residue left following heating of chitosan-saponin nanoparticle. (b) Thermal stability of chitosan-saponin nanoparticle as revealed by differential scanning calorimetry.

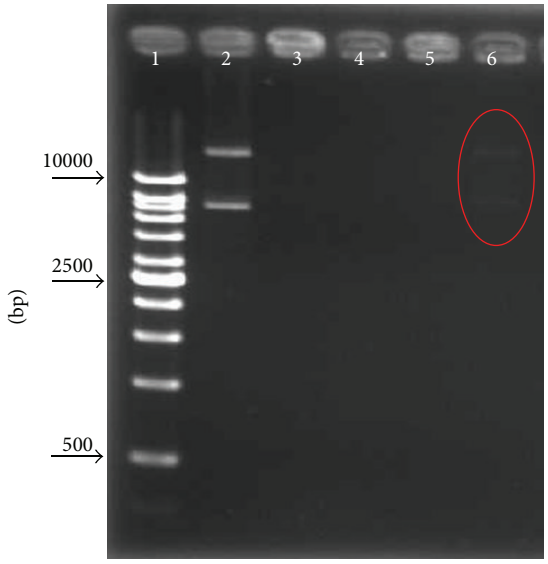


Figure 7: Protective capacity of CS-SP nanoparticle against enzymatic degradation of DNA plasmid. Lane 1: 1 kb ladder (Viventis, Malaysia); lane 2: naked untreated DNA; lane 3: CS-SP-DNA without enzymatic treatment; lane 4: naked DNA treated with DNASE1; lane 5: CS-DNA-SP treated with DNASE1; lane 6: CS-SP-DNA treated with chitosanase which removes the chitosan protection and exposes the DNA plasmid.

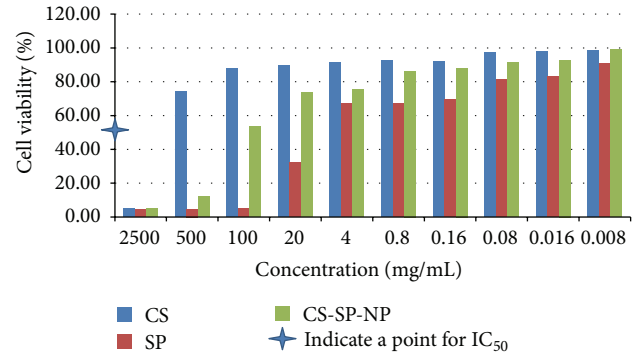


Figure 8: Determination of cell viability by MTT assay following treatment of DF1 cells with different concentrations of CS, SP, and CS-SP-DNA nanoparticle.

This may result from competitive displacement following encapsulation of the DNA [26].

In the thermal study, both TGA and DSC analyses confirmed that CS-SP DNA nanoparticle is thermostable, thus suggesting its potential application in plasmid vaccines used in a field situation. However, other studies have reported a much highly stable compound able to resist a temperature of 500°C [15]. The variation could be associated with disparity in water content between the two mixtures. It is expected that rapid weight loss may occur as a result of dehydration of the anhydrous glycosidic ring [27]. In addition, the use of tripolyphosphate (TPP) in Rejinold et al. [15] might have caused modification in the crystalline nature of both chitosan and saponin, thus making the two molecules more stable on heating. Other studies have also reported a good stability in either chitosan alone, as CS-SP, or in combination with other compounds [15, 21, 26].

Enzymatic degradation has been the major concern in plasmid DNA delivery [28]. In this study, encapsulation of plasmid DNA with CS-SP nanoparticle was shown to protect DNA from enzymatic degradation. This is thought to be majorly associated with a highly positive charged chitosan, surrounding both saponin and DNA molecules. In contrast, naked unprotected plasmid was shown to be degraded following enzymatic digestion. These findings agree with other reports demonstrating the ability of chitosan derived nanoparticle to protect DNA from DNASE1 enzyme *in vitro* [21, 29, 30].

In terms of toxicity, both CS and CS-SP nanoparticles were shown to be nontoxic at relatively high concentrations; in contrast, saponin was found to be toxic at a relatively high concentration. Several studies have reported the nontoxic

nature of chitosan in various cells, though most studies used a non-avian cell model [7, 21]. On the other hand, saponin alone or in combination with other molecules was reported to exhibit selective cytotoxicity in cancer cells and nontoxic at low concentration in normal cos-7, vero, L929, and NIH-3T3 cells [12, 15]. This suggests its addition as immunomodulator in a delivery system [22].

5. Conclusion

In this study, a CS-SP nanoparticle was synthesized and characterized. Based on the size, morphology, thermostability, protection against DNASE1 digestion, and cytotoxicity assays, it could be concluded that CS-SP nanoparticle has potentials for biological application as DNA delivery system.

Conflict of Interests

The authors of this study report no conflict of interests in the work.

Acknowledgments

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