ENCAPSULATION OF LEECH SALIVA EXTRACT (LSE) IN SODIUM ALGINATE (ALG) NANOPARTICLES USING ELECTROSPRAY TECHNIQUES

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Abstract: The use of nanotechnology in drug delivery could improve treatment by allowing targeted delivery and fewer adverse effects. The application of tailored nanomaterials as well as the development of delivery systems from nanoscale molecules are examples of nanotechnology drug delivery applications. Leeches have been chosen in this study as it has historically been shown to be a beneficial therapy for a variety of diseases, including war wound care. Leech saliva extract (LSE) contains complex mixture of different biologically and pharmacologically active substances which are beneficial for the mankind. **Objective:** This study was designed to encapsulate Leech Saliva Extract (LSE) in nanoparticles and to evaluate the effect of process parameters on the nanoparticle size, zeta potential, encapsulation efficiency and in vitro release profile. **Methodology:** Electrospray technique was used to fabricate the nanoparticles. The process parameters, namely applied voltage and flow rate were adjusted. The fabricated nanoparticles undergone characterization to evaluate how the process parameters affect the particle size, zeta potential, encapsulation efficiency and in vitro release profile. **Results and discussion:** LSE ALG nanoparticles have shown mean particle size and zeta potential of 205 nm and -7.32 mV respectively. Encapsulation efficiency (EE) of over 87% was obtained while maintaining payload integrity. In vitro release profile showed initial burst drug release (42%) was observed up to 6 hour and a constant slow LSE release was observed up to 48 hour. **Conclusion:** Adjustment of electrospray process parameters yielded optimum result of standard nanoparticles. LSE-loaded in alginate nanoparticles were successfully prepared using one-step-method.

Keywords: encapsulation; leech saliva extract (LSE); alginate; nanoparticles

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INTRODUCTION

A drug delivery system (DDS), is described as a formulant or device to introduce a medicinal material into the body by regulating the rate, timing and location of the release of medicines into the body, improving its effectiveness and safety [1]. In recent times, there has been a substantial increase in atomic, molecular, and macromolecular research and technological development, resulting in the controlled manipulation and study of structural ranges varying from 1 to 100 nm. The class of nanoparticle-based drug delivery systems is defined as colloidal drug delivery systems that, in terms of features and delivery mechanism, function as a whole unit. These are used to improve medication absorption and distribution in the body's cells. Because of their larger surface area per weight than microparticles, nanoparticles have become the main source of changes in the properties of many traditional materials, allowing them to be more active drug carriers [2].

Polymeric nanoparticles are constructed by combining a range of components, including proteins, lipids, polysaccharides, and synthetic polymers, to create nanospheres or nanocapsules. Nanocapsules are vesicular systems in which a drug is entrapped in a cavity enclosed by a polymer membrane, while nanospheres are solid colloidal particles in which a polymer matrix may entrap, encapsulate, chemically bind, or absorb an active. They have a hydrophobic surface and mucoadhesive qualities. Lipophilic chemicals may be integrated into nanocapsules' oily cores or solubilized in

nanospheres, while hydrophilic molecules can be adsorbed on the surface [3].

Due to their stability, safety, large number of reactive groups, abundance in nature, and cheap cost, natural polymers are extensively utilized in the production of nanoparticles. Alginate is a biomaterial with many uses in biomedical research and engineering owing to its advantageous characteristics such as biocompatibility and ease of gelation. To date, alginate hydrogels have proven especially appealing in wound healing, drug administration, and tissue engineering applications because they maintain structural resemblance to extracellular matrices in tissues and may be modified to perform a variety of important functions [4].

Electrospray technique uses electrohydrodynamic (EHD) or also known as electro-fluid-dynamics (EFD). A high-voltage power source, a syringe filled with a precursor solution, often a polymer solution and fitted with a metallic needle, a syringe pump regulating the solution feeding rate, and a ground collector are all used. A stable Taylor cone is produced during electrospray, which is stabilized by liquid surface tension, electrostatic force, and gravity [5].

Leeches (Phylum: Annelida, Class: Hirudinea) are found all over the globe in a variety of environments, including freshwater, oceans, deserts, and oases [6]. From the dawn of civilization, leeches have been thought to have significant therapeutic uses in clinical medicine. Clinical use of leech was described in Pharaohs' art, Roman texts, and Arabic Islamic literature. Leeches were also employed for phlebotomy, inflammatory treatment, rheumatism, cosmetic surgery, and tissue transplantation in the 18th and 19th centuries [7]. In Malaysia, the local leech has the following taxonomic status: Order: Hirudinea; Species: Hirudinaria manillensis; Local name: Buffalo leech or “lintah kerbau”[8].

Leech Saliva Extract (LSE) has shown prominent activity as antithrombin agent, platelet adhesion inhibitor, inhibitor of von Willebrand factor and antibacterial agent [9]. So, it had been thought as a valuable ingredient that give a lot of good effects towards the consumer. However, there is no study yet to be done about encapsulating of LSE into nanoparticles. In this study, the technique of electrospray is used to encapsulate LSE into nanoparticles. The findings of the study will provide evidence of how the parameter of electrospray will affect nanoparticles’ characterization in terms of particle size, surface charge (zeta potential), encapsulation efficiency and *in vitro* profile release.

MATERIALS AND METHODS

The leech saliva extract from Buffalo Leeches were donated by Biopep (M) Sdn. Bhd. The sampling and extraction of LSE were done by the company. It was thawed and centrifuged to remove mucus using ultrasonic at Biopep (M) Sdn. Bhd. Sodium alginate (Sigma-Aldrich, USA), Calcium Chloride anhydrous, Phosphate Buffer saline, BCA Protein Assay Kit (Thermo Scientific), distilled water and other analytical grade chemicals were utilised in the experiment. The apparatus used were weighing boat, measuring beakers, laboratory spatula, borosilicate glass rod, volumetric flasks, micropipette, centrifuge tubes, disposable cuvettes, pharmaceutical refrigerator, ice box, vials, stopper, hotplate stirrer, magnetic stirrer bar, weighing balance, syringe, syringe needle, oven, camera, Splab02 peristaltic syringe precision pump (Baoding Shenchen Precision Pump Co., Ltd, China), AC-DC High Voltage Power Supply (Analog Technologies Inc, U.S.A), Tecan Nano Quant Infinite M200 Pro Microplate Reader (Tecan Group Ltd, Switzerland), Supra 22k High Speed Centrifuge (Hanil Scientific Inc, South Korea), Zetasizer Nano Zs (Malvern Instrument, UK).

The preparation of polymeric solution started with preparing sodium alginate (ALG) solution with 2% w/v concentration. Then, LSE was incorporated into it by mixing the solutions homogenously. Electrospray setup was made with adjusting parameter of applied voltage and flow rate.

An unsharpened nozzle tip with a length of 1.5 cm and outer diameter of gauge 28 was used as electrospray (ES) needle. The LSE-ALG solution were filled into 10mL disposable syringe. A peristaltic syringe pump was used to pump the solution to the ES needle, which was linked via flexible tube. A petri dish containing calcium chloride solution was placed at distance of 3 cm from the nozzle tip, as a collecting bin for the produced LSE-ALG nanoparticles. The needle was charged with negative potentials of 5 kV and 7 kV with flow rate of 100 μL/h and 300 μL/h respectively and vice versa. The sample was prepared according to Table 2.1.

Table 2.1 Preparation of sample with respective process parameters

Sample	Flow rate (μL/h)	Applied voltage (kV)
A	100	5
B	300	5
C	100	7
D	300	7

For characterization of the LSE-ALG nanoparticles, the particle size and polydispersity index (PdI) measurement were carried out on a Zetasizer Nano ZS (Malvern Instrument, UK). The sample was prepared by dispersing nanoparticles with water which enclosed the viscosity of 0.8872 cP as a dispersant. The system temperature was maintained during the process at 25° C.

The Zeta potential distribution was determined between Zeta potential (mV) versus intensity (kcps) by using Zetasizer Nano ZS (Malvern Instrument, UK). The sample was prepared by dispersing nanoparticles with water which exhibited the viscosity of 0.8872 cP and the dielectric constant of 78.5 as a dispersant.

The amount of LSE encapsulated in the nanoparticles was determined by using indirect method of determining encapsulation efficiency. Sample were taken from the electrospray collector and were centrifuged using a centrifuge at 7000 rpm at room temperature of 25°C for 20 min to remove the polymeric debris. The supernatant produced was analyzed by using Tecan Nano Quant Infinite M200 PRO Microplate Reader (Tecan Group Ltd, Switzerland).

The in-vitro release profile was determined by resuspending the LSE-ALG nanoparticles sample in phosphate buffered saline (PBS) pH 7.4 for the in-vitro release profile study. A quantity of 16.14 µg LSE ALG NPs was obtained from the gelling solution and rinsed with distilled water before centrifugation at 7000 rpm for 20 minutes. The sample were resuspended in 5 mL PBS and incubated at 37 ◦C. At predetermined time points of 1, 3, 6, 24 and 48 hours, the sample were centrifugated at 7000 rpm for 20 minutes, and the supernatant was collected and then subjected to microplate reader to quantify the released LSE. The collected NPs after centrifugation were resuspended and replaced with fresh PBS.

RESULTS AND DISCUSSION

Effect of process parameters on particle size

The formulation and electrospray process parameters were adjusted in order to obtain LSE ALG NPs with acceptable particle size. Particle size is a major element affecting nanoparticle biocompatibility and bioactivity. It is a significant aspect since it has a direct impact on formulation stability.

Table 3.1. Results of process parameters effects on particle size.

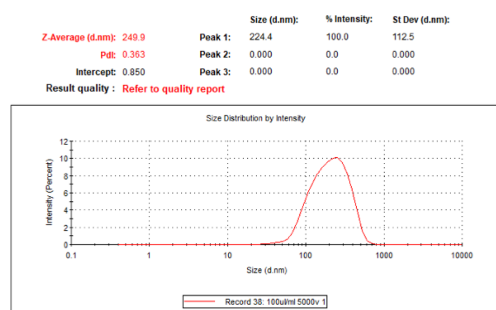
Sample	Flow rate (µL/h)	Applied voltage (kV)	Particle size (nm)	ANOVA	Post-hoc (Tukey)*
A	100	5	204.67 ± 19.73	$p < 0.05$	A & B
B	300	5	743.00 ± 145.33		B & A
C	100	7	276.33 ± 16.86		C & D
D	300	7	704.00 ± 41.33		D & C

a. One-Way ANOVA test

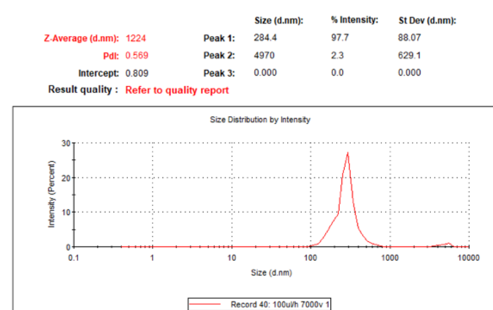
b. *pair wise showing the significant difference between the sample

The results of process parameters on particle size are presented in Table 3.1. The result of One-Way ANOVA test was significant (p< 0.05) to suggest that at least there was one pair among the sample were significantly different. From the results, the particle size decreased as the applied voltage rose. This finding ties well with previous studies of factors affecting size of Chitosan-Electrosprayed NPs [10]. The decrease in particle size was due to the fact that applying voltage to an electrosprayed solution generated charges at the nozzle tip. Only a few charges exist on the drop when the applied voltage is low. Thus, increasing the applied voltage causes a rise in charge, which causes coulombic repulsion forces to grow thus stretching the force on the jet segment. As a result, there is greater repulsion between adjacent droplets, thus LSE ALG nanoparticles with lower particle sizes are produced. However, increasing the flow rate resulted in a substantial increase in particle size, which is related to the droplet's polarisation time being shorter owing to quicker movement of the droplets toward the collector.

Aside from particle size, the Polydispersity Index (PdI) of the nanoparticles is an essential component in determining the dispersion of the nanoparticle size distribution. Most studies consider PDI levels of ≤ 0.3 to be optimal, however, values of ≤ 0.5 are also satisfactory [11].



[A]



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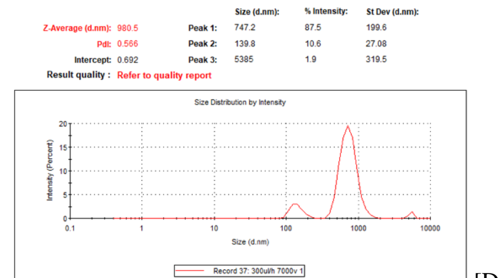

[D]

Effect of process parameters on Zeta potential

The presence of terminal carboxyl groups in the alginate polymer caused the zeta potential values for LSE ALG NPs formulation were negative. In the case of charged particles, when the zeta potential (positive or negatively charged) rises, the repulsive interaction becomes stronger, resulting in the production of more stable particles with a more uniform size distribution [12]. These findings suggest that the LSE was not adsorbed on the surface of the NPs. Zeta potential of the LSE ALG NPs was determined as a mean ± SD, n =3 as shown in Table 3.2.

Table 3.2: Results of process parameters effects on zeta potential

Sample	Flow rate (µL/h)	Applied voltage (kV)	Zeta potential (mV)	ANOVA	Post-hoc (Tukey)*
A	100	5	-7.32 ± 1.16	$p < 0.05$	A & C
B	300	5	-1.55 ± 0.46		B & A
C	100	7	-3.67 ± 0.28		C & A
D	300	7	-2.80 ± 0.14		-

a. One-Way ANOVA test
b. *pair wise showing the significant difference between the sample

The results of process parameters on zeta potential are presented in Table 3.2. The result of One-Way ANOVA test was significant (p< 0.05) to suggest that at least there was one pair among the sample were significantly different. Zeta potential has a direct impact on the colloidal structure's stability [13]. The LSE ALG solution droplets essentially had significant negative charges since the alginate chain was likewise negatively charged owing to the presence of hydroxyl and carboxyl groups in the alginate molecule's structure. The zeta potential (mV) of LSE ALG nanoparticles was found to be represented in Figure 3.2

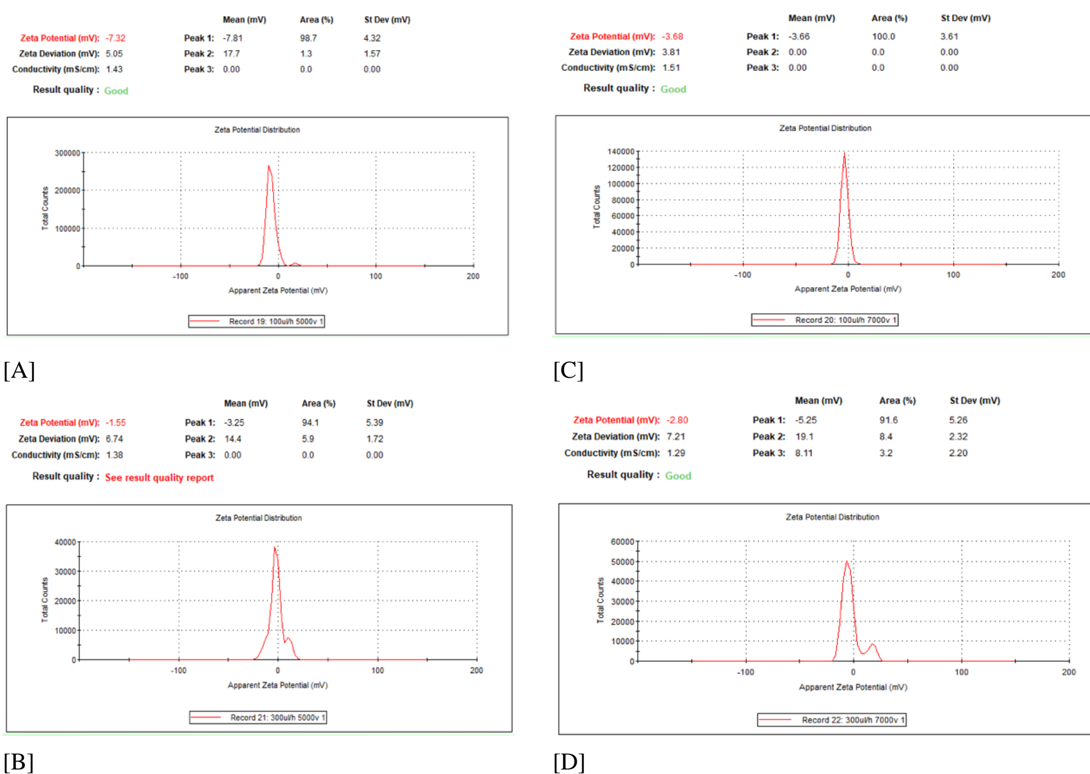

Figure 3.2: Effect of applied voltage and flow rate on the zeta potential of NPs [A] Flow rate: 100 µL/h & applied voltage: 5kV [B] Flow rate: 300 µL/h & applied voltage: 5kV [C] Flow rate: 100 µL/h & applied voltage: 7kV [D] Flow rate: 300 µL/h & applied voltage: 7kV.

Encapsulation efficiency (EE) of LSE ALG nanoparticles

Encapsulating hydrophilic compounds in alginate nanoparticles may be difficult owing to the encapsulated material's tendency to leak out into the gelling bath. Alginate nanoparticles often have a hydrogel structure with wide pores that allow the outflow of water-soluble tiny molecules from the particles. For instance, the EE of paracetamol in alginate beads quickly decreased from 33.92 ± 1.12% after 10 minutes of gelation to as low as 5 ± 1.32% after 30 minutes [14]. Similar results have been obtained [15] for macromolecules such as whey protein, where the EE generated was less than 60%.

Table 3.3. Results of process parameters effects on encapsulation efficiency

Sample	Flow rate (µL/h)	Applied voltage (kV)	Encapsulation efficiency (%)	ANOVA	Post-hoc (Tukey)*
A	100	5	38.18 ± 6.11	$p < 0.05$	A & C A & B
B	300	5	75.60 ± 15.53		B & A
C	100	7	79.37 ± 8.46		C & A
D	300	7	87.13 ± 6.99		-

a. One-Way ANOVA test
b. *pair wise showing the significant difference between the sample

The results of process parameters on encapsulation efficiency are presented in Table 3.3. The result of One-Way ANOVA test was significant ($p < 0.05$) to suggest that at least there was one pair among the sample with different process parameters were significantly different. The overall results showed that EE percentage increased with the increase in applied voltage and flow rate. This may be attributed to the high voltage, which could ramp up the affinity of cross-linking with CaCl2 gelling solution through a large number of sites for ionic cross-linking to entrap a large quantity of LSE and prevent LSE leaking from the polymer matrix. Astonishingly, Sample D with high applied voltage of 7 kV and flow rate of 300 µL/h demonstrated the best encapsulation efficiency of 87%.

In-vitro release studies

Figure 3.3 depicts the *in vitro* release data of LSE from ALG NPs during 48 hour timeframe and Figure 3.4 represents the detail of release during first 6 hour. LSE release behaviour from the polymer matrix followed a biphasic pattern, with an early burst followed by a slower sustained release. The release of LSE ALG NPs in Sample C formulation showed 100% at the end of 48th hours as depicted in Figure 3.3. An initial burst drug release (42%) was observed up to 6 hours in Figure 3.4 and a constant slow LSE release was observed up to 48 hour. The presence of surface drug on the surface area of the NPs may justify the first burst LSE release. LSE release was regulated by zero-order kinetics and a Fickian transport mechanism. LSE release was due to the polymer component, which had the impact of slowing drug release due to increased particle size and decreased surface area available for drug release. These findings clearly show that LSE ALG NPs exhibit prolonged release, which is needed to keep the therapeutic dosage stable.

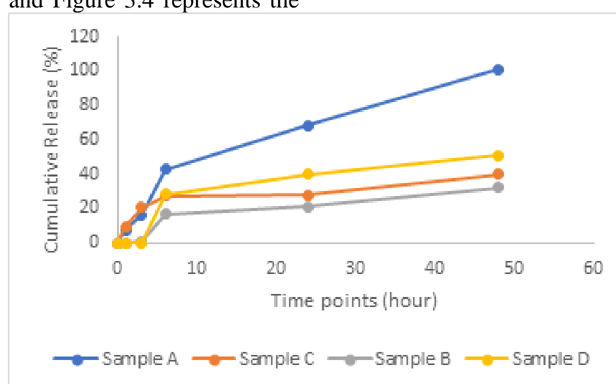

Figure 3.3 In vitro LSE cumulative release from LSE ALG NPs (mean ± SD; n=3) in phosphate buffered saline (PBS) (pH 7.4)

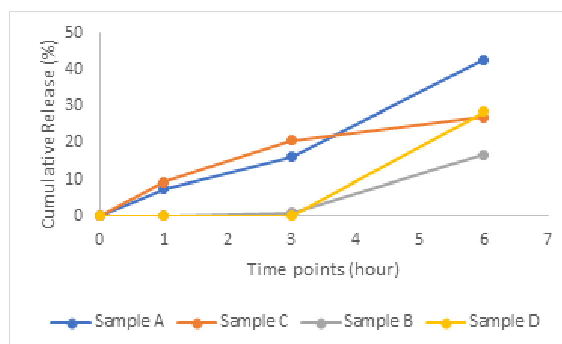

Figure 3.4. Detail of cumulative release during the first 6 hour

The drug release from alginate beads is dependent on dissolution media penetration into alginate beads, swelling and breakdown of the alginate hydrogel, and dissolution of the encapsulated drug after leakage through the swollen hydrogel [13]. In this context, the mechanism of alginate hydrogel swelling and dissolving includes diffusion (from the enlarged

pores of the hydrogel) and erosion (from the dissolved position) related to calcium depletion from the hydrogel. Alginate hydrogels may be dissolved by releasing calcium ions in the release media through exchange interactions with sodium ions. This transforms insoluble calcium alginate to a soluble salt of sodium alginate, causing the matrix to expand and dissolve, allowing LSE to be released.

CONCLUSION

The effect of process parameters of electrospray; applied voltage and flow rate on the particle size, zeta potential, encapsulation efficiency and *in vitro* release profile was studied. Different applied voltage (5 kV and 7kV) and flow rate (100 µL/h and 300 µL/h, respectively were chosen to fabricate the nanoparticles and yields optimum result of standard nanoparticles. LSE-loaded in alginate nanoparticles were successfully prepared using one-step-method; electrospray. This study shown that, in addition to flow rates and applied voltages, additional system parameters such as needle diameter, collector distance, solution concentrations, and conductivity must also be considered in order to achieve desired particle sizes. Further studies on Leech saliva extract (LSE) compatibility in nanoparticles are acquired to affirm its action and activity. Further in vivo investigation of the mechanism(s) of action and toxicity are also required before this protein or its constituents become a novel option to be used in the industry. Further research into these particles might aid clinical or industrial studies using alginate nanoparticles for biological purposes.

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