

Laser thrombolysis and *in vitro* study of Tissue Plasminogen Activators (tPA) Release Encapsulated by Chitosan Coated PLGA Nanoparticles for acute myocardial infarction (AMI)

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Abstract— The purpose of this study is to prepare cationic nanoparticles (NPs) by coating chitosan (CS) on the surface of PLGA NPs and evaluated laser thrombolysis capabilities and photomechanical drug delivery in a blood clot. The tPA encapsulated PLGA and PLGA/ CS nanoparticles were fabricated via the W/O/W double emulsion solvent evaporation surface coating method. The characteristics of NPs are determined by laser light scattering and zeta potential measurement. The CS coating was confirmed by zeta potential and fourier transform infrared spectroscopy. The surface morphology of NPs was also studied by transmission scanning microscopy (TEM). Differential scanning calorimetry was used for thermal analysis. *In vitro* drug release experiments of tPA encapsulated PLGA and PLGA/CS are determined by HPLC and showed a sustained release profile for three days with little initial burst release for PLGA/CS NPs. The mean particle size and encapsulation efficiency of tPA NPs were in the range of 280-360 nm and $46.7\% \pm 1.56$, $50.8\% \pm 1.09$, respectively. The encapsulation efficiency and the particles size were increased as a result of coating with CS. The release kinetics was evaluated by fitting the experimental data to standard release equations (Higuchie equation). This model was used to find the best fit for NPs. The results showed that the NPs for the highest weight percentages of digested clot is PLGA/CS NPs. Compared with tPA, the NPs significantly increase the weight of digested clots in the following order, PLGA/CS NPs (21.6%) > PLGA NPs (15.54%) > tPA (8.05%). Also, the thrombolysis process can be enhanced by delivering tPA into clot during laser ablation. In conclusion, laser thrombolysis and the NPs used in this experiment showed that they can significantly thrombolysis *in vitro* in this model, and may be useful for acute myocardial infarction (AMI).

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I. INTRODUCTION

THE Biodegradable nanoparticles (NPs) based on polyester such as PLGA (poly lactide-co-glycolide acid) or poly-(ϵ -caprolactone) (PCL), have attracted much attention as delivery systems for small molecules, DNA, peptides and proteins [1]-[3]. Developments in biotechnology have seen the growing use of proteins and peptides as therapeutic agents [4]. Significant advances in biotechnology have brought ever-increasing availability of recombinant peptide protein drugs in large quantities. Protein drugs carry out biological processes and reactions with high specificity and potency, so they will continue to be the choice for treating various diseases [5]. CS (chitosan) is an amino poly saccharide (poly 1,4-D-glucosamine) and it is insoluble in water and organic solvents, however, it is soluble in dilute aqueous acidic solution with $\text{pH} < 6.5$. Particle size, density, viscosity, degree of deacetylation, and molecular weight are important characteristics of CS which influence the properties of pharmaceutical formulations based on CS. Plasminogen activators (PAs) such as streptokinase (SK), urokinase, tPA (tissue plasminogen activators) have been administered effectively by intravenous infusion over a wide range of dosages. tPA is a protein involved in the breakdown of blood clots. Specifically, it is a serine protease found on endothelial cells, the cells that line the blood vessels. As an enzyme, it catalyzes the conversion of plasminogen to plasmin, the major enzyme responsible for clot breakdown. The clinical benefits of administering PAs for thrombolytic therapy may be markedly improved by developing new methods to promote clot lysis with reduced side effects. In this regard, a drug delivery strategy, such as encapsulating PAs (SK) into liposomes or polymeric microspheres as drug carriers to increase the therapeutic efficacy of conventional thrombolytic therapy has been demonstrated *in vitro* and *in vivo* animal models [6]-[9]. Many groups have developed methods for producing PLGA microspheres by dissolving polymers in a solvent and precipitating it into a sphere, e.g., using solvent evaporation, solvent removal, spray-drying or coacervation processes [10],[11]. The double- emulsion (water-in-oil-in-water), solvent evaporation/extraction method is one typical

method widely used for the preparation of PLGA microspheres loaded with hydrophilic drug such as therapeutic proteins.

Many parameters determine the drug release behavior from CS microspheres. These include concentration and molecular weight of the CS, the type and concentration of crosslinking agent, variables like stirring speed, type of oil, additives, crosslinking process used, drug CS ratio, etc.

Laser thrombolysis is an interventional procedure to remove thrombus in occluded arteries using laser energy. It offers cost, recovery time, and safety advantages over bypass surgery, in which surgeons must replace arteries but laser thrombolysis are limited because they cannot completely clear thrombotic occlusions in arteries, typically leaving residual thrombus on the walls of the artery. Therefore, using photomechanical drug delivery to enhance laser thrombolysis by delivering tPA into clot during laser thrombolysis [12].

A laser system capable of selectively targeting the thrombus is therefore desirable. This capability is offered by lasers emitting in the ultraviolet and visible regions, where the absorption by thrombus is much higher than that by artery. The principal chromophore of thrombus in the visible waveband is hemoglobin present in the red blood cells. Since higher absorption coefficients require less energy per unit area to achieve ablation, threshold for artery is higher than that for clot. Pulsed lasers operating in this waveband at radiant exposures between the thresholds for artery and clot can therefore selectively remove clot [13].

In this study, tPA-encapsulated PLGA and PLGA/CS were fabricated and drug delivery has been characterized in terms of particle size, thermal analysis, encapsulation efficiency, drug release profiles, weight loss (%) of clot, laser thrombolysis and photomechanical drug delivery. The release kinetics was evaluated by fitting the experimental data to Higuchi equation.

II. MATERIALS AND METHODS

2.1 PLGA-encapsulated tPA NPs

Nanoparticles were fabricated via the W/O/W double emulsion solvent evaporation surface coating method, as previously described [6]. Briefly, 3ml of de-ionized aqueous recombinant human tissue-type plasminogen activator solution (rtPA) (Actilyse, Boehringer Ingelheim Pharma KG, Germany) with albumin as an emulsifier were poured into dichloromethane solution (DCM) (Merck, Germany) containing PLGA (50:50, Resomer RG 504H, Mw 48000, Bohringer Ingelheim, Germany), and then emulsified using a probe ultra-sonicator (UP400S, hielscher, Germany) at 4°C to form an W/O emulsion. The W/O suspension at 4°C was added to 1wt% of polyvinyl alcohol (PVA; Mw 22000, Merck), and emulsified using the same sonicator in a pulse mode several times to produce W/O/W emulsion. 0.5wt% of PVA was added to the emulsion which was mechanically stirred. The suspension was evaporated at an ambient pressure to remove the solvent from the emulsified suspensions. The suspension that contained PLGA-encapsulated tPA NPs was centrifuged (Sigma, 3K30, RCF 25568, speed 16500 with rotor 12150H, Germany) at 4°C to separate the NPs from the suspension. Then, the NPs dried

at a freeze dryer (Chaist, Alpha 1-2 LD plus, Germany) for storage.

2.2 CS-coated PLGA-Encapsulated tPA NPs

To prepare the CS solution (low molecular, 80-85% deacetylation, Merck) for this work, CS was dissolved in 1% acetic acid solution and similarly PLGA NPs was followed, except that 0.1wt% CS solution and 0.5wt% PVA solution were added, instead of PVA 0.5% solution, to the aforementioned W/O/W emulsion with continuously stirring.

2.3 Measuring tPA Concentration using HPLC

Measuring tPA aqueous solution was analyzed using HPLC (BIO-TEK Kontorn Inst., Detector 535, Italy) equipped with a C₁₈ column at 37°C. The quantity of tPA was determined from the absorption intensity at the wavelength 254 nm. The same method was employed to determine the encapsulation efficiency (EE*) and release profiles of PLGA and PLGA/CS encapsulated tPA NPs.

2.4 Mathematical Analysis of the Drug Release

In order to study tPA release mechanism from the PLGA NPs and PLGA/CS NPs, the Higuchi equation which describes the Fickian diffusion of drug can be considered to fit the experimental data. Higuchi is the first to derive an equation to describe the release of a drug from a polymer as the square root of a time-dependent process based on Fickian diffusion (Eq. 1).

$$\frac{M_t}{M_0} = Q_t = \sqrt{2DS\varepsilon(A - 0.5S\varepsilon)} \times \sqrt{t} = K_H \sqrt{t} \quad (1)$$

Where, Q_t is the amount of drug released in time (t), D is the diffusion coefficient, S is the solubility of drug in the dissolution medium, ε is the porosity, A is the drug content per cubic centimeter of polymer, and K_H is the release rate constant for the Higuchi model.

2.5 Clot Preparation

Venous blood was repeatedly collected from a healthy volunteer and anticoagulated (by sodium citrate). Aliquots of 1ml of anticoagulated blood were calcified with calcium chloride. Tubes were incubated for 2 hr at 37°C in a water bath [14]. Clots were weighted just before and after each experimental. Thrombolysis was expressed as the relative reduction in clot weight (%). The thrombolysis was monitored using a digital camera with a CCD feature to observe the clot lysis. The weight percentage of digested clot is achieved by Eq. 2.

$$\text{weight of digested clot \%} = \frac{(\text{weight of clot at the start}) - (\text{weight of residual clot})}{(\text{weight of clot at the start})} \quad (2)$$

2.6 Nd:YAG laser

Drug delivery experiments were then performed by using a frequency-doubled Nd:YAG laser (wavelength 532 nm) (Top Rank-china) with 10 ns pulse duration.

As can be seen in Fig.1 the output of the laser was focused by a 500 μ m spot size on the clot surface. Clot removal measurements were made by exposing the sample to a predetermined number of pulses, n , at pulse repetition frequency of 2Hz and the weight of digested clot was measured before and after each experimental. The interaction process monitoring system included the time-resolved dynamics of laser- induced cavitations at different ablation stages .The first was done by a fast CCD Camera (Panasonic Super Dynamic WV-CP450) connected to an optical microscope (Prior-UK).

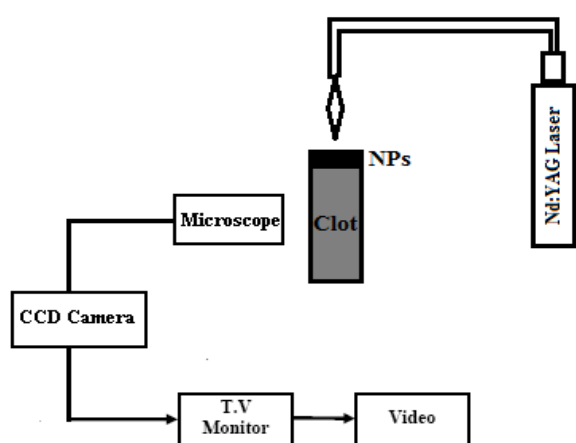


Fig 1. The experimental of set up

III. RESULTS AND DISCUSSION

The emulsion solvent method has been employed to fabricate hydrophilic agent and protein loaded PLGA micro and NPs for control release and drug delivery. In this study, the tPA encapsulated PLGA and PLGA/CS were fabricated by double emulsion solvent evaporation (W/O/W).

CS was used to coat PLGA NPs due to their cationic charge, biodegradability and mucoadhesive properties. In addition, CS coated PLGA NPs have been proposed for delivery of protein drugs. CS has been shown to possess mucoadhesive properties due to molecular attractive force formed by electrostatic interaction between positively charged CS and negatively charged surfaces. These properties may be attributed to: (1) strong hydrogen bonding groups like -OH, -COOH, (2) strong charges, (3) high molecular weight, (4) sufficient chain flexibility, and (5) surface energy properties. The electrostatic attraction is likely the predominant driving force especially in the formation of the first monomolecular adsorption layer [15]. The adsorption of CS continues even though a positively charged surface has been achieved, in which hydrogen bond (N-H) or van der waal's force can be involved. At high concentration of CS, it is possible that the subsequent layer of

CS could be adsorbed on the first layer and has no direct contact with the surface. CS was chosen as coating on the surface PLGA for encapsulation of tPA, mainly because of zeta potential of fibrinogen solution is -38.5 at pH of 7.4. Hence, it would be reasonable to postulate that the zeta potentials for fibrins in blood clots still maintain in negative charges that may facilitate the penetration of the aforementioned NPs into clots.

The reasons for encapsulating tPA as the thrombolytic drug are due to: i- the extremely short half life (<5min) renders the need for administering a high dose (1.25 mg/kg) of tPA. ii- due to administration of this high dose, clinical use of tPA is often associated with a high incidence of bleeding complication, and iii- in order to prevent restenosis for up to several months, prolonged attenuation of thrombus by utilizing a delivery carrier for thrombolytic drug has been suggested.

The spherical shape and the shell layer of the PLGA/CS NPs are shown by TEM micrographs (Fig. 2). TEM micrographs of tPA encapsulated PLGA and PLGA/CS NPs show that they were spherical with solid cores, and their diameters are in the rang 280-360 nm. The main component of the shell layer of NPs in Fig 2b is CS. This micrographs are shown size of the tPA encapsulated PLGA/CS NPs is larger than PLGA NPs (Table 1). The hydrodynamic diameter of CS coated PLGA NPs increases gradually with initial CS concentration. The increased particle size can be attributed to the increased viscosity of CS and the increased amount of adsorbed CS on the surface of PLGA NPs.

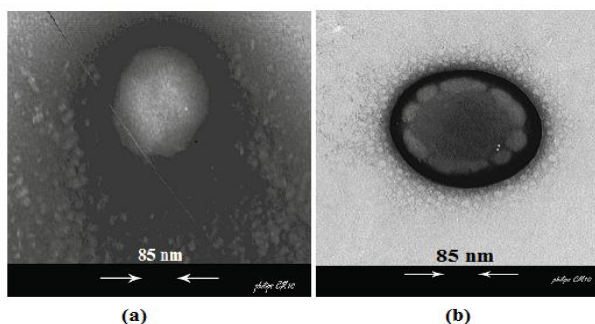


Fig 2. TEM micrographs of tPA encapsulated a) PLGA, and b) PLGA/CS NPs.

The positive zeta potential (Table 1) and FTIR spectroscopy of CS coated PLGA NPs confirmed the presence of CS on PLGA NPs (data not shown). The characteristic absorption bands of amine group (NH) and OH, CH, CN at CS at 3413, 2924, 2850 and 1629 cm^{-1} respectively were observed. The peaks at 1386, 1429, 1456, 1759 cm^{-1} and peaks around 3000 cm^{-1} are attributed to PLGA. The strong peak at 1759 cm^{-1} is due to the C=O stretch. The peaks at 3413 cm^{-1} corresponds to stretching and libational modes of hydroxyl.

Table 1. The particle size, zeta potential and encapsulation efficiency of the nanoparticles.

Sample	Size (nm)	Zeta potential (mv)	PDI	EE* (%)
PLGA NPs	282 ± 4.96	-8.92 ± 0.51	0.192	46.7 ± 1.56
PLGA/CS NPs	356 ± 2.94	+5.95 ± 0.17	0.334	50.8 ± 1.09

The data presented mean ± SD with n=3.

Fig. 3 illustrates the comparison between the Differential scanning calorimetry (DSC) curve for PLGA and PLGA/CS NPs and provides a qualitative and quantitative information about the physical state of drug in NPs. The pure PLGA exhibits an endothermic event (60 or 55 °C) referring to Tg. No melting point was observed, because PLGA appears amorphous in nature. The thermal decomposition, characterized by an endothermic event, has begun at approximately 355 °C. The DSC curve of CS shows an endothermic peak (160 °C) referring to Tg. The onset of thermal degradation of the CS is observed at 270 °C. The thermal degradation in nitrogen is exothermic, and corresponding is observed at 300 °C.

The DSC curve of PLGA NPs (Fig.3a) corresponds to Tg of PLGA (55 °C). The thermal decomposition of PLGA NPs begins approximately at 285 °C. Figure 3b shows two endothermic peaks of PLGA/CS NPs that correspond to Tg of PLGA (55 °C) and thermal degradation of the CS (275 °C). The melting point decreased with increasing CS content. The DSC curves of PLGA NPs and PLGA/CS NPs correspond to Tg of PLGA (55 °C). It can be observed that the nanoencapsulation process did not affect the polymer structure because the pure PLGA presented the same value for Tg. The DSC study did not detect any drug material in the NPs, i.e. the endothermic peak of tPA was not observed. Thus, it can be concluded that the drug incorporated into the NPs was in an amorphous polymer matrix.

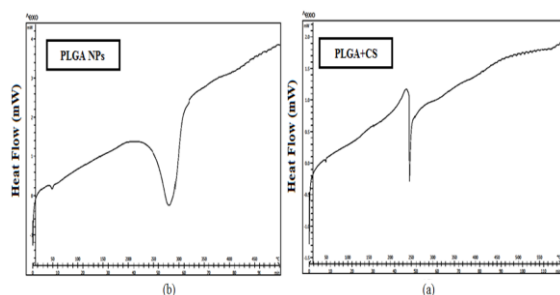


Fig 3. DSC curves for a) PLGA NPs, and b) PLGA/CS NPs.

The EE* of tPA encapsulated PLGA and PLGA/CS NPs are found to be 46.7% ± 1.56, 50.8% ± 1.09, respectively (Table 1). Effect of coating with CS on loading efficiency might be caused by an ionic interaction between tPA and CS and prevented leakage of tPA from emulsion droplet during evaporation process.

Release of the drug from NPs involves three different stages; the first stage is an initial burst followed by drug diffusion.

The second stage is governed by swelling of the polymer by inward diffusion of water during which the drug dissolved and can diffuse out. The third stage is characterized by the erosion phase, in which polymer degradation occurs. Initially, high release rate was observed due to the dissolution of surface adhered drug. The burst release of PLGA and PLGA/CS NPs were monitored during the first hour of release which accounted for 11% and 7.8 % respectively. This difference is explained by the fact that it takes some times for CS to degrade and let tPA to diffuse as well as higher hydrodynamic pressure is experienced in the opposite direction (i.e. acting as resistance) in the latter. However, after this short time some degradation and PBS uptake is taken place as a result of which the release trend gradually increases. After the initial release burst, it continued significantly for 2 days where 55% and 65% of tPA for PLGA and PLGA /CS was released respectively (Fig.4). Longer drug release time due to the diffusion process is much slower compared with the initial release. Several concurrent processes such as interactions between tPA and CS or between CS and PLGA, most probably influence the release of tPA. Therefore, the ratios of the different components in each particle type and the influence of the increased CS solubility in acidic media (pH=5) affect the release of the drug. The final stage was the release of drug for few days where the process was completed. The complete release of the drug from the NPs occurred only after a complete erosion or degradation of the NPs.

The release kinetics was evaluated by fitting the experimental data to standard release equation (Higuchi equation). From the analysis of the first phase (<10% of drug released) and the last phase (>65% of drug released), can be deduced that Higuchi square-root of time model ($R^2 > 0.99$) for PLGA/CS NPs (Table 2). The drug release mechanism is Fickian diffusion and The best fit was obtained for this model. The new NPs carrier for thrombolysis treatment may benefit many patients who are undergoing conventional thrombolytic therapy.

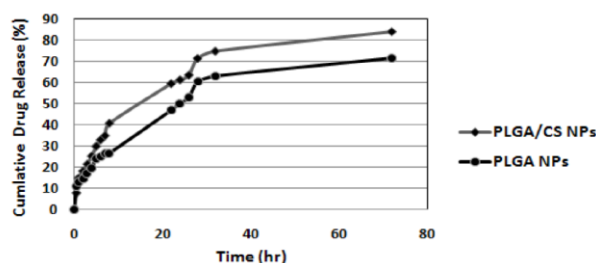


Fig 4. The cumulative release profiles of tPA encapsulated PLGA and PLGA/CS NPs.

Table 2. The release parameters calculated using by Higuchi equation.

Sample	Higuchi equation		
	R ²	K _H	K _{H theory}
PLGA NPs	0.9829	10.627	11.16
PLGA/CS NPs	0.9925	12.868	13.2

The thrombolysis of clots in an occlusive tube was examined by adding free tPA, PLGA NPs and PLGA/CS NPs into PBS solution. The digested clot obtained with PLGA/CS NPs was largest (21.6%) while that for free tPA was smallest (8.05%), suggesting the effect of the interactions between the CS and blood clots (Fig.5).

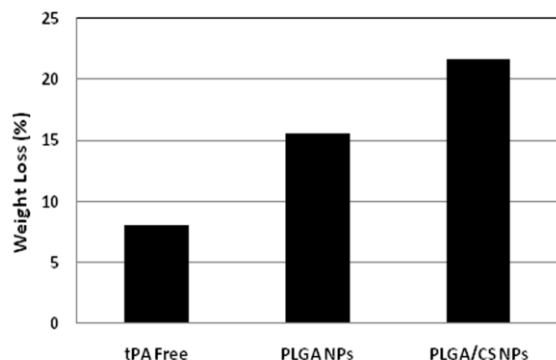


Fig 5. The weight loss percent and thrombolysis of clots after exposure to NPs.

Laser thrombolysis of clots by NPs was investigated at room temperature. Mean weight of clots before treatment was 100 mg in group free tPA , 299.3 mg in group PLGA NPs, and 316.2 mg in group PLGA/CS NPs. Weight reductions of clots in each group for 1h are summarized in Table 3. Laser thrombolysis obtained with PLGA/CS NPs was largest (33.2%). This demonstrated a frequency-doubled Nd:YAG laser can enhance delivering tPA into clot. The main operating mechanism with 532 laser is due to combined effects of photothermal because of vaporization and photomechanical (ie photoacoustical) effect. Most explanations have focused on cavitation-related processes such as microstreaming around the bubbles in stable cavitation, or microjetting in unstable cavitation, both of which can alter the structure of clots. In all cases, microbubbles may also increase the number of available binding sites for fibrinolytic enzyme molecules by stretching or damaging clot fibers. Efficient thrombolysis appears to be dependent upon transport of the tPA into clot, as well as adsorption or interaction with the fibrin substrate and it is a function of both diffusion and convection.

Table 3. laser thrombolysis of clot after exposure to NPs.

Group	Initial weight, mg	Final weight, mg (after treatment)	Weight loss, %
tPA	100	83	17
PLGANPs	299.3	206.1	31.13
PLGA/CS NPs	316.2	211	33.27

IV. CONCLUSION

The PLGA-encapsulated tPA NPs and the CS-coated PLGA-encapsulated tPA (PLGA/CS) NPs have been synthesized by the W/O/W method. The EE* and size of NPs was increased by coating with CS. The PLGA/CS NPs reduced the initial burst of tPA release. *In vitro* drug release experiments showed a sustained release profile for 3 days and the release kinetics was evaluated by fitting the experimental data to standard release equation (Higuchi equation).

PLGA/CS NPs successfully thrombolysis in a clot-occluded tube and they have the highest weight percentages of digested clots. Also, this study demonstrated the thrombolysis process can be enhanced by delivering tPA into clot during laser ablation. Therefore, this methods and PLGA/CS NPs may be employed in future clinical studies.

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