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Encapsulation of staphylokinase and *Leucasaspera* plant extracts using chitosan nanoparticles.

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Abstract: Encapsulation of the most potent combination of staphylokinase and *Leucasaspera* plant extract was done using chitosan nanoparticles. Chitosan is a biodegradable and non-toxic polysaccharide that can act as a drug carrier. Chitosan nanoparticles were prepared based on the ionic gelation of chitosan with tripolyphosphate(TPP) anions. The physicochemical properties of the nanoparticles were determined by atomic force microscopy (AFM), FTIR analysis and XRD.

Introduction

CVDs are projected to remain the single leading cause of death[1.] With over 3 million deaths owing to cardiovascular diseases every year, India is set to be the 'heart disease capital of the world' as it is estimated that by 2020 cardiovascular disease will be the cause of over 40 per cent deaths in India as compared to 24 per cent in 1990[2]. Thus, many thrombolytic agents are used to dissolve the fibrin of blood clots which are potentially fatal, especially those in the arteries of the heart and lungs. It is also used against the clots formed in shunts during kidney dialysis and multiple pulmonary emboli. Alteplase, anistreplase, streptokinase (SK), urokinase (UK) and tissue plasminogen activator (tPA) are some of the commonly used thrombolytics used for dissolving the blood clots[3]. In India, though SK and UK are widely used due to lower cost as compared to other thrombolytics but their use is often associated with numerous side effects like hemorrhage, allergic reactions, severe anaphylactic reaction and also they lack specificity[4]. Thus almost all the thrombolytic agents that are currently in use have significant side effects including bleeding tendency, limited fibrin specificity, and the requirement of large doses to be maximally effective and are highly expensive. Moreover, as a result of immunogenicity multiple treatments with SK in a given patient are restricted. This search has led to the exploration of the therapeutic properties of various medicinal plants that may act as thrombolytic agents. The main advantage of using such medicinal plants is that they are 100% natural[5.]

Leucasaspera is a common aromatic herb found as weed in Africa, Asia-temperate and Asia-tropical countries and is known to possess antifungal, antioxidant, antimicrobial and cytotoxic activity[6]. Therefore, to synthesize an effective thrombolytic drug with minimal side effects, combining the properties of conventional thrombolytic agents with natural plant extracts may yield a highly potent thrombolytic drug. Sustained-release properties of nanoparticles (NPs) have paved the way for a promising new therapy that delivers the thrombolytics using a nano carrier. Chitosan is a biodegradable polysaccharide that has been extensively examined in the pharmaceutical industry for the development of a drug delivery system. Nanoparticles are prepared via self-assembled chitosan and tripolyphosphate (TPP), an ionic linker and the thrombolytic agent when incorporated in this nanoparticle can for a highly effective thrombolytic therapy. This study aims in the synthesis and characterization of NPs produced from staphylokinase-*Leucasaspera* medicinal plant extract combination.

Materials & Methods

Chitosan preparation

Chitosan was prepared from shrimp cells using a method followed by Duttaetal[7]. Chitosan was first suspended in 4 % HCl at room temperature in the ratio of 1:14 (w/v) for 36 hours. This caused the demineralization of shells and thereafter they were washed with water to remove acid and calcium chloride. Deproteinization of shells was done by treating the demineralized shells with 5 % NaOH at 90 for 24 h with a solvent to solid ratio of 12:1 (w/v). After the incubation time the shells were washed to neutrality in running tap water and sun dried. The product obtained was chitin. Deacetylation of chitin was done to get chitosan. It involves the removal of acetyl groups from chitin and that was done by employing 70 % NaOH solution with a solid to solvent ratio of 1:14 (w/v) and incubated at room temperature for 72 h. stirring was must to obtain a homogenous reaction. The residue obtained after 72 h was washed with running tap water to neutrality and rinsed with deionised water. It was then filtered, sun dried and finely grinded to obtain chitosan.

Preparation of Cross linked Chitosan nanoparticles

CS-NPs were prepared by ionic gelation of CS with TPP anions. CS was dissolved in acetic aqueous solution at 3.0 mg/mL. Then a solution of TPP at 1.0 mg/mL was prepared with deionised water. Finally, 25 mL of the CS solution was added drop wise under constant stirring to 10 mL TPP solution. An opalescent suspension was formed spontaneously in the aforementioned conditions. Nanoparticles were separated by centrifuging at 13,000 rpm at 14°C for 30 minutes followed by discarding the supernatant. The pellet was washed with 20, 75 and 100% ethanol respectively and then it was freeze-dried and stored at 4–8◦C for further studies. The staphylokinase and staphylokinase- *Leucasaspera* plant extract loading nanoparticles were prepared with incorporation of CS solution into TPP solution containing 1mL of the enzyme and enzyme- plant extract combination.

Characterization of nanoparticles

XRD analysis

The prepared chitosan was characterized by X-Ray diffraction(XRD) technique using an X-ray diffractometer (Bruker Germany, D8 Advance, 2.2 KW Cu Anode, Ceramic X-ray) with CuKa radiation (k=1.5406 A˚). The measurement was in the scanning range of 5–70 at a scanning speed of 50 S^{-1}.

FTIR analysis

The samples of prepared chitosan were characterized in KBr pellets by using an infrared spectrophotometer in the range of 400–4,000 cm-1.

AFM analysis

The chitosan nanoparticles were visualized with an atomic force microscope (AFM). A thin film of the sample was prepared on a glass slide by dropping 2 drops of the sample on a clean glass slide and was allowed to dry for 5 minutes. The slides were then scanned with the AFM (Nanosurf AG, Switzerland, and Product: BT02089, v1.3R0). Nanosurf Easyscan-2 software was used for the AFM analysis.

Results & Discussion

XRD analysis of chitosan and chitosan nanoparticles

An X-Ray powder diffraction pattern of chitosan is shown in fig.1. There were strong characteristic peaks in the diffractogram of chitosan at (2h)=9.04°, 20.255° and 30.418° and many small peaks at different angles indicating the high degree of crystallinity of chitosan.. Thus, the XRD of chitosan was the characteristic of an amorphous polymer.

Fig.2 represents the X-Ray powder diffraction patterns of chitosan nanoparticles. Less significant peaks with very low intensity were found in this diffractogram of chitosan nanoparticles showing a dense network structure of interpenetrating polymer chains cross-linked to each other by TPP counter ions. Thus this diffractogram with lesser peaks showed formation of chitosan nanoparticles having strong interaction between chitosan and TPP counter ions.

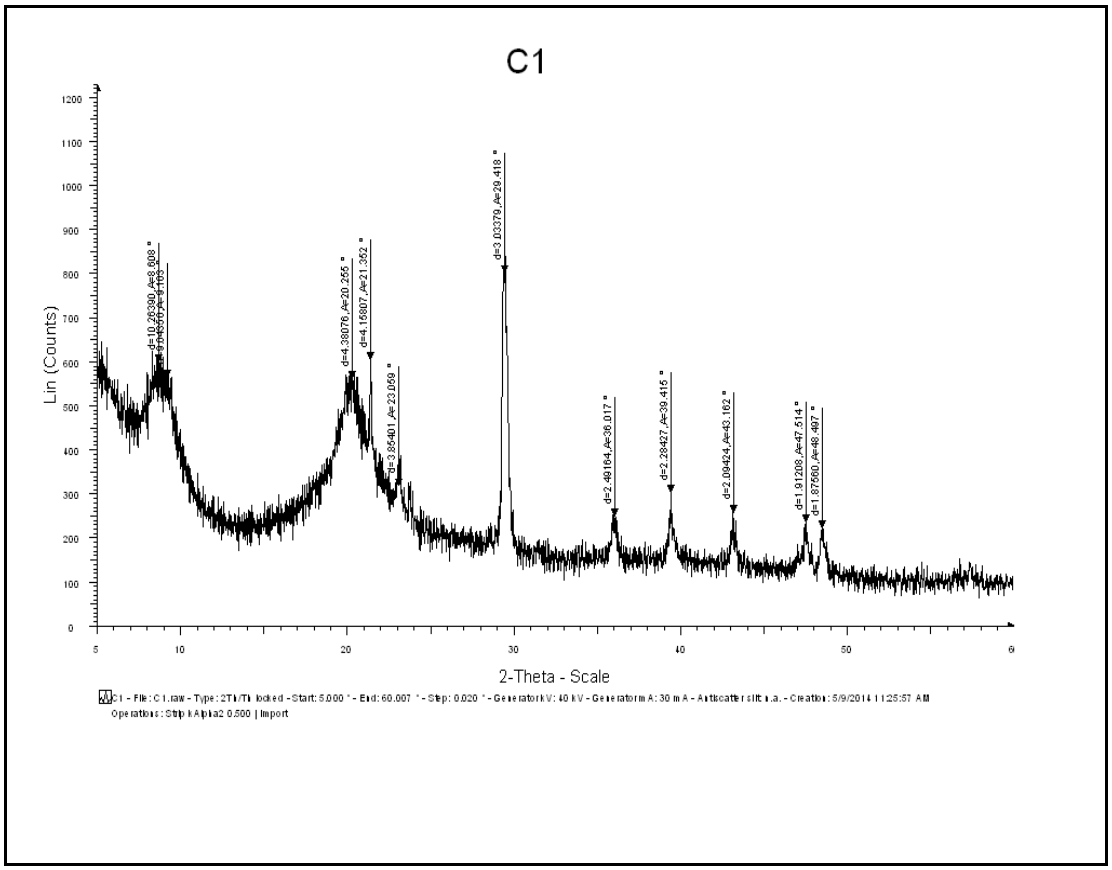


Fig.1. XRD of chitosan

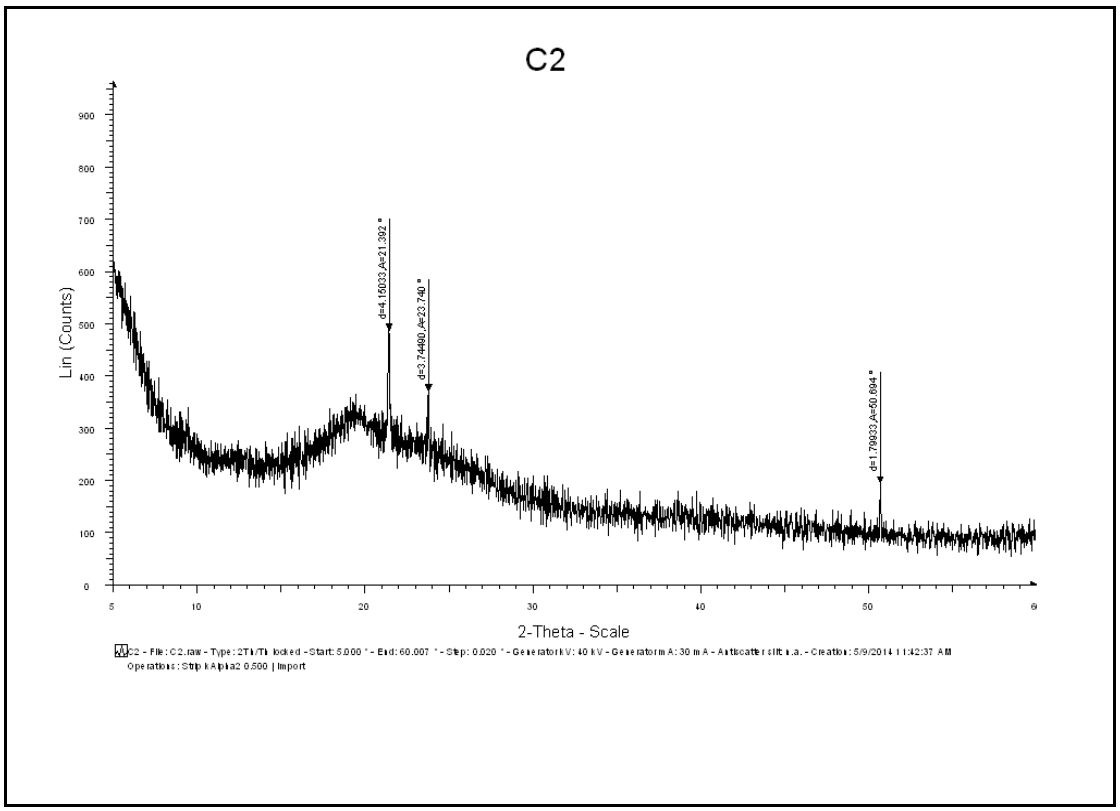


Fig.2. XRD of chitosan nanopaticles

The X-ray diffractograms obtained for chitosan and chitosan nanoparticles were showing similar results as were shown by previous experiments related to preparation of chitosan nanoparticles thereby confirming the orderly preparation and characterization of nanoparticles formed[8].

FTIR Analysis

IR spectrum of chitosan and chitosan nanoparticles

IR spectrum for chitosan polymer is represented in fig.3. An infrared spectrophotometer in the range of 400–4,000 cm-1 was used. The structure of chitosan was confirmed by FTIR analysis. The spectra of chitosan show the following absorption bands:

A broad absorption band in the region of 3,437.15 and 3128.54 cm-1 corresponded to the OH stretching vibrations of water and hydroxyls broadened by hydrogen bonding and NH stretching vibrations of free amino groups[9]. The band observed at 2,926.01 and 2,856.58 cm-1 corresponded to the asymmetric stretching of methylene C-H group (CH3 and CH2)[10]. The peak around 1,641.42 and 1627.92 cm-1 corresponds to bending vibration of secondary amines (NH2) which was also a characteristic feature of chitosan polysaccharide and also indicated the occurrence of deacetylation[9]. The narrow band at 1400.32 cm-1 corresponded to the OH bend of phenol and C–H bending and 1,066.64 cm^{-1} indicates C–O stretching[11]. The minor peaks from 873.75 cm-1 to 513.07cm-1 showed the presence of alkyl halide groups and C-H bends of alkynes.

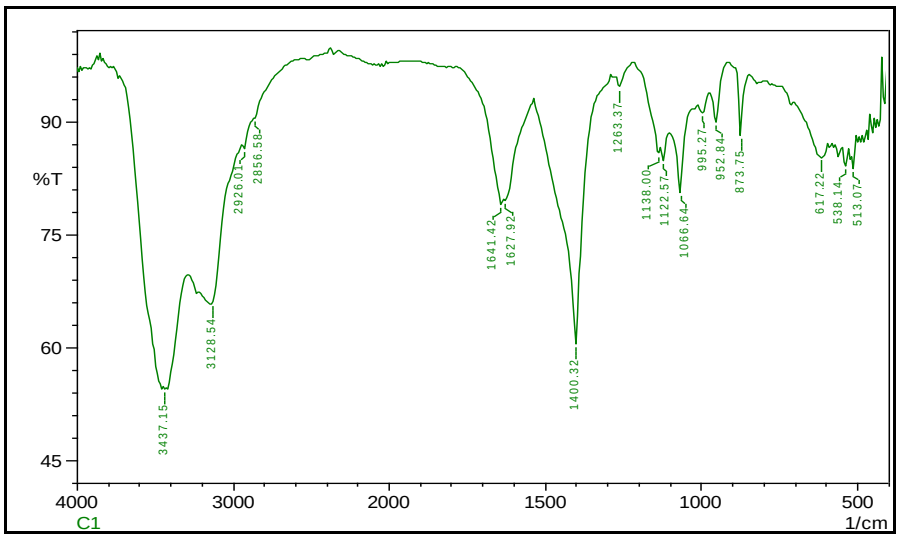


Fig.3. IR spectrum for chitosan polymer

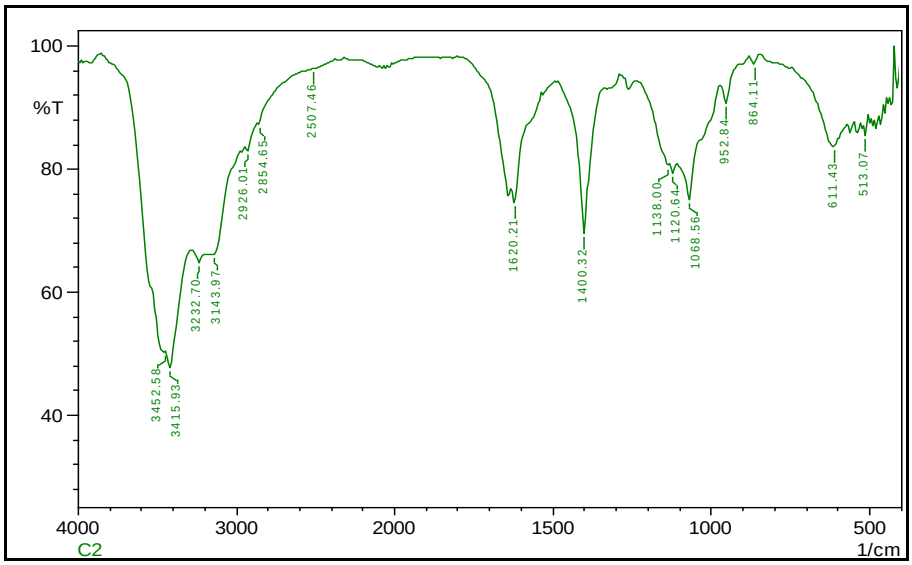


Fig.4. IR Spectrum of chitosan nanoparticles

Fig.4 represents the IR spectrum of chitosan nanoparticles. A shift from 3437.15 cm-1 to 3452.58 cm-1 showed that the peak was sharper in the chitosan nanoparticles, which indicated that the hydrogen bonding was enhanced. The intensity of secondary amine band at 1641.42 cm-1 in pure chitosan has decreased to 1620.21 cm-1 which showed that the ammonium groups were cross linked with tripolyphosphate (TPP) molecules.Thus it is postulated that polyphosphoric groups of sodium polyphosphate interacted with the ammonium groups of

chitosan, which served to enhance both inter and intra molecular interaction in chitosan nanoparticles[8].The peaks present in the right side of the spectrum from 1066.64 to 513.07 cm-1 in chitosan have decreased significantly in the chitosan nanoparticle depicting decrease in impurities present in chitosan and also strong bonding between TPP and chitosan.

IR spectrum of chitosan nanoparticles with staphylokinase

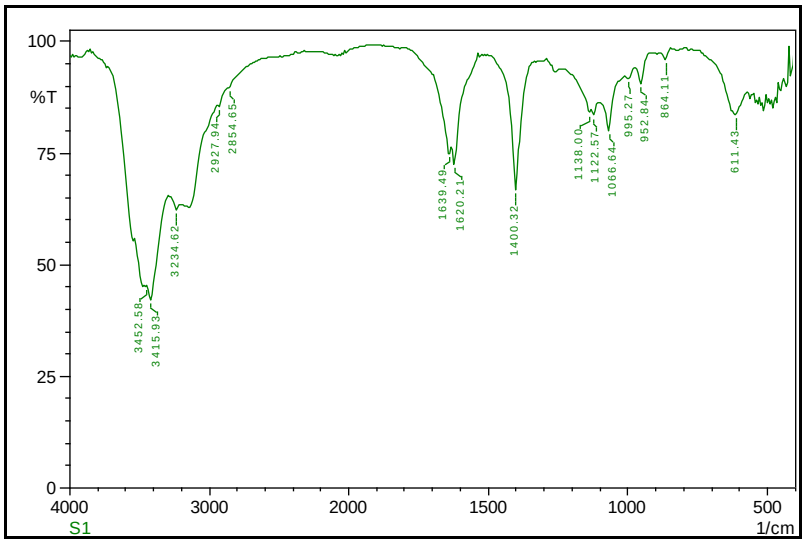


Fig.5. IR spectrum of chitosan nanoparticles with staphylokinase

Fig.5 represents the IR spectrum of chitosan nanoparticles coated with enzyme. A shift from 3437.15 cm-1 to 3452.58 cm-1 in the combination of staphylokinase with chitosan nanoparticle again corresponded that the peak has become sharper which indicates that the hydrogen bonding was enhanced. A small peak at 3234.64 cm-1 corresponded to the presence of C-H bond stretch. Also the intensity of secondary amine band at 1641.42 cm-1 in pure chitosan has decreased to 1620.21 cm-1 which showed more interaction between the nanoparticle and enzyme due to more crosslinking in ammonium groups. Thus it may be postulated that enzyme has properly adhered to the surface of the prepared chitosan nanoparticles.

IR spectrum of chitosan nanoparticles with staphylokinase and *Leucasaspera*plant extract

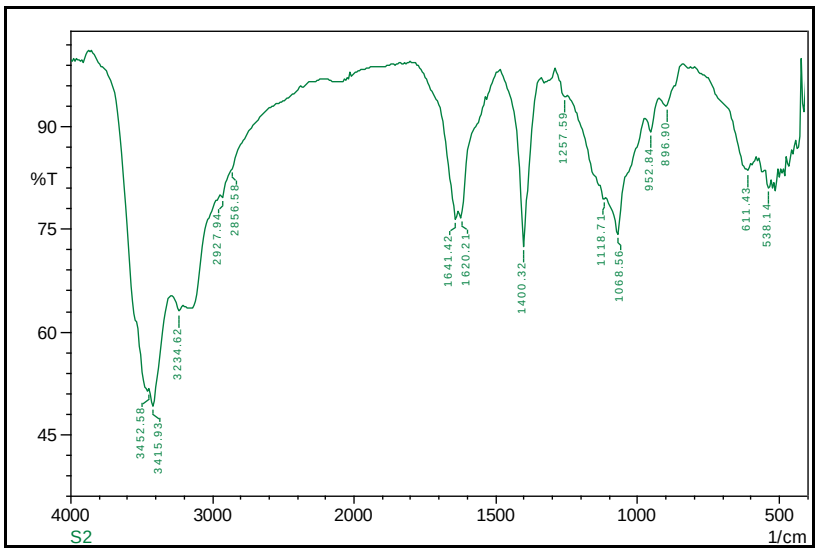


Fig.6. IR spectrum of chitosan nanoparticles with enzyme and plant extract

Fig.6 represents the IR spectrum of the combination of chitosan nanoparticles with enzyme and plant extract. The shift from 3437.15 cm-1 to 3452.58 cm-1 corresponded that the peak became sharper which indicated that the hydrogen bonding was enhanced. A small peak at 3234.64 cm-1 corresponded to the presence of C-H bond stretch. Also the intensity of secondary amine band at 1641.42 cm-1 in pure chitosan has decreased to 1620.21 cm-1 which showed more interaction of the nanoparticle with the staphylokinase and

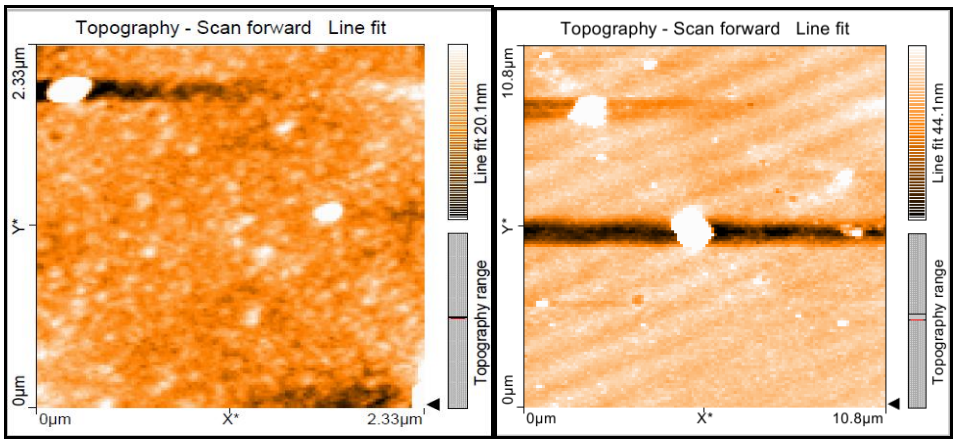
plant *Leucasaspera*. Also the peaks at 3415.93 cm−1 and1257.59cm−1 may correspond to a sharp peak for alcohols and phenols, indicating possible polyphenol encapsulation. The peaks at 1641.42 cm−1 may correspond to primary amines correlating to proteins. This may prove to an extent that polyphenols and proteins of the plant might be encapsulated agents[6].

The FTIR results obtained for chitosan and chitosan nanoparticles were similar to those obtained in previous experiments[8]. Preparation of chitosan nanoparticles has been done previously but with metals like copper forming chitosan-copper nanoparticles[11]. The results for chitosan nanoparticles with enzyme and chitosan nanoparticles with enzyme staphylokinase and extract of *Leucasaspera*were purely novel and there were no reports related to current study.

AFM analysis

AFM analysis of chitosan nanoparticles

The AFM micrograph of chitosan nanoparticles reported the formation of nanoparticles shown by white colored spheres having a non-homogenous surface. (fig.7) Thus the topographic image corresponded to the presence of non-uniformly synthesized chitosan nanoparticles.



Fig,7. AFM micrographs of chitosan nanoparticle

AFM analysis of chitosan nanoparticles with staphyokinase

The micrograph of chitosan nanoparticles with enzyme staphylokinase is represented in fig.8. The white colored spherical structures represented the chitosan nanoparticles formed coated with enzyme staphylokinase shown by transparent regions surrounding the nanoparticles in the micrograph. Thus the micrograph showed that the enzyme was unevenly coated over the nanoparticles.

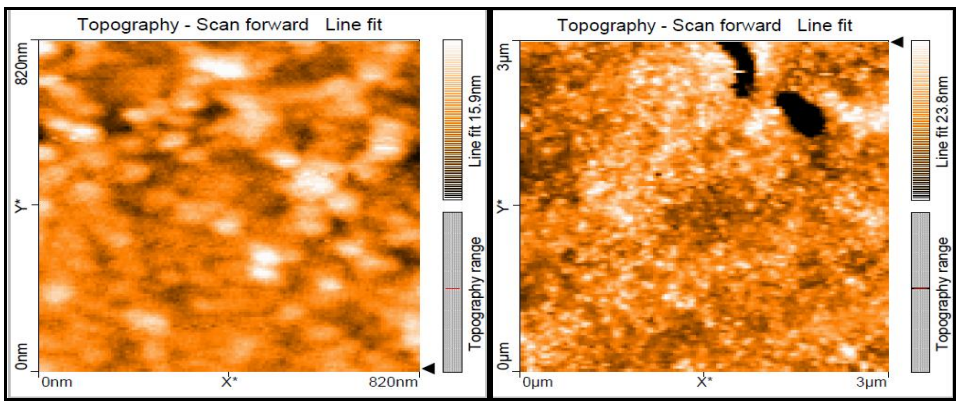


Fig.8. AFM micrographs of chitosan nanoparticles with staphylokinase

AFM analysis of chitosan nanoparticles with enzyme and plant extract

The AFM micrograph of chitosan nanoparticles with enzyme staphylokinase and plant extract of *Leucasaspera*is shown in fig.9. The white dots represented the chitosan nanoparticles formed coated with

enzyme staphylokinase and plant extract of *Leucasaspera*shown by black colored slightly transparent regions surrounding the chitosan nanoparticle in the micrograph. Thus the micrograph showed that the enzyme and plant extract were unevenly coated over the chitosan nanoparticles.Nanoparticles were synthesised using the enzyme-medicinal plant extract, it showed a highest activity i.e 50µL Staphylokinase + 50 µL methanol extract of *Leucasaspera*combination.

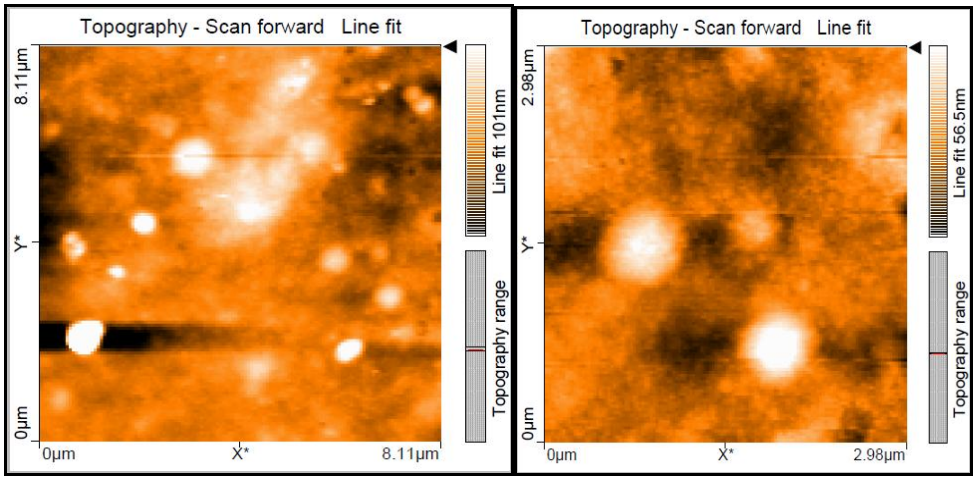


Fig.9. AFM micrographs of chitosan nanoparticles with enzyme and plant extract

The AFM results depicted the formation of non-homogenous chitosan nanoparticles similar to the nanoparticles synthesized for previous studies[8]. The micrographs obtained for combinations of chitosan nanoparticles with enzyme and chitosan nanoparticles with enzyme and extract are the new reports. Earlier studies were conducted only on metallic nanoparticles (silver nanoparticles) formed by plant extracts (*Pimentadioica*leaf extracts) [12], and chitosan-copper nanoparticles formed by microorganism strains of four species of bacteria, including two Gram-positive species, i.e., a pathogenic strain of MRSA (methicillin-resistant *S. aureus*) and *B. subtilis*, two Gram-negative species, i.e., *S. choleraesuis* and *P. aeruginosa*, and one yeast species (*C. albicans*)[11]. The most potent combination was found to be 50 µLstaphylokinase + 50 µL*Leucasaspera* extract and this was used for the preparation of chitosan nanoparticles with the motive of faster drug delivery. Nanoparticles were prepared using chitosan which is non-toxic and biodegradable making it safe for clinical applications. XRD and FTIR confirmed the formation of chitosan nanoparticles which were used to encapsulate the thrombolytic combination. This synergistic activity of a clot buster enzyme and a plant extract would represent a novel and effective *in vitro* thrombolytic therapy. Further studies are required regarding the physico-chemical properties of these nanoparticles like SEM, zeta potential, loading capacity etc. The synergistic activity was done with an aim to reduce the post treatment complications that arise in the present day thrombolytics without affecting the treatment efficiency. The plant extract-enzyme combination combines the efficiency of the enzyme and the safety of the plant extract and this principle could be used to find more potent thrombolytic therapies by combining different plant extracts and clot buster enzymes.

Conclusion

Presently the use of nano carriers is a highly researched and implemented area but not much has been done regarding the transport of thrombolytic drugs and so there is a huge potential to improve the drug delivery of thrombolytic drugs using nanoparticles as carriers. Thus this combinational therapy coupled with the nanoparticle approach has the potential to change the face of thrombolytics. Thus plant extracts provide a natural and safer alternative to the existing thrombolytic agents which are although quite efficient as clot busters but may lead to unwanted post treatment complications and also drug delivery and targeting may be improved by using nanoparticles as carriers of which chitosan nanoparticles are a non-toxic and effective option.

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