

TITANIUM NANOPARTICLES CONJUGATED WITH STREPTOKINASE AS A MODIFIED THROMBOLYTIC AGENT

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ABSTRACT

Objective: Blood clots are the main cause of death worldwide by stroke and myocardial infarction. Streptokinase a thrombolytic agent that is used in the treatment of circulatory disorders.

Methods: Titanium Nanoparticles was supplied from Changsha Santech Co. Its characterized were studied using (FT-IR, XRD, AFM, FE-SEM). Streptokinase at concentration 0.1 mg/ml was conjugated with Titanium nanoparticles using PH equal to 5.2 with continuous stirring. Formation of Streptokinase loading Titanium nanoparticles confirmed using FT-IR, Ninhydrine's test and Bradford protein assay. Physicochemical Properties were studied *in vitro*. Thrombolytic activity *in vitro* was determined using d-dimer indicator and weight of blood clot after treatment as indicators of thrombolytic activity.

Results: Titanium nanoparticles show particle size at range 31 nm. The thrombolytic activity of streptokinase loading Titanium nanoparticles shows significant value in d-dimer and weight of blood clot compared with the control group and non-significant compared with an equivalent amount of streptokinase alone.

Conclusion: Titanium nanoparticles conjugated with streptokinase show high thrombolytic activity against blood clots *in vitro*.

Keywords: Titanium, Nanoparticles, Streptokinase and Thrombolytic

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INTRODUCTION

Blood clots are the main cause of death worldwide by stroke and myocardial infarction. The aim of emergency medicine in such cases to dissolve the blood clots in the vessel as quickly and safely as possible and restore blood flowing. An essential requirement is an agent that target the blood clots and dissolve theirs without causing harms in blood vessels [1].

Streptokinase (SK), consider as a thrombolytic agent that used in the treatment of circulatory disorders like stroke and myocardial infarction. Streptokinase immunogenicity and short half-life of the enzyme make it has limited clinical uses [2, 3]. It has isoelectric point at range 4.7, in addition, consist of (414) amino acids and there are no disulphide bridges. Streptokinase converts plasminogen molecules to plasmin. Plasmin acts as a proteolytic agent and cleaves the insoluble fibrin polymer at a specific location then result insoluble fragments. A problems in streptokinase using in clinical applications are its immunology response as recognized and cleared by antibodies from the blood of the body [4].

Titanium nanoparticles (NpTiO₂) consider as a carrier material for different drugs, such as valproic acid, sodium phenytoin and daunorubicin. After an initial higher release rate, a nearly constant release rate can be obtained over a long period of time. These new agent delivery systems of Titanium nanoparticles allow high delivery efficiency, specific control of dose, long time periods such as days, weeks, or months [5, 6].

This study describes how titanium nanoparticles conjugated with streptokinase can offer an effective method to dissolve blood clots through increasing biological half-life of streptokinase, in addition, using nanoparticles as delivering system can increase acting of streptokinase drug to dissolve blood clots.

MATERIALS AND METHODS

Streptokinase was supplied from Sigma (USA) in concentration (250 KU). Titanium nanoparticles (NpTiO₂) supplied from Changsha Santech Co. (China) in particles size 30±5 and purity 99.8%. Its characterized was studied using the following test:

- 1- Fourier Transform Infrared Spectroscopy (FT-IR): using Bruker Tensor 27 FT-IR Spectrum System (Germany).
- 2- Atomic Force Microscopy (AFM) using Bruker BIOAFM instrument (Germany).
- 3- X-ray Diffractometers (XRD): using Philips X'Pert instrument (Holland).
- 4- Field Emission Scanning Electron Microscopy (FE-SEM) using TESCAN instrument (Czech Republic).

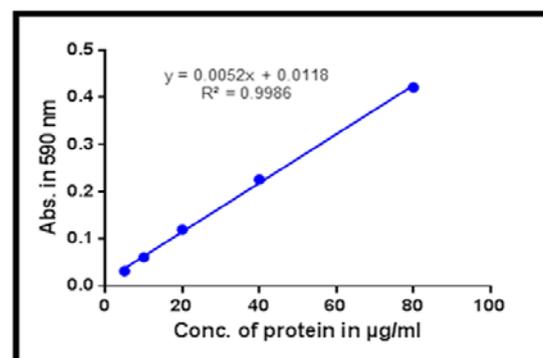


Fig. 1: Standard curve for a series of bovine albumin concentration using Bradford protein assay

Preparation of streptokinase loading titanium nanoparticles (NpTiO₂-SK)

0.1 mg of NpTiO₂ was dissolved in 1.0 ml of double distilled water (ddH₂O) in PH=5.2 (by added few drops of acetic acid in 0.1M and stirring for about 2 h). 100 µl Streptokinase in concentration 1.0 mg/ml was added to 0.1 mg/ml NpTiO₂ drop wise with stirring for about 4 hour in 4 °C [7, 8]. Then mixed were centrifuged in 10000 rpm [9], supernatant was removed and pellet washed three time with double distilled water than FT-IR analysis and Ninhydrine's test were carried out as qualitative tests whereas Bradford protein assay (Coomassie Brilliant Blue) were used to estimation concentration of streptokinase in nanoparticles as quantitative tests [10, 11] using series of bovine albumin in concentration from (5 µg/ml to 80 µg/ml) as in fig. (1) to confirmed streptokinase was loading in nanoparticles. The standard curve linear regression equation was gotten by using the GraphPad prism program (version 6), then the OD values of the samples streptokinase were applied to calculate the corresponding sample's concentration.

Physicochemical properties of streptokinase loading titanium nanoparticles

Entrapped efficiency (EE) and loading capacity (LC) were determined depending on the concentration of nanoparticles as in equation (1) and (2). Seven samples of nanoparticles having series different concentration (0.05 mg/ml) to (1 mg/ml). Streptokinase concentration was at range (1 mg/ml) and PH=5.2 [12]. streptokinase released from Titanium nanoparticles were studied using streptokinase loading nanoparticles that immersed in 5 ml of Phosphate buffer saline (PBS) at PH (7.4) then concentration of streptokinase in supernatant was determined in seven different interval times for 24 h [13] using Bradford protein assay. Cumulative streptokinase release was measured.

$$\text{Entrapped efficiency\%} = \frac{(\text{Total amount of SK} - \text{Amount of SK in supernatant})100}{\text{Total amount of SK}} \quad (1)$$

$$\text{Loading capacity\%} = \frac{(\text{Entrapped drug})100}{\text{Nanoparticles weight}} \quad (2)$$

Thrombolytic activity of streptokinase loading Titanium nanoparticles

Thrombolytic activity study was carried out *in vitro* depending on previous studies method of thrombolytic agent [14, 15]. 2.0 ml of blood samples were collected from human volunteer (N=10) and the

consent form was signed by all patients volunteer. These (2.0 ml) were divided and transferred in to four micro tubes (500 µl/tube) incubated at 37 °C for 45 min until blood clot is formation, than the blood clot in tubes are treated as following.

1. 100 µl of sterile distilled water was added to tube No.1 as a negative control
2. 100 µl of Titanium nanoparticles (NpTiO₂) in concentration 2.0 mg/ml was added to tube No.2 as positive control
3. 100 µl of streptokinase (SK) in concentration 29 mg/ml was added to tube No.3 as standard
4. 100 µl of streptokinase loading Titanium nanoparticles (NpTiO₂-SK) in concentration 0.2 mg/ml was added to tube No.4

Incubation at 37 °C for 120 min, D-dimer was measured using (D-dimer strep by using ichromate II instrument-Boditech-Korea) every 60 min (for two times) as indicator of thrombolytic agent activity [16, 17]. Then serum is removed completely, weight of blood clot in these samples were measured using sensitive balance depending on the following equation (4).

$$\text{Clot weight} = \text{weight of clot containing tube} - \text{weight of tube} \dots\dots (3)$$

Statistical analysis

Statistical analysis processes, fig. and Tables were carried out using GraphPad prism version (6) depending on ANOVA test method and T-test independent samples method.

RESULTS AND DISCUSSION

Titanium dioxide nanoparticles (NpTiO₂) are manufactured worldwide in large quantities for using in a wide range of medical and chemical applications. Titanium nanoparticles show different physicochemical characterizes [18]. FT-IR spectroscopy analysis of Titanium nanoparticles (NpTiO₂) in fig. (2) Show a broadband in (3400 cm⁻¹) revealed to the interaction of hydroxyl groups of water molecules with TiO₂ surfaces. The peak at range (1620 cm⁻¹) is revealed to bending vibration of hydroxyl groups, whereas, broad peaks at range (1650 cm⁻¹-500 cm⁻¹) refer to bending vibration of interaction (Ti-O-Ti) bond [19, 20].

Titanium nanoparticles (NpTiO₂) were studied using atomic force microscopy (AFM) that show in fig. (3) and (4). Atomic Force Microscopy (AFM) are emerging as fundamental tools to extremely investigate the morphology and structural properties at micro and sub-micrometric scale [20].

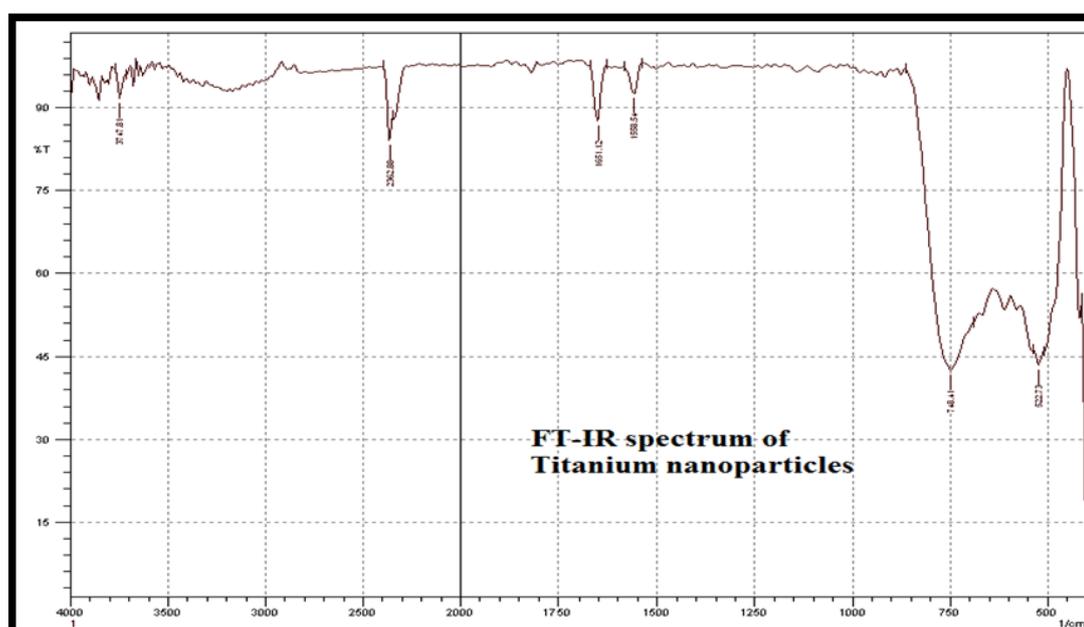


Fig. 2: FT-IR spectrum of titanium nanoparticles

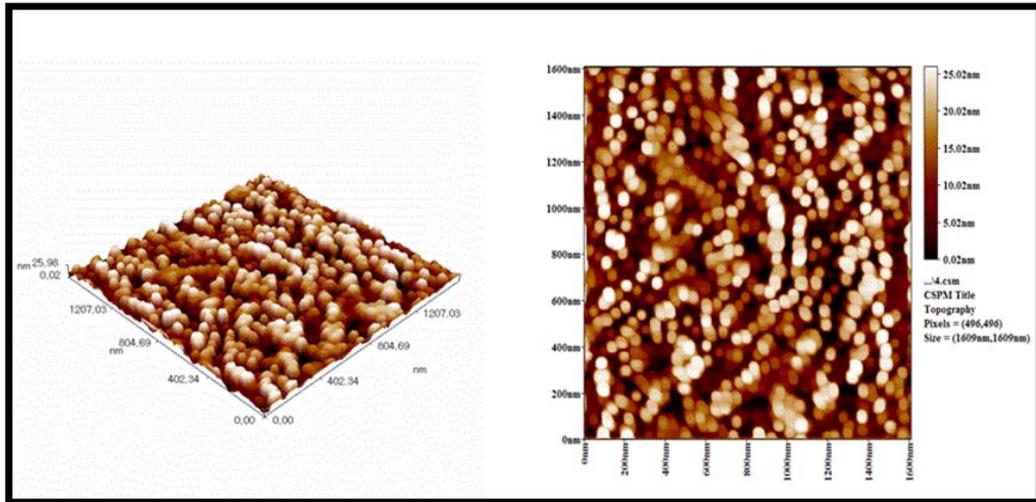


Fig. 3: Atomic force microscopy (AFM) picture of titanium nanoparticles

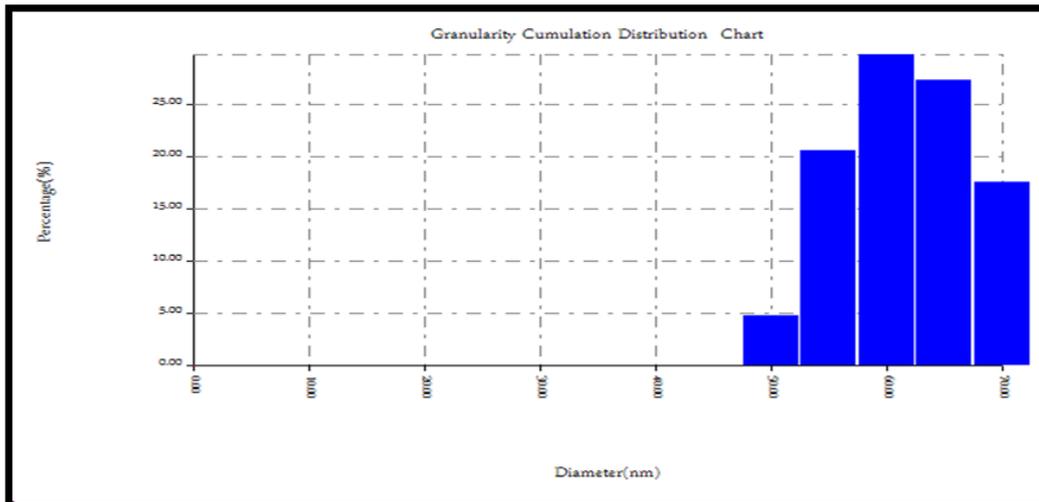


Fig. 4: Show granularity cumulating distribution of titanium nanoparticles by AFM analysis

Titanium nanoparticles were analysis by using Emission scanning Electron Microscopy (FE-SEM) in different resolutions as in fig. 5.

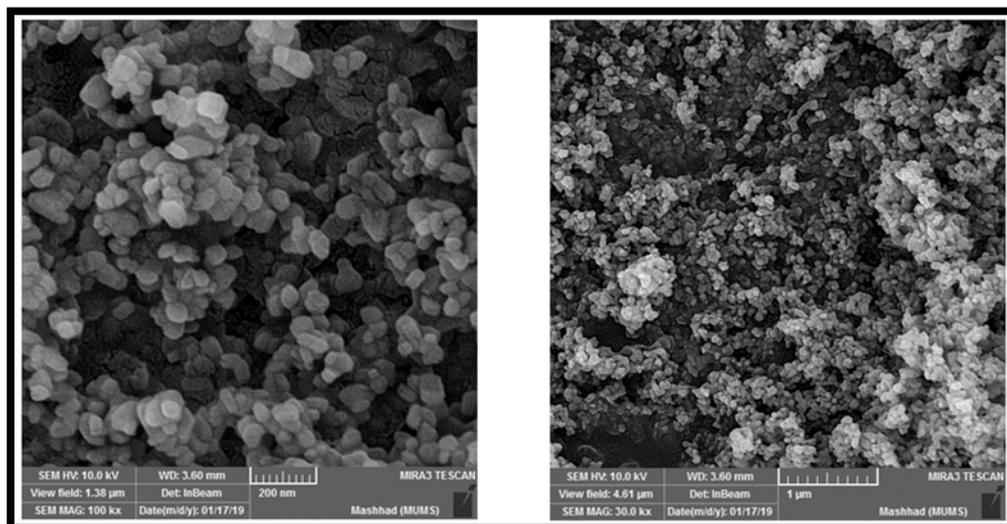


Fig. 5: Show FE-SEM of Titanium nanoparticles in 200 nm field (left) and in 1 µm field (right)

X-ray Diffract meters (XRD) of Titanium dioxide nanoparticles showed in fig. (6), according to reference (00-001-1292) and the peaks at positions 27.507° , 36.041° , 41.187° , 54.233° and 68.999° corresponding to (110), (101), (111), (211) and (301)

respectively reflection of tetragonal with α and $\beta = 4.5800^\circ$ [22]. The size of nanoparticles crystal was (31 nm) calculated by (Xpert high score plus program 3th version) using the Sherrer equation.

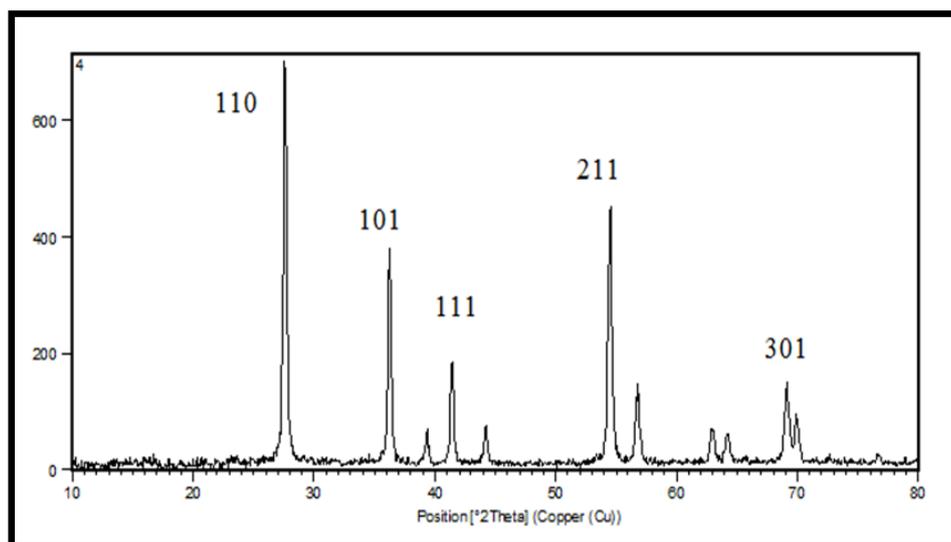


Fig. 6: XRD spectra of titanium nanoparticles

Qualitative tests were used to confirmed interaction and loading between streptokinase and nanoparticles. Ninhydrine's tests results of Streptokinase (SK) loading titanium nanoparticles ($\text{NpTiO}_2\text{-SK}$)

were giving a positive result by formation blue color with samples that show in fig. (7) and table (1) and these results confirmed the presence of streptokinase protein in nanoparticles.

Table 1: Result of streptokinase (SK) loading titanium dioxide nanoparticles ($\text{NPs-TiO}_2\text{-SK}$) with ninhydrine's tests

Type	Solution	Color	Result
Blank	NpTiO_2 in conc. 0.1 mg/ml	No change	Negative
Sample solution	$\text{NpTiO}_2\text{-SK}$ in conc. 0.1 mg/ml	Light Blue	Positive (+)



Fig. 7: Result of ninhydrine's tests with (left) $\text{NpTiO}_2\text{-SK}$ and (right) with NpTiO_2 alone

FT-IR spectrum of streptokinase loading Titanium nanoparticles as in fig. (8), the broad peak at range (3400 cm^{-1}) revealed to (O-H) stretching of hydroxyl group interfering with (N-H) stretching which refer to presence amine and hydroxyl group, peaks at (2960 cm^{-1}) and (2928 cm^{-1}) revealed to (C-H) stretch vibration of aliphatic groups,

whereas, peak at (1653 cm^{-1}) revealed to (C=O) stretch vibration of amide group in addition peaks at (1535 cm^{-1}) revealed to (N-H) bending vibration of amino group and this spectrum compared with Titanium nanoparticles spectrum in fig. (2) Confirmed to presence streptokinase and loading it on nanoparticles [23, 24].

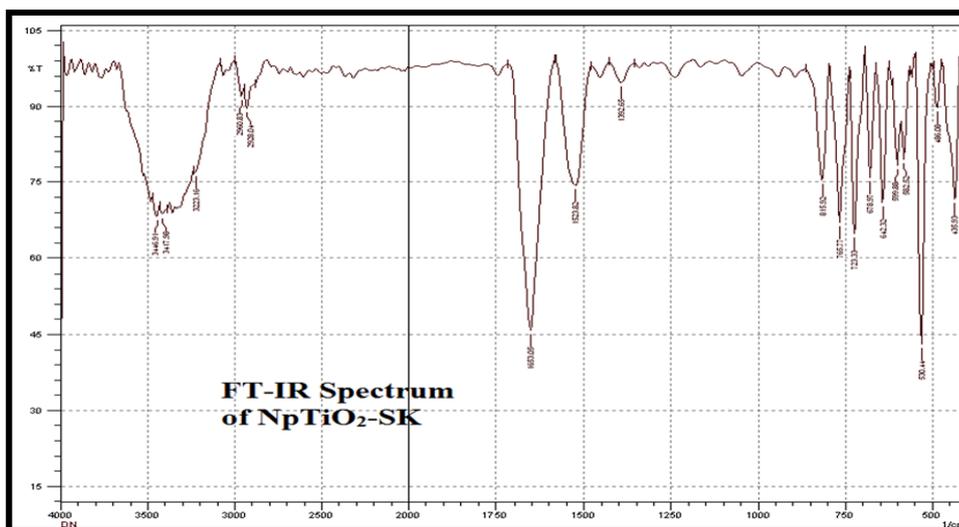


Fig. 8: FT-IR spectrum of streptokinase loading titanium nanoparticles (NpTiO₂-SK).

Quantitative tests results of streptokinase (SK) loading Titanium nanoparticles were carried out depending on the Bradford assay method (Coomassie Brilliant Blue). Concentration of sample were determined depending on standard curve linear regression equation in fig. 1. The concentration of streptokinase loading titanium nanoparticles were in range (11µg/ml) and these results agreed with result of qualitative tests of streptokinase loading nanoparticles by testing in FT-IR spectroscopy and Ninhydrine's test.

Entrapped efficiency (EE) and loading capacity (LC) of Streptokinase loading Titanium nanoparticles (NpTiO₂-SK) show in table (2).

Entrapped efficiency was increased with increasing concentration of Titanium nanoparticles where the highest value of entrapped efficiency was 60% with (1.0 mg/ml) concentration of Titanium nanoparticles whereas the lowest value of entrapped efficiency was 6% with (0.05 mg/ml) concentration of Titanium nanoparticles. The highest loading capacity (14.5 %) was gotten with a concentration of Titanium nanoparticles (0.2 mg/ml). Loading capacity is decreased with increased concentration of Titanium nanoparticles above (0.2 mg/ml) as show in table (2). Also, a decrease concentration below (0.2 mg/ml) causes a decreasing loading capacity of streptokinase in Titanium nanoparticles.

Table 4-16: Results of entrapped efficiency and loading capacity of streptokinase loading titanium nanoparticles (NpTiO₂-SK)

No.	Conc. of NpTiO ₂	Streptokinase	Entrapped efficiency%	Loading capacity%
1	0.05 mg/ml	0.1 mg	6	12
2	0.1 mg/ml	0.1 mg	14	14
3	0.2 mg/ml	0.1 mg	29	14.5
4	0.4 mg/ml	0.1 mg	36	9
5	0.6 mg/ml	0.1 mg	53	8.833
6	0.8 mg/ml	0.1 mg	58	7.25
7	1 mg/ml	0.1 mg	60	6

Study of streptokinase release from Titanium nanoparticles using a physiological phosphate buffer saline (PBS) at PH (7.4) for 24 hour as in fig. (9) Which show high rate release of streptokinase from Titanium nanoparticles due to physical bonding between them.

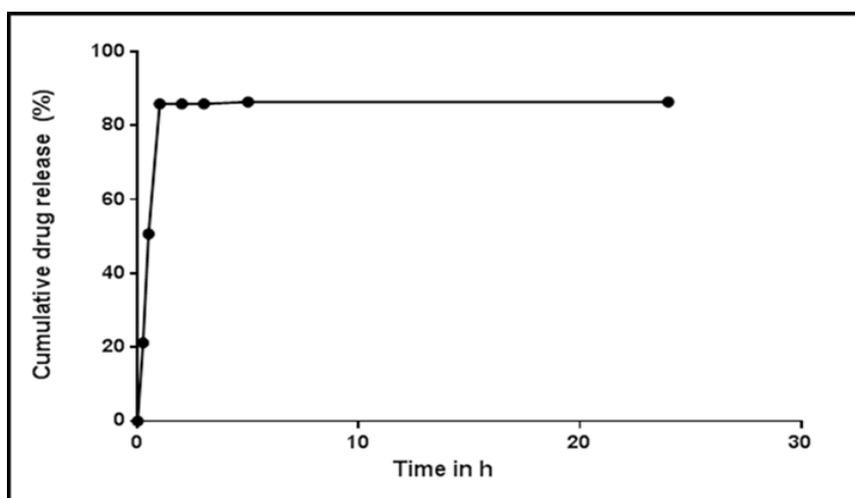


Fig. 9: Curve of cumulative streptokinase release from Titanium nanoparticles for 24 h

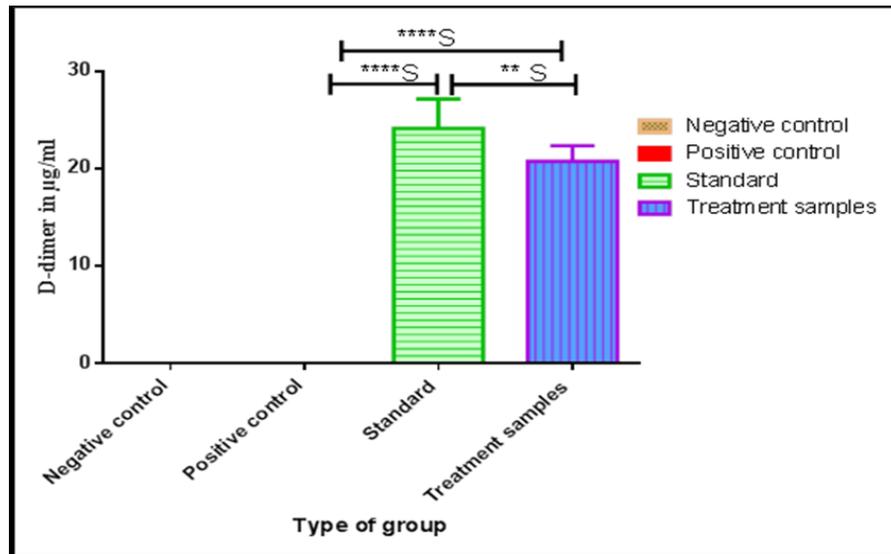


Fig. 10: D-dimer value in blood samples after 60 min of treatment with NpTiO₂-SK, n=10, p-value ≤ 0.05. S= significant, NS= non-significant

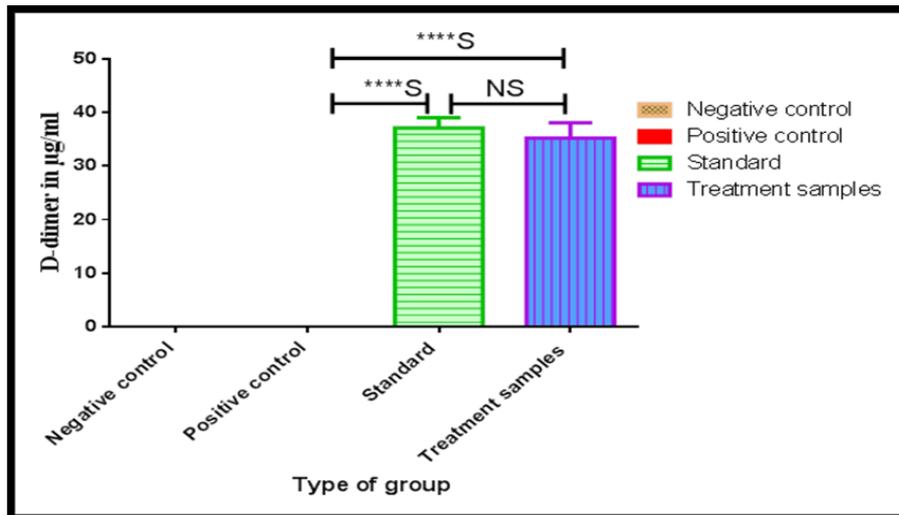


Fig. 11: D-dimer value in blood samples after 120 min of treatment with NpTiO₂-SK, n=10, p value ≤ 0.05. S= significant, NS= non-significant

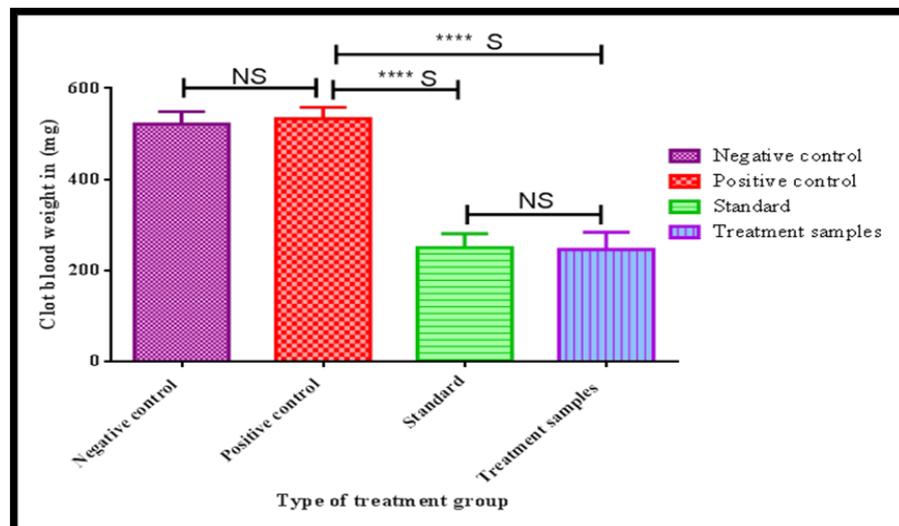


Fig. 12: Clot blood weight in mg of blood samples after treatment with NPs-TiO₂, n=10, P value ≤ 0.05 S= significant, NS= non-significant

The thrombolytic activity of streptokinase loading titanium nanoparticles

Thrombolytic activity is a processes to dissolve blood clots in blood vessels to improve blood flow and prevent damage to tissues and organs. Thrombolytic activity of the samples was determined using two indicators, the first one is D-dimer as fibrin degradation product (or FDP), a small protein fragment result in the blood after a blood clot is lysis by fibrin. D-dimer is so named because it consists of two D fragments of the fibrin protein that joined by a cross-link [25]. The second indicator is the weight of blood clot *in vitro* which used as an indicator to determined thrombolytic activity according to Prasad method. D-dimer value was negative in both negative and positive control whether measured after 60 min or 120 min as in fig. 10) and

11, whereas there are no significant differences in the weight of blood clot of samples after treatment (521.42±27.46 and 533.44±24.71) for both negative and positive control respectively as in fig. 12. D-dimer value was significant (**) after 60 min (24.124±3.03 and 20.752±1.61) for standard streptokinase and streptokinase loading Titanium nanoparticles respectively and also it was significant (****) to positive control as in fig. 10 and 13. D-dimer value after 120 min was no significant (37.202±1.93 and 35.262±2.86) between standard streptokinase and streptokinase loading nanoparticles respectively but they were significant (****) to positive control as in fig. (11) and (13). The weight of blood clots show no significant difference (250.18±30.82 mg and 146.18±37.81 mg) between standard streptokinase and streptokinase loading nanoparticles but they were significant (****) to positive control show in fig. 12 and 13.



Fig. 13: Show results of thrombolytic activity for (1) Negative control (2) Positive control (3) Streptokinase (4) Streptokinase loading titanium nanoparticles

CONCLUSION

Titanium nanoparticles conjugated with streptokinase show high thrombolytic activity against blood clot *in vitro*.

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AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

CONFLICT OF INTERESTS

Declare none

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