RESEARCH PAPER

Thrombolysis based on magnetically-controlled surface-functionalized \( \text{Fe}_3\text{O}_4 \) nanoparticle

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ABSTRACT
In this study, the control of magnetic fields to manipulate surface-functionalized \( \text{Fe}_3\text{O}_4 \) nanoparticles by urokinase coating is investigated for thrombolysis in a microfluidic channel. The urokinase-coated \( \text{Fe}_3\text{O}_4 \) nanoparticles are characterized using particle size distribution, zeta potential measurement and spectroscopic data. Thrombolytic ratio tests reveal that the efficiency for thrombus cleaning is significantly improved when using magnetically-controlled urokinase-coated \( \text{Fe}_3\text{O}_4 \) nanoparticles than pure urokinase solution. The average increase in the rate of thrombolysis with the use of urokinase-coated \( \text{Fe}_3\text{O}_4 \) nanoparticles is about 50%. In vitro thrombolysis test in a microfluidic channel using the coated nanoparticles shows nearly complete removal of thrombus, a result that can be attributed to the clot busting effect of the urokinase as it inhibits the possible formation of blood bolus during the magnetically-activated microablation process. The experiment further demonstrates that a thrombus mass of 10.32 mg in the microchannel is fully removed in about 180 s.

KEYWORDS
\( \text{Fe}_3\text{O}_4 \) nanoparticles; magnetic-control; magnetically-activated microablation; surface-functionalized; thrombolysis; urokinase

Introduction

Many human cardiovascular diseases (CVDs) are caused by the accumulation of vascular blockages such as thrombus and fat. Upon detection traditional treatments implement the removal of the blockage by surgery or medicine. The prospect of using magnetic nanoparticles (NPs) for the treatment of CVDs via thrombolysis lies on their ability to be magnetically-controlled in order to carry out targeted drug delivery or non-invasive microsurgery within the blood stream.\(^1,2\) It has been shown recently that uncoated magnetic \( \text{Fe}_3\text{O}_4 \) NPs, exposed to a low gradient and oscillating magnetic field, can be driven magnetically in a microchannel.\(^3\) The motion of the NPs creates a velocity field that can be harnessed to facilitate non-contact microablation of thrombus occlusions in microscopic channels. However, one of the possible tradeoff of the microablation process is the removal of small debris, which can form new occlusions in neighboring tiny blood vessels in the absence of clotting inhibitors. Therefore, a suitable drug agent with hemolytic functions in combination with magnetic NPs is desirable to allow the complete cleaning of thrombus via non-contact microablation.

In this study, the use of urokinase as a surface coating of \( \text{Fe}_3\text{O}_4 \) NPs is investigated for thrombus removal. Co-precipitation method was used to prepare the bare \( \text{Fe}_3\text{O}_4 \) NPs before surface-coating the NPs with agarose-gel. The thrombolytic drug urokinase was immobilized onto the agar-coated \( \text{Fe}_3\text{O}_4 \) nanoparticles by covalent bonding via EDC/sulfo-NHS.\(^4,5\) The hemolysis performance between pure urokinase powder and magnetically-controlled urokinase-coated \( \text{Fe}_3\text{O}_4 \) nanoparticles were investigated experimentally. An in vitro thrombolysis test using a microfluidic channel was also employed. About 50% increase in efficiency of the surface-functionalized magnetic nano-drugs is achievable in the static thrombolytic comparison experiment compared to the pure urokinase solution. The in vitro experiment reveals that although the urokinase coating can weaken the response of the nanoparticles to an externally applied
magnetic field, the clot busting effect of the urokinase results to faster and nearly complete thrombus cleaning process in the microchannel. The observed enhancement in thrombolysis rate using urokinase-coated Fe₃O₄ unwraps their potential as biocompatible nanodrug for efficient and complete thrombus removal in microchannels.

**Magnetic control of nanoparticles**

In ref.3, the generalized formulation describing the behavior of NPs in a viscous fluid assumes negligible electrostatic and van der Waals forces between the nanoparticles. In addition, Brownian movement can be neglected due to the agglomeration tendency and relative size of the surface-coated NPs. Further, by approximating a Newtonian flow throughout the channel, the propulsion mechanism of the NPs are induced solely by the motive force from a magnetic gradient source. The translational magnetic force \( F_x \) acting on the NPs parallel to the longitudinal direction of the microchannel is found to be dependent on the vacuum permeability \( \mu_0 \), the material susceptibility \( \chi \), the volume \( V \) of the surface-coated NP, the magnetic field strength \( H_y \), and the gradient field \( \frac{\partial H_y}{\partial x} \) as shown by the following relation

\[
\bar{F}_x = \mu_0 \chi H_y V \frac{\partial H_y}{\partial x}
\]

(1)

Given the hydrodynamic Stokes’ drag force for spherical particles of diameter \( d \), and liquid viscosity \( \eta \), i.e. \( \bar{F}_d = -3\pi d^2 \eta \dot{\omega} \), the NP acquires a linear velocity along the gradient field given by

\[
\bar{v} = \frac{\mu_0 \chi V}{3\pi d \eta} H_y \frac{\partial H_y}{\partial x}
\]

(2)

Equation (2) predicts the linear dependence of the terminal velocity of a magnetic NP to the combined attributes of the oscillating magnetic field amplitude and the field gradient, \( \left( \frac{\partial H_y}{\partial x} \right) \).

Moreover, the rotational motion of the NPs is influenced by the induced magnetization and the external oscillating magnetic field. The magnetic torque on the induced dipole on the NP is given by

\[
\tau_m = V \frac{\chi^2}{2(2 + \chi)} \mu_0 H^2 \sin(2\theta)
\]

(3)

where the angle \( \theta \) is between the dipole moment \( m \) and the magnetic field induction \( B \). Equation (3) reveals that the torque needed to rotate the NPs is dependent on the square of the amplitude of the magnetic field strength, that it can be increased increasing the particle volume or by introducing a slight misalignment between \( m \) and \( B \).

The rotation of the NPs is also subjected to a viscous drag force, where the drag torque induced on a particle of radius \( r \) and rotation speed \( \omega \) is proportional to \( \tau_D = 3\pi \eta r^4 \dot{\omega} \). It is thus possible that the rotating NPs under low Reynolds regime reach terminal angular velocity instantaneously as a result of the superposition of the magnetic and induced drag torques. As such, the angular speed can be expressed as

\[
\omega = \frac{V\chi^2 \mu_0 H^2 \sin(2\theta)}{3\pi^2 \eta r^4}
\]

(4)

**Preparation of bare and urokinase-coated Fe₃O₄ NPs**

The preparation of uncoated Fe₃O₄ NPs was implemented at room temperature. The reaction process made use of the co-precipitation of iron (II) chloride tetrahydrate and iron (III) chloride hexahydrate with sodium hydroxide as given by

\[
FeCl₂ + 2 FeCl₃ + 8 NaOH = Fe₃O₄ + 8 NaCl + 4 H₂O.
\]

(5)

To synthesize the urokinase-coated Fe₃O₄ nanoparticles, agar-coated Fe₃O₄ nanoparticles were first prepared.4,5 Agar powder (2 g) was dissolved in deionized water (100 ml) to make 2% agar/H₂O solutions. The solutions were mixed using a heating stirrer and then heated at 90°C for 1 h. The agar gels were formed upon cooling at room temperature and then immersed in a pre-mixed solution (i.e., 6.75 g iron (III) and 2.5 g iron (II) dissolved in 100 ml of deionized water of pH = 7) of 1M iron (III) ions and 0.5M iron (II) ions for 12 h.
Next, the agar gels were rinsed 2 times in deionized water and soaked in 2.5 mol/L sodium hydroxide solution for 30 min. Finally, the agar gels were rinsed 5 times in deionized water and oven-dried for 24 h at 70°C. The agar-coated Fe₃O₄ (Fe₃O₄@agar) nanoparticles were then processed into powder form by grounding the dried gels using an agate mortar. Figure 1 shows the size distribution of aggregate size of the Fe₃O₄@agar nanoparticles, ranging from 40 nm to 200 nm. In Fig. 1A, the average size in the distribution is 119 nm and the approximate shape is close to a spherical polyhedron.

A covalent binding method was used to bond the urokinase with the magnetic Fe₃O₄@agar nanoparticles. Carboxylates (-COOH) of agar gels may interact with Sulfo-NHS in the presence of a carbodiimide such as EDC. The result is a semi-stable Sulfo-NHS ester, which can easily react with primary amines (-NH₂) to form amide crosslinks and fix urokinase on the surface of the Fe₃O₄@agar nanoparticles. 0.4 M EDC solutions were first mixed with 0.1 M sulfo-NHS solution and stirred for 5 min to obtain the carrier activator EDC/sulfo-NHS. A solution of EDC/sulfo-NHS was added into a beaker containing the prepared suspension of Fe₃O₄@agar and then shook for 30 min. The Fe₃O₄@agar nanoparticles were then attracted to the bottom of the beaker via a magnet and the supernatant was removed to obtain surface activated Fe₃O₄@agar nanoparticles. The activated nanoparticles were cleaned with deionized water, then uniformly mixed with 10 ml of urokinase solution (i.e. 6000 I.U./ml dissolved in deionized water of pH = 7), and allowed the reaction to proceed for 2 h. The urokinase-coated Fe₃O₄@agar nanoparticles were again separated from the supernatant using a magnetic source. Finally, the surface-coated nanoparticles were stored in deionized water at 4°C to keep the activation of the materials. Figure 1B shows the size distribution of the urokinase-coated nanoparticles. Figure 1B shows the size distribution of the urokinase-coated Fe₃O₄@agar nanoparticles, ranging from 40 nm to 250 nm. Compared to the uncoated nanoparticles, the averaged size of the distribution is 169 nm. Figure 2 shows the zeta potential of the nanoparticles. For the agar-coated Fe₃O₄, the zeta potential is very low about 0.0191 mV at pH 7, which is indicative of higher tendency to form aggregates as opposed to the urokinase-coated Fe₃O₄ nanoparticles with zeta potential of −8.13 mV at the same pH. The slightly improved potential implies that in addition to its thrombolytic functionality, the urokinase provides a shielding effect between aggregating nanoparticles.

Fourier transform infrared spectroscopy (FTIR) was used to verify the adsorption of urokinase coating to the Fe₃O₄ NPs. The urokinase is a protein in nature and its absorption peaks on the FTIR plot are found at 1656 cm⁻¹, 1541 cm⁻¹, 1438 cm⁻¹, 1311 cm⁻¹, and 1085 cm⁻¹. As shown in Fig. 3, the FTIR spectrum of
surface-coated Fe₃O₄ NP is consistent with the characteristic peaks of the urokinase powder. Therefore, the surface bonding of urokinase to the magnetic Fe₃O₄ NPs is verified.

**Results and discussion**

A comparison of hemolysis performance between pure urokinase powder and magnetically-controlled urokinase-coated Fe₃O₄ nanoparticles was first made. We prepared 2 sets of glass sample bottles, each bottle containing 15 ml deionized water and a little amount of thrombus. Ten mg of urokinase powder was added on one set of bottles and 10 mg of urokinase-coated Fe₃O₄ NPs was added on the second bottle set. Thrombolytic tests were repeated 5 times for each set. Through visual observation, we measure the elapsed time between the addition of the drug agents up to nearly complete removal of thrombus in each bottle. **Figure 4** shows the observed results of the experiment, where the average thrombolytic rate is increased from 0.0026 mg/min (without the NPs) to 0.0039 mg/min (with surface-coated NPs). The results clearly show that the performance of the magnetically-controlled nano-drugs is roughly 50% higher than of the pure urokinase setup. Furthermore, it can be observed that there is a slight decline in the hemolysis rate with increasing thrombus weight. This may be attributed to the reduction of the urokinase concentration with the drug reaction time; therefore a larger thrombus needs more operation time.

The second experiment is an *in vitro* test of thrombolysis. To represent a microvessel, a microchannel (length = 25 mm, width = 0.8 mm) was adopted as shown in Fig. 5. **Figure 6** is the set-up used for the *in vitro* test. A programmable microinjection system was used to simulate the blood flow. The deionized water solution containing urokinase-coated Fe₃O₄ NPs was injected into the microchannel with the help of the microinjection pump. The flow speed of the NPs was gradually reduced until stoppage because of the thrombus. The external magnetic field was then driven to manipulate the movement of the urokinase-coated Fe₃O₄ NPs. To capture the movement of the NPs during the process of thrombolysis, an X-Stream XS-3 high-speed camera was installed and attached to an optical microscope. The NPs were observed to form agglomerates of elliptical-like microstructures, which were recorded at 10x to 100x magnification. However, the size of individual surface-coated magnetic NPs is roughly 150~250 nm, which is substantially below the diffraction limit of the optical microscope. Even at
100x magnification, the motion of the agglomerated NPs was very difficult to observe. Therefore, the images were generally captured at 10x magnification for the sake of a bigger field of view.

Figure 7 shows the image sequence of a 180 s experiment demonstrating the microablation of a thrombus occlusion via magnetic-controlled steering of the urokinase-coated Fe₃O₄ NPs. The NPs were injected on the right hand side of the microchannel and were controlled to move toward the occlusion target to the left of the microchannel. The magnetic control system used for this investigation consisted of a static magnetic field of 624 Am⁻¹ from NdFeB magnets and a gradient of 3.2 Tm⁻¹ generated by a magnetic coil. The velocity field created by the rotating NPs initiates the thrombus ablation resulting to a 'pathway' through the occlusion area. The width of this pathway is gradually increased due to the contact of the urokinase-coated NPs and thrombus. As shown, the cleared areas in the microchannel are not uniform, which may be accounted for by the inhomogeneous thickness of the occlusion and the non-uniform distribution of the velocity field. However, the thrombolysis process was roughly completed in 3 min in our experiment.
Finally, the fluid in the microchannel was also inspected at 100x magnification and no micro blood clots were found. The thrombus in the microchannel was weighed before and after the in vitro thrombolysis experiment. An amount of 10.32 mg thrombus was found to be removed in 180 s. Compared to existing medical procedures for thrombolysis, the most common uses direct hemolytic agent infusion into the vein or artery. The time of treatment can last for a few hours to days or even longer because the system relies on the agent being aided by the natural blood flow toward the occlusion. However, the presence of such occlusions can slow or even stop the blood circulation. Consequently, the drug action would be delayed requiring patients to endure the ailment longer. The use of functionalized nanoparticles controlled by an external magnetic force could guide the drug to reach the target quickly.

A thrombus removal experiment based on agar-coated Fe₃O₄ NPs without urokinase was also carried out. The magnetic control condition was the same as the urokinase-coated Fe₃O₄ NPs experiment. An image sequence of 400 s demonstrating the thrombolysis process by magnetic steering of the Fe₃O₄@agar NPs is shown in Fig. 8. As shown, there were still occlusions that cannot be removed from the boundary of the microchannel after 400 s. This shows that the thrombus cannot be completely cleaned without the action of a hemolytic agent.

Conclusions

The use of an oscillating magnetic field system to control the motion of urokinase-coated Fe₃O₄ NPs for complete removal of thrombus in a microchannel was presented. The velocity field generated by the rotating NPs enabled the microablation of thrombus occlusion. The surface-functionalization of the NP’s using urokinase was investigated to improve the efficiency of the cleaning process. As a new approach for microsurgery, we demonstrate that the controllable movement of surface-coated magnetic nanoparticles is much simpler compared to traditional invasive devices or slow-reaction drug for thrombus cleaning.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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