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Nanoscaled ultrasound contrast agents for enhanced sonothrombolysis

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ABSTRACT

Sonothrombolysis, the enhancement of thrombolysis with ultrasound (US), is widely used in clinical practice. The use of an ultrasound contrast agent can lead to a further reduced recanalization time of the occluded blood vessel and thus to better outcome for the patient. In this study the sonothrombolytic efficacy of our new nanoscaled ultrasound contrast agent (NUSCA) was investigated. This new contrast agent has a size of less than 100 nm and should thus be able to penetrate the thrombus and achieve a thrombolysis from inside out. In this study human whole blood clots were exposed to US, US and NUSCA, US and recombinant tissue plasminogen activator (rt-Pa) or urokinase (UK), or a combination of US, NUSCA and thrombolytic drug in a closed-loop flow model. We sonicated with diagnostic US at a frequency of 2.85 MHz for 30 min. Clot mass loss of 50.6 ± 6.0% for the combination of US, NUSCA and rt-PA was found. Using UK as thrombolytic drug 57.7 ± 9.0% clot mass loss could be seen. Thus the weight loss exceeded the conventional values of up to 30%. Scanning electron microscopy (SEM) images revealed changes of the fibrin network on the thrombus surface. The NUSCA was able to loosen the network and induce large pores in the thrombus surface. The high rates of clot mass loss and the obvious changings of fibrin structure make our NUSCA a promising tool for sonothrombolytic therapy.

1. Introduction

Cardiovascular diseases belong to the most frequent causes of death in the developed world. In the federal republic of Germany 38.5% of all death in 2015 were provoked by cardiovascular diseases (Statistisches Bundesamt). Among them thrombotic occlusive diseases like myocardial infarction, stroke, deep vein thrombosis or pulmonary embolism are frequent and accompanied with severe health problems and mortality [1]. Thrombolytic therapy with drugs like streptokinase (SK), urokinase (UK) or recombinant tissue plasminogen activator (rt-Pa) was able to reduce the mortality in the last years [1]. The primary aim of thrombolytic therapy is an early recanalization of the occluded blood vessel. For ischemic stroke it has been proven that an early recanalization is strongly associated with improved clinical outcome and reduced mortality after three months [2]. The limitation of thrombolytic therapy is the high frequency of severe side effects like intracerebral hemorrhage (ICH). To overcome these problems fibrin specific thrombolytic agents like rt-Pa were developed. Contrary to the expectations, some studies revealed increased bleeding complications after treatment with rt-Pa [1]. Enhancement of fibrinolytic activity and prolonged lytic effect by complexing fibrin degradation products were found as reason for these observations. Another possibility to reduce the incidence of ICH, catheter-directed intra-arterial delivery of pro-urokinase, was tested with promising results [3,4]. A big drawback for intra-arterial therapy is that specific equipment and skilled operators are needed. Thus, only stroke units can use this technique as standard method.

In recent years the ultrasound (US) has been discussed as a potential factor increasing thrombolysis. High rates of early recanalization were observed by Alexandrov et al. after monitoring thrombolysis with 2 MHz ultrasound [5]. Several studies showed increased thrombolysis after the use of US alone or in combination with thrombolytic drugs [6–11]. The thrombolytic efficacy depends on the US frequency and seemed to be better with low-frequency US (20 kHz to 1 MHz) than with diagnostic US [6]. Due to these findings a phase II clinical trial regarding sonothrombolysis with low-frequency US for acute stroke was conducted [12]. This trial was prematurely stopped due to significantly more hemorrhages in the US group. Another randomized study utilized

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1.8 MHz US in combination with standard rt-Pa treatment [13]. In this study only a non-significant trend towards more severe hemorrhages was found, while the total number showed no differences between the two groups.

To further enhance the thrombolytic effect of US an ultrasound contrast agent (UCA), normally used to improve the image quality in clinical routine, can be combined with the thrombolytic drug and the insonation [14–20]. These agents reduce the threshold for cavitation and cavitation related processes like mircostreaming or microjetting. Stable cavitation seems to lead to better outcome than inertial cavitation [15,21]. Commercially available UCA are normally sized in the micrometer range (e.g. 2 to 8 µm for SonoVue®) [22]. This size hinders their deep penetration into the fibrin network of a thrombus. We developed a nanoscaled ultrasound contrast agent (NUSCA) with a size of less than 100 nm [23]. This small size and the good contrast enhancement related to SonoVue® make it a promising tool for sonothrombolysis [24]. Due to its size, our NUSCA should be able to penetrate the clot and degrade the fibrin network from the inside.

In this *in-vitro* study we investigated the thrombolytic effect of our new NUSCA. Therefore its thrombolytic capacity was tested in combination with diagnostic US and two thrombolytic drugs (rt-Pa and UK).

2. Materials and methods

2.1. Materials

1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) was a gift from Lipoid GmbH (Ludwigshafen, Germany), polyethylene glycol (40) stearate (PEG40S) was purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Stock solutions were prepared in a mixture of chloroform:methanol (2:1, v:v) and stored at 4 °C. Chloroform (HPLC-grade) and methanol (HPLC-grade) were purchased from Fisher Scientific (Fisher Scientific UK Ltd, Loughborough, UK). The thrombolytic drugs used were rheothromb®-Actavis (Actavis Group PTC ehf., Hafnarfjördur, Iceland) as UK and Actilyse® (Boehringer® Ingelheim Pharma GmbH & Co. KG, Ingelheim am Rhein, Germany) as rt-Pa. All other chemicals were of analytical grade and were used as received.

2.2. Preparation of nanoscaled ultrasound contrast agent

The NUSCA composed of DSPC and PEG40S with a molar ratio of 96:4 was prepared using the thin film hydration method [25,26]. Briefly, from the stock solution the lipids were mixed in a round bottom flask. The lipid mixture was subsequently dried to a lipid film using a rotary evaporator (Heidolph Laborota 4000 efficient, Heidolph Instruments GmbH & Co.KG, Schwabach, Germany). The resulting film was rehydrated with phosphate-buffered saline (PBS) pH 7.4 (0.15 mol/l). After vigorous shaking the lipid dispersions were sonicated in a bath-type sonicator (Bandelin Sonorex RK 100H, Bandelin Electronics, Berlin, Germany) for 20 s at 65 °C. After incubation for 60 min at the above mentioned temperature the dispersion was again sonicated in the bath-type sonicator for 2 min. Immediately after this the warm NUSCA was sonicated at the air-water interface with a probe sonicator (Branson sonifier 250, G. Heinemann Ultraschall-/ Labortechnik, Schwäbisch Gmünd, Germany) for 20 s, using maximum US output level and a duty cycle of 20%. After cooling to room temperature the lipid dispersion was transferred into a tube leaving the produced foam in the flask and stored at 4 °C.

2.3. Characterization of NUSCA

2.3.1. Dynamic light scattering

The hydrodynamic diameter of the NUSCA was determined by Dynamic Light Scattering (DLS) using a Zetasizer Nano ZS (Malvern Instruments, Herrenberg, Germany) as described previously [23]. The device is equipped with a 10 mW HeNe laser at a wavelength of 633 nm and the measurements were performed at a temperature of 25 °C. Scattered light was detected at a 173° angle with laser attenuation and measurement position adjusted automatically by the Malvern software. The particle size is calculated automatically based on the scattered light and the Brownian Motion of the particles using the Stokes-Einstein equation

$$R_h = k_B T / 6\pi\eta D.$$

With the radius of the particles R_h, the Boltzmann constant k_B, the absolute temperature T, the solvent viscosity η and the diffusion coefficient D. Values given are the means ± standard deviation of five independent experiments with each experiment comprising three measurements of the same sample with at least 10 runs, as determined by the Malvern software. The average values were calculated with the volume distribution data of five samples ± standard deviation.

2.3.2. Cryo-transmission electron microscopy

For visualization of the NUSCA cryo-Transmission Electron Microscopy (cryo-TEM) was utilized. The samples were analyzed without dilution on Quantifoil® S7/2 Cu 400 mesh, holey carbon film grids (Quantifoil Micro Tools GmbH, Jena, Germany). Shock-frozen samples were prepared by dipping the grid into liquid ethane (90 K). After transfer to the microscope (Leo 912 Ω-mega, Leo Elektronenmikroskopie GmbH, Oberkochen, Germany) the samples were examined as described elsewhere [27].

2.3.3. Determination of echogenicity

The echogenicity of the NUSCA was determined as mean grey value related to SonoVue® (set as 100%) in an *in-vitro* flow model as published previously [24]. In this model (different to the closed loop model used in this study) a silicon tube (C-flex®, Cole-Parmer Inc., Illinois, USA) was embedded into an agar gel. With an US probe mounted to the gel surface the echogenicity of the NUSCA was determined.

2.4. Clot preparation

Human venous blood was repeatedly drawn from one healthy volunteer by sterile venipuncture without any anticoagulant therapy. Immediately 500 µl of the blood were transferred into 1.5 ml tubes to form blood clots. To prevent sedimentation of the blood cells the tubes were shaken after 20 min and after 40 min. For a maximal retraction and lytic resistance the clots were incubated 3 h at room temperature and afterwards stored at 4 °C for 3 days [28].

2.5. Sonothrombolysis model and US device

For the thrombolytic experiments a custom build Teflon chamber (inner diameter 30 mm) was included in a closed loop flow system (Fig. 1). A blood clot was placed inside the chamber so that the included

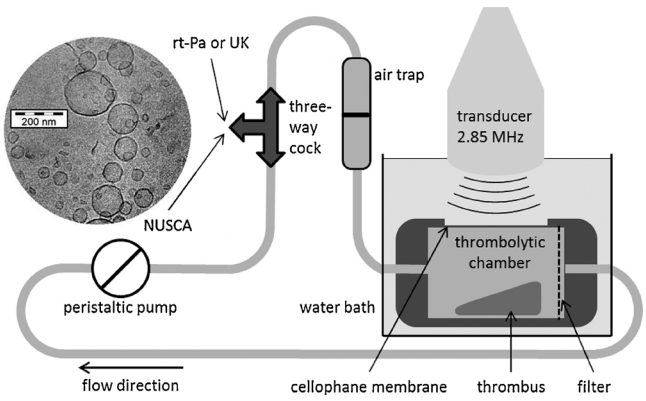

Fig. 1. Schematic diagram of the closed-loop flow system.