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Research article

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Feasibility study of positronium application for blood clots structural characteristics

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Abstract: Positron-electron annihilation in living organisms occurs in about 30% via the formation of a metastable ortho-positronium atom that annihilates into two 511 keV photons in tissues because of the pick-off and conversion processes. Positronium (Ps) annihilation lifetime and intensities can be used to determine the size and quantity of defects in a material's microstructure, such as voids or pores in the range of nanometers. This is particularly true for blood clots. Here we present pilot investigations of positronium properties in fibrin clots. The studies are complemented by the use of SEM Edax and micro-computed tomography (μCT) to evaluate the extracted thrombotic material's properties. μCT is a versatile characterization method offering in situ and in operando possibilities and is a qualitative diagnostic tool. With μCT the presence of pores, cracks, and structural errors can be verified, and hence the 3D inner structure of samples can be investigated.

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Introduction

Spontaneous intravascular blood clot formation has been a problem and a potential cause of fatal events in patients with cardiovascular risk over the years. It is a common complication and the root cause of many diseases and conditions, including thrombosis, atherosclerosis, trauma, stroke, and cancer amongst others [1,2]. Clot formation is intended to achieve hemostasis and tissue repair. However, there are some factors which accelerates fibrin clot formation such as genetic predispositions and environmental factors [2].

Positron annihilation lifetime spectroscopy (PALS) is widely recognized as a method to analyze free volume and defects in functional and other materials like microstructure of condensed matter, biological matter, and nanocomposite [3]. PALS provides for the examination of material structure at the nano and sub-nanometer scales. The lifespan and intensity of ortho-positronium atoms in free volumes of specific structures are used in this technique. Recently a method of positronium imaging was introduced [3] that combine the PALS and positron emission tomography (PET) enabling imaging of positronium properties in the human body during the PET diagnosis [4–8]. Positronium is considered as a potential biomarker of hypoxia [9–11], uterine cancer [13] and cardiac masses. Here we perform pilot studies aiming at testing whether it can also be considered as a diagnostic parameter for the vascular masses [3, 5]. The purpose of the presented study is to evaluate the protocol for fibrin clot examination with the use of PALS technique and be able to characterize the produced blood clot with scanning electron microscopy techniques (SEM) and micro computed tomography (μCT). PALS allows the examination of the structure of materials at nano and sub-nanometer scales and μCT at the micrometer scale.

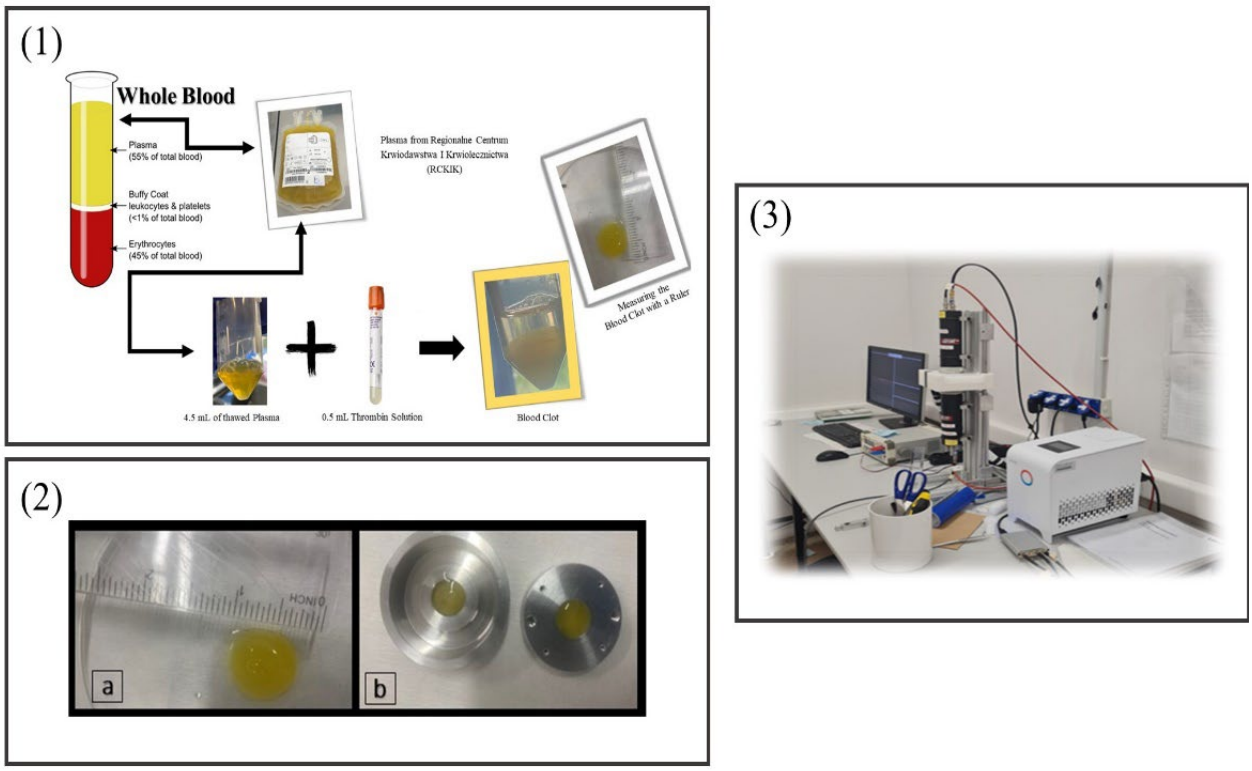

Figure 1: (Figure 1.1) Schematic diagram of the Blood Clot formation using 9:1 ratio of Blood Plasma and thrombin working solution respectively, (Figure 1.2) Schematic diagram showing (1.2a) Blood Clot suspended in a open plate and being measured by a ruler, (1.2b) Blood clot being fitted into 100µm aluminum chambers, (Figure 1.3) Photograph showing the experimental setup used in performed PALS measurements. Two scintillator detectors with BaF_2 crystals were used.

Materials and methods

Blood clot formation

Human blood plasma for clot formation was obtained from Regionalne Centrum Krwiodawstwa I Krwiolecznictwa (RCKIK) w Krakowie. For clotting 9 parts of plasma and 1 part of thrombin working solution (10IU/mL) dissolved in Tris-HCl 7.4 pH buffer ware used. Figure 1 shows a summarized process of how blood clot is formed. Blood clots formed were then washed 3 times by working Tris-HCl buffer.

PALS measurement

The fresh blood clot of around 10 mm in size (Figure 1.2a) was measured with the PALS within 1 hour of ex-vivo production. The blood clot sample was cut into two pieces to fit to the two, 100 µl aluminum chambers equipped with the ^{22}Na radioactive positron source with the activity of approximately 1.3 MBq. The radioactive source was wrapped in a 0.6 µm Kapton foil and the detectors used are made up of two BaF_2 scintillators (Figure 1.3). The PALS measurement uses the digitizer and data acquisition system (DAQ) [12]. Temperatures used for this experiment were 22°C and 37°C (Figure 1. 3).

PALS Avalanche program [12] was applied to decompose positron annihilation lifetime spectra in the form of a set of exponential functions convoluted with the experimental resolution that correspond to the contributing positron lifetime components.

Micro Computed Tomography (µCT)

Blood clot produced was fixed with 2.5% Glutaraldehyde buffered with Tris-HCL and subsequently washed in Tris-HCL 3 times for 30 minutes. The fixed blood clot was then dehydrated in a subsequent concentration of Ethanol from 10% to 99%. In solutions of ascending concentrations of ethanol 10,20, 30, 50, 70, 80, 99% for 15 min each time, and were dried using critical point drying. Dried samples were then put in a desiccator containing silica gels to avoid any wet environment and structural change. Finally, the prepared sample was then sent for µCT and measurements were done under 32kV voltage,

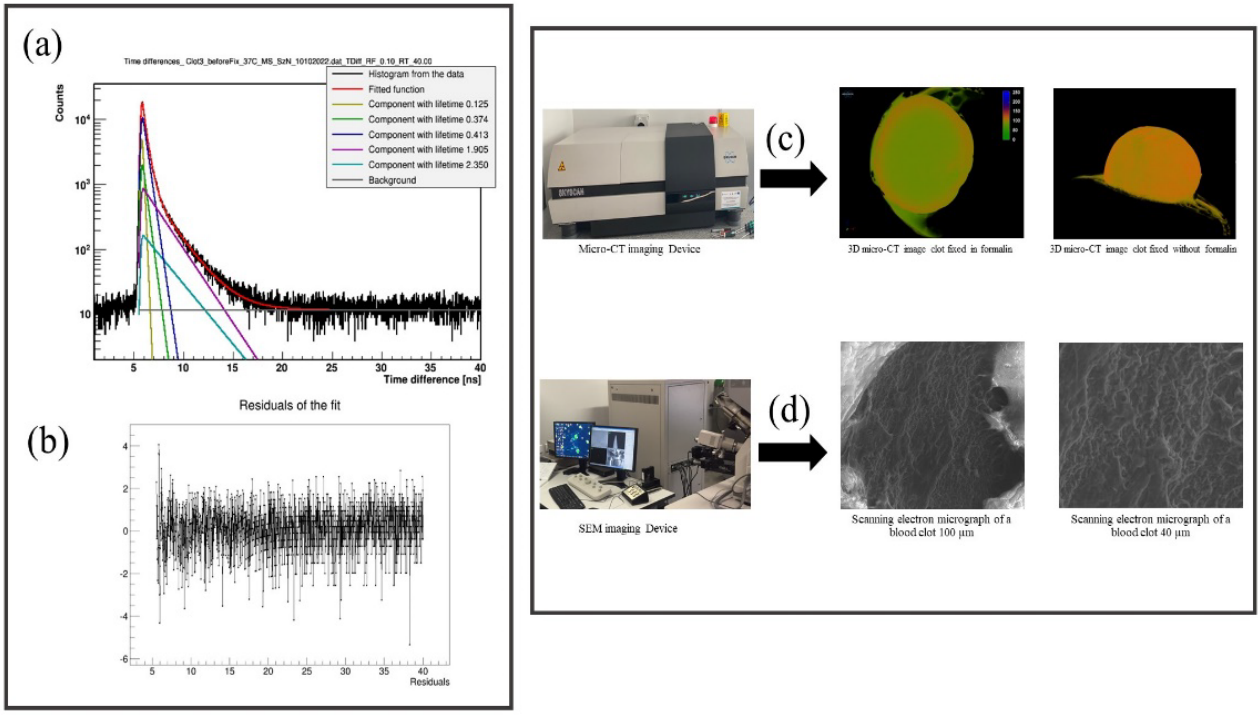

Figure 2: Results from fitting simulated PAL spectra (a) Blood Clot positronium lifetime spectrum, (b) Residual of the model of the fit to the distribution from (a) where the goodness of the fit was measured by the chi squared value divided by degrees of freedom χ^2_ν. (c) Blood Clot analysis via Micro-computed tomography (micro-CT; µCT) showing 3D color visualization of a Blood Clot which was stained with lugol's solution for 24 hours before and scanned via SkyScan 1172. NRecon (Bruker, Kontich, Belgium) software was used for the reconstruction of the projections. (d) SEM micrographs for the structure and composition of the Blood Clot, showing the exterior of morphology of the blood clot.

100Ua current, with 13.1µm image pixel size.

Scanning Electron Microscopes Energy Dispersive X-ray Analysis (SEM Edax)

The Blood clot which was dehydrated and kept in a Desiccator for 24 hours was taken to SEM analysis at the Solid-state Physics laboratories at Jagiellonian University. The samples where mounted on SEM stubs and measurements were performed at 10 keV acceleration voltage with working distance of 7.2 µm.

Results

Figure 2 (a) is showing a exemplary experimental lifetime spectra for fixed Blood Clot with black histogram indicating data and supposed curves result from the fit of the sum of the exponential function convoluted with the detector resolution performed by PALS Avalanche program [12]. The spectrum shows different parameters with the first one represented by a (yellow line) showing the contribution of p-Ps (with mean lifetime of 0.125ns), second component (green line) originates from annihilation in source (Kapton foil) (0.34 ns). Third component (dark blue line) indicates the free annihilation (0.392 ns), the fourth component represented by a (purple line) illustrates contribution from o-Ps (with mean lifetime of 2.118 ns) and the fifth line which is in (light blue color) indicates contribution from annihilations in the parafilm covering the Kapton foil and source (2.350 ns). Red curve indicates model fitted to the positron lifetime distribution. The differences in the o-Ps mean lifetime will determine which blood clots has the smaller free inter-molecular voids.

Discussion

This research shows that it is possible perform the measurements of positronium properties in fibrin clots. It paves the way for testing the hypothesis that positronium lifetime differentiates fibrin clots and blood clots of different origin, and will also allow the study of raw and

fixed blood clots under various environmental conditions, such as temperature, etc.

By using μCT we have seen that it is an imaging technique currently used in biomedical research to visualize the structure of calcified and soft tissues without damaging the sample [14]. It provides the methodology, based on the attenuation of X-rays enabling to achieve micrometric resolution and generating 3D anatomical and morphological data with micron resolution to characterize very small cell clusters like cell spheroids [15]. μCT has been used as an X-rays based imaging techniques, to characterize blood clot composition, its etiology and its amenability for pharmacological treatment, and a better understanding of thrombus structure and composition. In this study, we stained the blood clot with Lugol's iodine solution which increases and enhance μCT to evaluate blood clot imaging [16].

SEM imaging of blood fibrin network provides critical information about a clot structure including fiber orientation, branching diameter, length, fiber density, and the pore size or porosity [2]. Thrombi that occur on the surface of atherosclerotic plaques primarily include fibrin. Their development is aided by the deposition of fibrin because of local blood coagulation activity [17]. These microscopic characteristics are related with the macroscopic clot properties such as permeability resistance to lysis or viscoelasticity which are associated with the diseases [18].

Clots with thinner fibers, fewer pores and more branch points have a stiffer network reduced permeability and are more resistant to fibrinolysis. In contrast, clots formed from thicker fibers tend to have looser and less stiff networks which are more permeable and more susceptible to fibrinolysis [1].

Arterial and venous thrombotic diseases have been associated with plasma clots with increased mechanical stiffness and resistance to fibrinolysis and the structure and stability of clots may also be used to diagnose and treat bleeding disorders such as hemophilia A and B [19].

Conclusion

In conclusion we strike the feasibility that positronium can be used to analyze fibrin and blood clot structure in vitro. We hope to translate our research to in vivo studies for positronium imaging to introduce the new biomarker in cardiovascular research.

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