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Optical detection of nanoparticle agglomeration in a living system under the influence of a magnetic field

Robert Müller [a,*], Ondrej Stranik [a], Florian Schlenk [b], Sebastian Werner [b], Daniéll Malsch [a], Dagmar Fischer [b], Wolfgang Fritzsche [a]

[a] Leibniz Institute of Photonic Technology, Albert-Einstein-Str. 9, 07745 Jena, Germany
[b] Department of Pharmaceutical Technology, Institute of Pharmacy, Friedrich Schiller University, Otto-Schott-Str. 41, 07745 Jena, Germany

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ABSTRACT

Nanoparticles are important in diagnosis and therapy. In order to apply their potential, an understanding of the behavior of particles in the body is crucial. However, *in vitro* experiments usually do not mimic the dynamic conditions of the *in vivo* situation. The aim of our work was an *in vivo* observation of particle transport in chicken egg vessels in the presence of a magnetic field by particle tracking. For that we demonstrate the spatial resolution of our observations in a vein and a temporal resolution by observation of the cardiac cycle in an artery. Microscopic images were recorded in dark field reflection and fluorescence mode.

1. Introduction

Manufactured particles are attracting increasing importance in diagnosis, for drug delivery in therapy as well as in prevention [1,2]. Differences between the *in vitro* test conditions under static conditions e.g. in cell culture, and the dynamic *in vivo* conditions in the blood stream were suggested as one of the reasons for the low number of particle applications making their way through clinical to the market [3]. Blood flow may compromise the interaction between the particles and their targets. Most of the *in vitro* experiments do not mimic the dynamic conditions of the *in vivo* situation, therefore real-life extrapolations are challenging.

Chicken-based test systems using eggs and embryos represent one option for medical and toxicological research exploring nanoparticle *in vivo* behavior, but were only rarely employed [4]. So information about the use of hen egg models as *in vivo* test system to investigate the flow conditions of manufactured particles is still limited.

Blood flow has exceptional rheological properties which are due to blood composition consisting of plasma and cellular components, mainly red blood cells (RBCs). The deformation and orientation of RBCs and their ability to form rouleaux in the absence of shear stress are the key cellular factors affecting blood viscosity [5]. So far, the blood flow in the presence of nanoparticles has mainly been studied using theoretical flow simulations or artificial microchannel systems [6]. It was shown that red blood cells are moving substantially in the center of the blood stream and are showing a tumbling effect. By volume displacement and the tumbling effect of RBCs the concentration of nanoparticles is increased in the peripherial areas of the vessels [6].

The existing velocimetry studies on chick embryo were performed on candled eggs, where the eggs are backlighted by a bright light source and details show through through the shell [7,8]. It utilized μPIV (micro particle imaging velocimetry), a method for the characterization of flow conditions based on the imaging and correlation investigations of consecutive frames. These studies were focused on the calculation of wall shear stress forces. For artificial tracers like liposomes or polystyrene microparticles, fluorescence microscopy is necessary. The artificial tracers have to be injected intravenously with a glass micro needle by penetration of the vitelline membrane.

The influence of the magnification as key optical parameter in μPIV measurements was investigated for particle transport in chicken egg vessels [9]. Deviations in the measured velocities of blood cells and fluorescent polymer beads at different magnifications increase with higher magnification, caused by differences between centerline velocity and depth-averaged velocity. A magnification $< 15\times$ is recommended.

In [10] a study of the dynamics of CdSe/ZnS quantum dots and polystyrene nanospheres in the blood vessels of the chicken embryo chorioallantoic membrane was undertaken.

* Corresponding author.
E-mail address: robert.mueller@ipht-jena.de (R. Müller).

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The transport of magnetic particles in rat blood vessels was observed macroscopically using a small distance between magnetic field source (permanent magnet) and particles [11]. Three transport modes of MNP in vessels could be distinguished: velocity dominated, magnetically dominated, and a boundary layer formation. The latter one occures already in the presence of magnetic forces (by an external magnetic field gradient) of about 0.005% of the centerline drag forces (by blood flow). In the considerations a constant hydrodynamic size, i.e. no agglomeration, was assumed.

Aim of our work is the first *in vivo* observation of magnetic particle transport in chicken egg vessels in presence of a magnetic field by single particle tracking (SPT). For that we demonstrate the spatial resolution of our observations by means of non-magnetic fluorescence beads.

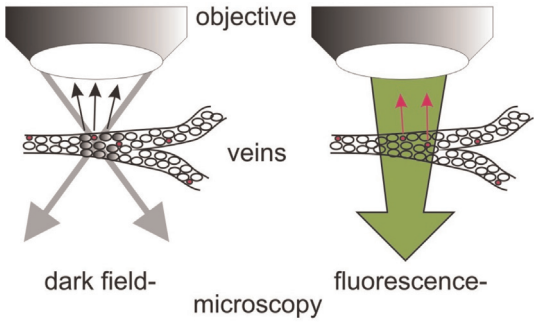

Fig. 1. Scheme of the detection modes: observation of erythrocytes and vessel topology by scattering in dark field illumination (left) and observation of fluorescence labeled particles (red) after excitation. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2. Materials and methods

For the HET-CAV (hen's egg test–chick area vasculosa), fertilized eggs of White Leghorn chicken were incubated at 37 °C and at 80% relative humidity in horizontal position. After 72 h, the eggs were poured into a Petri dish containing Ringer solution and incubated for further 24 h. Particles (25 mg/ml) were injected intravenously with a borosilicate glass capillary (World Precision Instruments, Inc., Sarasota, USA) at a volume of 1 μL using a micromanipulator MM33-R (Märzhäuser Wetzlar GmbH, Wetzlar, Germany) and a dosing volume XenoWorks microinjector (BRI 217; Sutter Instrument Co., Novato, USA) connected to a 100 μL syringe (Hamilton, Bonaduz, Switzerland). Only embryos with an intact yolk sac membrane, well-developed blood vessels and constant heart beat (0.5 Hz), were selected for the experiments. Immediately after particle injection the embryos were transferred to the microscope for flow analysis.

Two different types of particles with different physicochemical properties were used for the biological tests. Commercially available carboxylate-modified polystyrene latex beads, (L4530, Sigma-Aldrich, Germany) with a diameter of 2 μm were compared to fluorescent-labeled superparamagnetic iron oxide nanoparticles (SPIONs) with starch coating with a hydrodynamic size of 150 nm (nano-screenMAG/R-D, chemicell GmbH, Berlin, Germany), according to the suppliers information. The latex beads had a fluorescence extinction maximum and emission maximum at 470 and 505 nm, respectively. The magnetic particles showed their maxima at 578 and 613 nm.

Hydrodynamic diameter (HD) and polydispersity index (PDI) of the magnetic particles were measured at a concentration of 0.05 mg/ml by photon correlation spectroscopy (PCS) at 25 °C with a Zetasizer Nano ZS (Malvern Instruments, Herrenberg, Germany) using the Zetasizer software 7.02. The viscosity (0.8872 mPa s) and the refractive index (1.330) of distilled water and the refractive index (2.420) of iron oxide at 25 °C were used for analysis of the data. Results were calculated from three independent samples with each sample measured in five runs. Zeta potential measurements were carried out under the same conditions in a standard capillary electrophoresis cell (DTS1070, Malvern Instruments) of the Zetasizer Nano ZS.

Optical images were recorded with an optical microscope AxioImager Z1.m (Carl Zeiss, Jena, Germany) using a tungsten lamp and a PCO Sensicam camera. The microscope is operated in the dark field reflected light and fluorescence mode, respectively (see Fig. 1). The scattered light was captured only from a measuring spot that scales with the magnification of the microscope. In combination with a 5× objective the observed area had a diameter of about 1.7 mm. The picture size is 688/520 (horizontal/vertical) pixel. One pixel of the video corresponds to 2.58 μm of the object. The used camera settings are: Gain setting: normal, Binning horz./vert.: 2/2, Delay: 0 ms, Exposure: 40 ms.

Analysis is realized with TrackMate [12], a versatile SPT plugin for the image processing package Fiji [13]. It performs image segmentation, feature-based filtering, particle-linking and the visualization of the results. We employ the built-in LoG (Laplacian of Gaussian) filter for image segmentation. Tracking is based on the LAP tracker (Linear Assignment Problem) [14] with splitting and merging activated where applicable. Spots and tracks are filtered in order to exclude stagnant particles and uncertain results.

3. Results and discussion

In previous studies the blood flow in heart [7] and blood vessels [9] of chicken embryos has been studied using fluorescent artificial tracers injected into a candled egg placed in a water bath. In the present work, we selected a different setup using a shellless, planar embryo model demonstrating several advantages. The planar surface of the open egg model made the whole blood circulation accessible for investigation. Incubating the embryo in open conditions, the same vessels can be investigated for several hours to days. The velocimetry measurements are focused on the particle speed distribution over the vessel.

Erythrocyte aggregation and hemolysis depending on the particle concentration were investigated in order to see possible effects that could alter the transport properties.

The hemocompatibility of the particles indicated their suitability for administration directly into the systemic circulation. The hemolytic behavior of the particles was tested according to the ASTM F756-08 standard [15]. None of the particles did show any detectable disturbance of the red blood cell membranes (hemolysis $<2\%$) under the chosen conditions. No aggregation of red blood cells was induced by particles up to 10-fold higher concentration than reached in the embryo model (data not shown).

The hydrodynamic diameter measured by photon correlation spectroscopy was 191 ± 2 nm with a polydispersity index of 0.207 ± 0.008. The zeta potential was -5.5 ± 0.4 mV.

The behavior of the magnetic particles in a flowing medium exposed to an external magnetic field was investigated in a microfluidic channel in order to see whether particles form agglomerates irreversibly caused by magnetic interaction. The results are described below.

In vivo transport experiments in the absence of a magnetic field were done with carboxylate-modified polystyrene latex beads. In Fig. 2 (top) a dark field image revealing a large vein, smaller vessels and the red blood cells as well as a fluorescence image with the latex beads are shown. The width of the image is about