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# Effects of Blood Type and Number Concentration on the Circulation Time of Micro-sized Erythrocyte-Derived Optical Particles in Mice

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## ABSTRACT

Erythrocyte-derived optical microparticles containing near infrared (NIR) dyes such as indocyanine green (ICG) present a promising platform for fluorescence imaging and laser treatment of abnormal vasculature, including port wine birthmarks. Herein, we have investigated the effects of blood type utilized in fabricating these microparticles, and the number density of the particles on their circulation time in mice by real-time NIR fluorescence imaging of the dermal vasculature. We find that the emission half-life of microparticles engineered from human O<sup>+</sup> blood type increases by approximately two-fold as compared to those engineered from B<sup>+</sup> blood type. Increasing the number density of the microparticles fabricated from O<sup>+</sup> blood type from ~0.5 millions/ $\mu$ l to 1.6 millions/ $\mu$ l is associated with nearly a fourfold increase in the emission half-life of the particles. These findings emphasize the importance of blood type and number density in engineering erythrocyte-derived particles for clinical applications as treatment of PWBs.

**Keywords:** drug delivery, fluorescence imaging, indocyanine green, near infrared, port wine birthmarks, red blood cells

## 1. INTRODUCTION

Port wine birthmarks (PWBs) are congenital dermal capillary malformations occurring in approximately 3-5 infants per 1000 births. Initially appearing as flat red macules, over time they can darken and may result in asymmetry, distortion, and hypertrophy of bone and tissue. Patients with PWBs tend to feel different and unattractive which can result in low self-esteem and mental stress [1], [2]. The current treatment based on visible pulsed dye laser (PDL) irradiation, which targets hemoglobin, only results in about 21% of patients receiving >75% clearance [3]. About 20% of patients hardly see any lightening of their PWBs [4]. Particular shortcomings of PDL irradiation which delivers visible wavelengths in the range of 585-600 nm are non-specific light absorption by epidermal melanin, especially in patients with darker complexions, and insufficient depth of dermal vasculature photocoagulation, limited to about 300  $\mu$ m below the skin surface) [3], [5]. Therefore, there is an unmet clinical need to develop safe and effective laser therapeutic methods to treat all patients with PWBs, regardless of complexion and depth of the lesion.

Indocyanine green (ICG) is an FDA-approved near infrared (NIR) dye, and has a long-established history of usage in photomedicine due to its nontoxicity [6]. There is reduced absorption of light by melanin at NIR wavelengths. For example, at 755 nm, there is nearly a 2.6-fold reduction in light absorption by eumelanin as compared to 585 nm [7], [8]. Therefore, use of ICG as the target chromophore, administered into the vasculature, in conjunction with NIR laser irradiation may provide an alternative approach to PDL treatment.

ICG, however, has a short half-life of about 3-5 minutes in bloodstream [9]. To extend the circulation time of ICG, our group has encapsulated ICG into hemoglobin-depleted erythrocyte ghosts (EGs) [10]–[13]. The fabricated EGs retain key glycoproteins CD 47, CD 55, and CD 59 that protect the erythrocytes from phagocytosis by macrophages and attacks by the complement system [14], [15]. We refer to EGs doped with NIR optical agents such as ICG, as near infrared erythrocyte-derived transducers (NETs). In this study, we investigate the effects of blood type utilized in fabricating the NETs, and number concentration of NETs on the resulting circulation kinetics of the particles in a C3H mouse model.

## 2. METHODOLOGY

To fabricate NETs, erythrocytes were isolated from human whole blood by centrifugation (1000g at 4°C for 5 minutes), and then subjected to hypotonic treatment by incubation in 80 mOsm phosphate buffer saline (PBS) for 15 minutes at 4 °C followed by centrifugation (20,000g at 4°C for 15 minutes). This process was repeated six times to produce

hemoglobin depleted EGs. To encapsulate ICG, the EGs were incubated in a loading buffer containing Sørensen's buffer and ICG dissolved in water at 1.1 mM for 20 minutes at 4°C in the dark. The solution was then centrifuged to obtain the NETs pellet, which was then stored in isotonic PBS. This fabrication methodology was consistently applied to both human B<sup>+</sup> and O<sup>+</sup> positive blood samples. Prior to each experiment, the sample NET concentration was determined using a hemocytometer to ascertain the number density. Isotonic PBS was added or removed to create the specified number density for each trial.

We used C3H mice to investigate the circulation dynamics of the various NETs samples. Animal experiments were performed under a protocol approved by the Institutional Animal Care and Use Committee at University of California, Irvine (Protocol number AUP-23-018). A dorsal window chamber was placed on the back of the mice [12], [13]. The NETs suspension (~150 μl) was administered by retro-orbital injection. Following injection, near infrared fluorescence images were obtained by an EMCCD in response to excitation at 785 nm and emission in the range of 815-845 nm every second for the first 5 minutes, then every minute for 10 minutes, and finally every 5 minutes for the final 45 minutes.

### 3. RESULTS

An illustrative sequence of NIR fluorescence images of a mouse vasculature in response to administration of μNETs fabricated from O<sup>+</sup> blood type (O<sup>+</sup>-μNETs) is shown in Figure 1. The maximum emission intensity was between 1- and 10-minutes following injection. At 60 minutes, post-injection, the vasculature could still be resolved with respect to background.

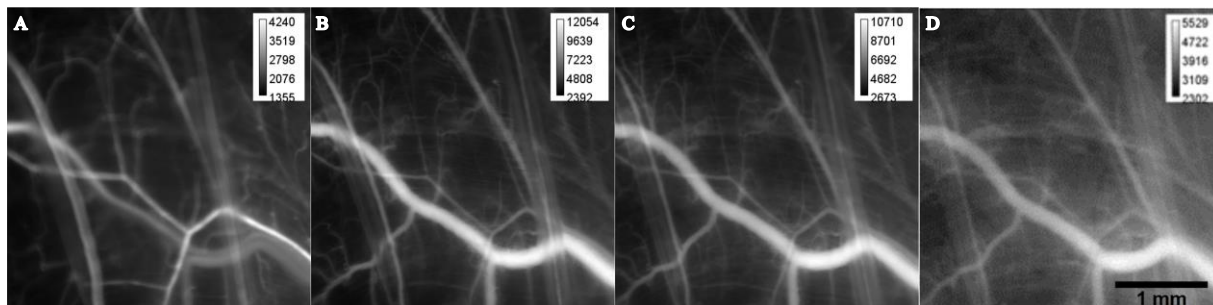


Figure 1: Illustrative NIR fluorescence images of a C3H mouse vasculature at (A) 1 minute, (B) 10 minutes, (C) 30 minutes, and (D) 60 minutes following retro-orbital injection of O<sup>+</sup>-μNETs. The number density of O<sup>+</sup>-μNETs was ~1.6 millions/μl.

Increasing the number density of O<sup>+</sup>-μNETs was associated with increased half-life of the emission intensity (Figure 2A). For example, the intensity half-life of O<sup>+</sup>-μNETs administered at number density of ~0.5 millions/μl was increased from ~11.7 minutes to ~38.6 minutes when administered at ~1.6 millions/μl. The blood type used in fabricating

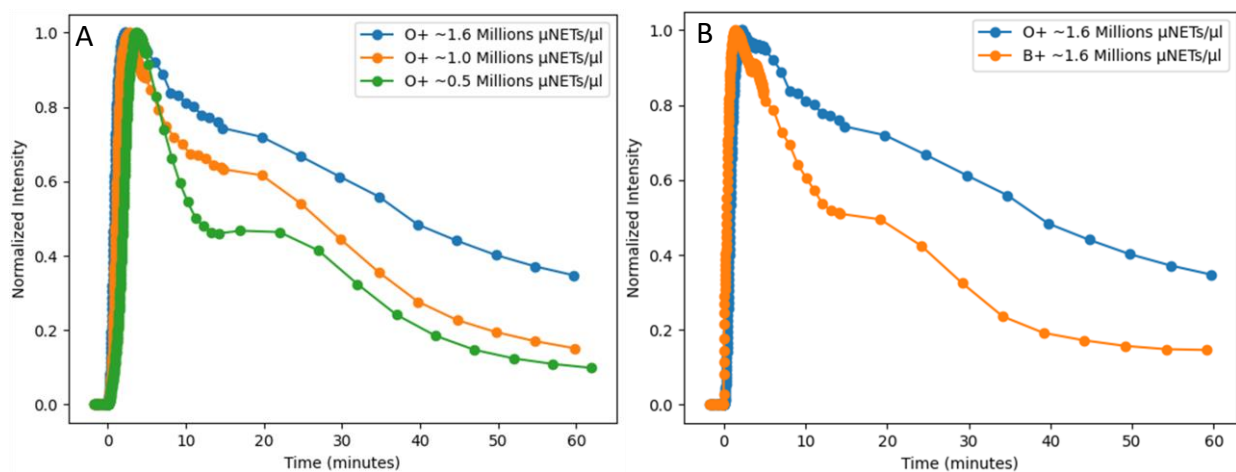


Figure 2: Illustrative fluorescence emission decay kinetics of (A) O<sup>+</sup>-μNETs administered at number densities of ~0.5, 1.0, and 1.6 millions/μl, and (B) O<sup>+</sup>-μNETs and B<sup>+</sup>-μNETs administered at number density of ~1.6 millions/μl from dermal vasculature of C3H mice.

the  $\mu$ NETs also influenced the emission kinetics of the circulating particles from the bloodstream (Figure 2B). For the same number density of  $\sim 1.6$  millions/ $\mu\text{l}$ , the intensity half-life of  $\mu$ NETs fabricated from B<sup>+</sup> blood type (B<sup>+</sup>- $\mu$ NETs) was  $\sim 16.7$  minutes as compared to  $\sim 38.6$  minutes for O<sup>+</sup>- $\mu$ NETs.

#### 4. CONCLUSION

Our intravital NIR fluorescence imaging of C3H mice vasculature suggest that number density and blood type used in fabricating  $\mu$ NETs influence the circulation kinetics of  $\mu$ NETs. Increased circulation times of  $\mu$ NETs are associated with use of higher number densities of  $\mu$ NETs, and  $\mu$ NETs fabricated from O<sup>+</sup> blood type. These results also suggest that O- $\mu$ NETs may serve as a promising universal erythrocyte-based delivery system. Our findings also contribute to advancing the development of effective treatments for individuals with PWBs using erythrocyte-derived particles containing NIR chromophores.

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