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Accumulation of nano-sized particles in a murine model of angiogenesis

Thomas R. Wittenborn [a,*,1], Esben K.U. Larsen [b,c,d,1], Thomas Nielsen [a,b], Louise M. Rydtoft [e], Line Hansen [b,c,d], Jens V. Nygaard [b,f], Thomas Vorup-Jensen [d,g], Jørgen Kjems [b,c,d], Michael R. Horsman [a], Niels Chr. Nielsen [b,d,h]

[a] Department of Experimental Clinical Oncology, Aarhus University Hospital, Noerrebrogade 44, 8000 Aarhus C, Denmark
[b] Interdisciplinary Nanoscience Center (iNANO), Aarhus University, Gustav Wieds Vej 14, 8000 Aarhus C, Denmark
[c] Department of Molecular Biology and Genetics, Aarhus University, C.F. Moellers Allé 3, 8000 Aarhus C, Denmark
[d] The Lundbeck Foundation Nanomedicine Center for Individualized Management of Tissue Damage and Regeneration (LUNA), Aarhus University, Aarhus, Denmark
[e] Center of Functionally Integrative Neuroscience (CFIN), Aarhus University Hospital, Noerrebrogade 44, 8000 Aarhus C, Denmark
[f] Department of Engineering, Aarhus University, Finlandsgade 22, 8000 Aarhus, Denmark
[g] Department of Biomedicine, Aarhus University, Bartholins Allé 6, 8000 Aarhus, Denmark
[h] Center for Insoluble Protein Structures (inSPIN) and Department of Chemistry, Aarhus University, Gustav Wieds Vej 14, 8000 Aarhus C, Denmark

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ABSTRACT

Purpose: To evaluate the ability of nm-scaled iron oxide particles conjugated with Azure A, a classic histological dye, to accumulate in areas of angiogenesis in a recently developed murine angiogenesis model.
Materials and methods: We characterised the Azure A particles with regard to their hydrodynamic size, zeta potential, and blood circulation half-life. The particles were then investigated by Magnetic Resonance Imaging (MRI) in a recently developed murine angiogenesis model along with reference particles (Ferumoxtran-10) and saline injections.
Results: The Azure A particles had a mean hydrodynamic diameter of 51.8 ± 43.2 nm, a zeta potential of −17.2 ± 2.8 mV, and a blood circulation half-life of 127.8 ± 74.7 min. Comparison of MR images taken pre- and 24-h post-injection revealed a significant increase in R_2^* relaxation rates for both Azure A and Ferumoxtran-10 particles. No significant difference was found for the saline injections. The relative increase was calculated for the three groups, and showed a significant difference between the saline group and the Azure A group, and between the saline group and the Ferumoxtran-10 group. However, no significant difference was found between the two particle groups.
Conclusion: Ultrahigh-field MRI revealed localisation of both types of iron oxide particles to areas of neovasculature. However, the Azure A particles did not show any enhanced accumulation relative to Ferumoxtran-10, suggesting the accumulation in both cases to be passive.

1. Introduction

Angiogenesis has been established as a common feature of many pathological conditions including diseases such as atherosclerosis and cancer [1,2]. During the angiogenetic process and in the initial phase of neovascular maturation, the vessels tend to be fragile and leaky because of a discontinuous or missing basal membrane [3,4]. These leaky vessels introduce a potential route of transport from the blood into the extravascular extracellular space (EES) and extracellular matrix (ECM) [5–7]. The ECM contains an abundance of heavily glycosylated proteins, in particular sulphated glycosaminoglycans [8], which under physiological conditions are negatively charged.

These combined features can be exploited for drug delivery or imaging [5–7,9], and has made angiogenesis a desired target for new nanotechnological approaches. The field of nanotechnology has expanded markedly within the past decades and has now developed to such an extent that it has become possible to manufacture tailor-made nm-scaled particles to target specific biomarkers of biological processes [10–15].

Azure A, a small positively charged metachromatic dye used for decades in histology [16], has been known to stain DNA, azurophilic granules of leukocytes, and other negatively charged molecules [17–20]. By conjugating this small dye to a nano-sized particle it could enable binding of the particle to the negatively charged proteoglycans in the ECM.

Here, we describe the physicochemical characterisation and accumulation properties of such an Azure A-conjugated iron oxide

* Corresponding author. Address: Department of Experimental Clinical Oncology, Aarhus University Hospital, Nørrebrogade 44, Building 5, 2nd floor, DK-8000 Aarhus C, Denmark.
E-mail address: Wittenborn@oncology.dk (T.R. Wittenborn).
[1] These authors contributed equally to this paper.

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nano-sized particle. Hydrodynamic size, zeta potential, and blood circulation half-life were estimated using standard methods, and its ability to localise to areas of angiogenesis was assessed by ultrahigh-field MRI in a recently described murine angiogenesis model [21].

2. Materials and methods

2.1. Materials

Diethyl ether, 3-Amino-7-(dimethylamino)-phenothiazin-5-ium (Azure A) and (3-Amino-propyl)-triethoxysilane (Si-NH_2) were purchased from Sigma–Aldrich, St. Louis, MO, USA. NHS-PEG-Maleimide (PEG molecular weight of 2000 Da) was purchased from IRIS biotech GmbH, Marktredwitz, Germany. Methoxy PEG succinimidyl active ester (NHS-PEG; PEG molecular weight of 2000 Da) was purchased from Rapp-polymere GmbH.

2.2. Synthesis of Azure A conjugated nano-sized particles

The synthesis of oleic acid-coated iron oxide particles is described by Larsen et al. [7]. Iron oxide particles (50 mg) coated with oleic acid were dissolved in 5 mL toluene. Under constant stirring, 10 μL Si-NH_2 (55.8 μmol), 38 mg NHS-PEG (50.7 μmol), 0.8 mL triethylamine (TEA) and 80 μL H_2O were added. After overnight reaction particles were heated to 105 °C for 1 h and washed in toluene/pentane three times. To conjugate Azure A, 15 mg particles were first conjugated to 90 mg NHS-PEG-Maleimide (45 μmol) and reacted together with 90 μL DIC (714 μmol) and 200 μL TEA in toluene for 24 h. Particles were washed in toluene/pentane three times. Then 9 mg Azure A was added to the particle formulations in 1 mL DMSO with 60 μL TEA for 24 h. Finally, the particles were precipitated with diethyl ether. Particles were purified by dialysis (6–8 kDa cut-off) against PBS for 5 days, changing the solute twice a day. Following dialysis the particles were centrifuged to remove aggregates.

2.3. Characterisation of functionalized particles

The hydrodynamic size and zeta potential of the Azure A particles were assessed by dynamic light scattering (DLS) using a Zetasizer Nano ZS (Malvern Instruments, Malvern, UK). Measurements were made in phosphate buffered solution at pH 7.0 and 25 °C. Three consecutive measurements were performed and the mean ± standard deviation was calculated.

To determine blood circulation half-life, particles were diluted in saline and injected intravenously (i.v.) into male CDF1 mice. Three mice were injected with the Azure A particle formulation and six mice were injected with the Ferumoxtran-10 formulation. A volume of 10 μL/g of mouse body weight was administered at a dose of 5 mg Fe/kg. A control group with three mice received i.v. saline injections also in a volume of 10 μL/g. Blood samples were drawn from the sub-orbital sinus of animals prior to injection and 5, 60, 360 and 1440 min after injection. Blood was collected in EDTA tubes, and blood samples were centrifuged to obtain EDTA-plasma. Samples were digested using a solution of 5.5 mL Nitric acid (HNO_3) and 0.5 mL Hydrochloric acid (HCl, 12 M), which was heated to speed up the process. Iron concentrations in the samples were analysed by inductively coupled plasma atomic emission spectroscopy (ICP-AES) using a Plasma 2000 (Perkin-Elmer, USA) as described elsewhere [22]. Blood circulation half-life was calculated based on linear regression of the exponential washout of the injected particles.

2.4. Angiogenesis model

Fifteen male CDF1/Tac mice (12–20 weeks old) were included in the experiment. Poly-capro-lactone (PCL) discs were made in-house and prepared according to Andersen et al. [23]. The procedure for implantation of porous PCL discs has been previously described [21]. In brief, a small incision (1.5 cm) was made in the skin on the back, through which the PCL discs (measuring 8 mm in diameter and 2–3 mm in height) were subcutaneously implanted. After surgery the mice were caged individually for 3–4 weeks, which was the optimal time point after implantation for a maximum amount of neovessels [21]. Mice were distributed equally into three groups: a saline group, a reference group (Ferumoxtran-10), and an Azure A-conjugated group (Azure A).

2.5. MRI of injected particles

A 16.4 T spectroscopy/imaging system (wide-bore Bruker 700 Avance-II, Bruker Biospin, Rheinstetten, Germany) equipped with a GREAT 60 gradient system with a maximum gradient strength of 1.5T/m and a Micro 2.5 probe (coil inner diameter 25 mm) was used for the in vivo MRI. Mice were anaesthetized by intraperitoneal (i.p.) injection (10 μL/g of a mixture of 10 mg/mL ketamine and 1 mg/mL xylazin) and supplemented with top-up doses (5 μL/g) when needed. An i.p. line was connected to a syringe primed with anaesthesia to administer top-up doses. A respiration-monitoring pad was attached to the abdomen of the mice using tape and connected to a monitoring system. Mice were placed with the tissue of interest aligned to the middle of the coil. Mice were restrained using tape and the entire setup was inserted into the vertical MR scanner. Here mice were kept warm by a circulating water system around the gradients (30 °C). A pre-scan was performed and after that particles were diluted in saline and administered i.v. at a dose of 2.5 mg Fe/kg (resembling a concentration of 0.25 μg/μL injected at 10 μL/g). In the saline group, saline was administered in equal amounts (10 μL/g), and 24 h post-injection all mice were scanned again (24 h post-scan).

R_2 and R_2^* measurements were performed before and 24 h after administration of the particles using gradient echo and spin echo sequences. The measurements were performed on 10 transverse slices of 0.5 mm thickness and 0.5 mm spacing. For both gradient echo and spin echo experiments additional scanning parameters were: T_R = 4000 ms, field of view = 25 × 25 mm, acquisition matrix 256 × 192 reconstructed to 256 × 256, acquisition matrix 256 × 128 reconstructed to 256 × 256, or acquisition matrix 128 × 96 reconstructed to 128 × 128, and number of averages = 1. In the gradient echo sequence, the flip angle was 90°, and 12 T_E within the interval 3.4–58.4 ms were used, and in the spin echo sequence, 12 T_E values within the interval 8.1–117.3 ms were used.

Data analysis was performed using MATLAB 7.11 (The MathWorks, Inc., Natick, MA, USA). R_2 and R_2^* maps were produced by nonlinear least squares fitting of the image signal S for different echo times (T_Es) to the equation $S(T_E) = S(0)\exp(-T_E \cdot R_2^{(*)})$. Regions of interest (ROIs) were drawn manually on a raw image to contain the entire implanted scaffolds. From the characterisation of the angiogenesis model [21] we learned that the vasculature covered most, but not all, of the implant after 3–4 weeks. To avoid non-vascularized areas in our analysis we did the following: for the pre-scan ROIs, voxels with $R_2 < 25\ s^{-1}$ and $R_2^* < 95\ s^{-1}$ were removed for R_2 and R_2^* analysis, respectively. These values were based on the bimodal shape of the pooled ROI histograms for all the animals (Fig. 1). Because the mice were repositioned before the 24 h post-scan, and new ROIs were drawn, we assumed that the same ROI percentage was vascularised at this time point. From the post-scan ROIs we then excluded the lowest R_2 or R_2^* values leaving the same ROI voxel percentage as in the pre-scan for analysis.

Some of the obtained images suffered from water-fat shift, susceptibility or motion artefacts, resulting in poor image quality and overestimated relaxation rates. In some spin-echo images the