Development, in vitro characterization & in vivo testing of multimodal nanoparticles in an animal model

PhD thesis

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1. Introduction

The term “Quality by Design” (QbD) is highlighting the need of built-in-quality into goods and services by closely detecting five critical steps: costumers and their needs must be determined, followed by product features, development reaching to the final step – transferring it into practice and manufacturing. The quality and process-oriented perspective of the pharmaceutical industry helped in the early adoption of the aforementioned approaches and methods. Nevertheless, the concept of QbD has only been taken over in the beginning of the 21st century, by the biggest regulatory authorities – the U.S. Food and Drug Administration (FDA) and the European Medicines Agency (EMA).

The tools of drug development are sophisticated: molecular dynamics, protein interactions and routes of metabolism can be calculated, in silico assays for human organs are widely available and used. The development of fine-tuned nanocarriers and nano drug delivery systems (nDDS) have only been investigated since the late 90s, showing a strongly increasing tendency in the field of successful licensing, therapeutic efficacy and safety. The toxicology as well as the detailed examination of such novel, nano-sized materials, however, still requires extensive screening not only in computer simulations and in silico testing, but in vivo experiments too, as the most complex, multivariate system is still the living body.

The development of such materials for imaging purposes is required: MRI, as a powerful imaging method for functional and morphological investigation, is a widely used whole-body diagnostic device used in the daily clinical practice, however, in most cases the intrinsic contrast of the different tissues is insufficient for the desired spatial resolution, thus requiring the application of a variety of extrinsic (injected) contrast agents (CAs). The most commonly used Gd (III) materials lack the in vivo stability, causing toxic side-effects for the patiens; materials other than Gd (III) available lack the required in vivo contrast for the MRI imaging.

A possible candidate could be the FDA authorized Prussian blue (PB), which is used to treat heavy metal poisoning since 2003. Its uniquie structure allows the control of size, shape and biocompatibility, nevertheless, non-functionalized PBNPs show less significant T1 and T2 signal changes in vitro; their measured longitudinal and transversal relaxation times did not suggest their in vivo use. Therefore, functionalizing PBNPs, to achieve multimodal contrast would highly increase their impact for preclinical applications, thus in the routine procedures MRI is oftentimes coupled with other modalities providing greater functional contrast.

2. Objectives

Our studies were focused on creating a multimodal platform with fluorescent labelling of Prussian blue nanoparticles (PBNPs) with commonly used dye-candidates, with the following aims:

I. To create a nanoparticle platform, with quality requirements as follows:
   - a stable nanosystem, which does not aggregate over 8 weeks of examination period;
   - the emission of a fluorescent signal that could be detected in living organisms to make the particle suitable for in vivo imaging;
   - a clearance mechanism to reduce possible side effects but also to slow down accumulation in the monocyte-macrophage system.

II. To create a fluorescently labeled Prussian blue nanoparticle (PBNP) based nanosystem for preclinical fluorescent imaging;

III. To also take advantage of the biocompatible coating of the particle, thus connecting the potential fluorescent dyes and stabilizers to PBNPs

IV. To investigate the fluorescent imaging capabilities of the successfully labelled PBNP in a model system over 3 hours, in clinically relevant, translational study.

Furthermore, based on the obtained knowledge on the fluorescent labelling of PBNPs, we aimed to enhance the multimodal relevance of the PBNP platform, by aiming:

V. To develop and characterize a Prussian Blue based biocompatible and chemically stable T1 magnetic resonance imaging (MRI) contrast agent with near infrared (NIR) optical contrast for preclinical application.
   - As part of the in vivo studies, investigating the physical properties of beforementioned nanoparticulate system by DLS, Zeta-potential measurements, AFM and TEM as well as the MRI contrast enhancement capabilities

VI. To investigate the T1-weighted contrast of the prepared PBNPs in vivo, furthermore,

VII. To measure the NIR fluorescence of the samples with optical imaging modality after intravenous administration into NMRI-Foxn1 nu/nu mice.

3. Materials & Methods

3.1. Methods of nanoparticle synthesis

3.1.1. Production of uncoated Prussian Blue nanoparticles

Native PBNPs were synthesized according to as described by Shokouhimehr [37], with modifications. As first step, the reactant solutions were prepared with Solution A containing Fe(III) chloride anhydrous with 6 drops of 1 N HCl while Solution B containing 20 mL of 1.0 mM potassium ferrocyanide anhydrous with 6 drops of 1 N HCl (Merck KGaA). Secondly, these solutions were mixed slowly under vigorous stirring for 10 min at 60 °C (88).

3.1.2. Citrate coated Prussian Blue production

Citrate-coated PBNPs were produced with the process as described by Shokouhimehr [37]. A two-step PBNP preparation was made.

3.1.3. Fluorescent labelling of the particles with Eosine Y, Rhodamine B and Methylene Blue

Eosin Y and Rhodamine B were adsorbed to the particles. For fluorescent labelling, the concentrated methylene blue (MB) stock solution was diluted twofold and filtered through a 0.22 µm pore size membrane filter. Further, 200 µL of this filtered solution was added to 2 mL of the PBNP solution. This would result in absorbed MB on the mesoporous surface of the biocompatible PBNPs.

3.1.4. PEGylation of Prussian Blue nanoparticles

For the PEGylation, PEG 3000 was available in monodisperse solution, while PEG 6000 and PEG 8000 were commercially sold in solid form. At first, these power-based agents were dissolved in 50% ethanol–water mixture the final PEG content was set to 10 w/w%. PBNP-MB solutions (2 mg/mL) were prepared by adding distilled water to the stock MB-labelled PBNP solution. After incubation at room temperature, different PEG solutions (PEG 3000, PEG 6000, and PEG 8000) were added to the PBNP-MB solutions and dialyzed for 24 h (14 kDa filter) in phosphate buffer saline solution (pH = 6.8; Ph.Eur. 8.). The particles were prepared and characterized in the following with DLS and Zetasizer instruments.

3.1.5. Preparation of fluorescent Prussian Blue nanoparticle complexes with labelled IR820 dye

Following the coated and uncoated particle syntheses, the two different types of PBNPs were mixed under vigorous stirring for 10 min at 60 °C. With 10 minutes passed, 5 g Chelex (chelating ion exchange resin,/100 mL solution was applied to eliminate the superfluous metal or alkali metal ions from the system. This suspension was stirred and incubated for one hour, whereby the styrene divinylbenzene copolymer beads were separated from the PBNP solution. In the next step, PBNPs were isolated from the complex suspension using ultracentrifugation at 4 °C for 30 min.

To achieve fluorescence in the PBNPs, 0.1 mg/mL IR820 NIR dye was filtered through a 0.22 µm pore size membrane filter. 10 µL of this filtered dye solution was adsorbed to the particles in 300 µL PBNP solution for a one-hour incubation.

3.2. Methods of particle characterization in vitro

3.2.1. Dynamic light scattering (DLS) and zeta-potential measurement

The surface charge and hydrodynamic diameter of the particles were determined using a Malvern Nano ZS and Anton Paar Litesizer 500. DLS measurement was performed at 25 °C in automatic mode using a 633 nm He-Ne laser. Samples were measured in Omega cuvettes. Measurement of zeta-potential was performed under similar conditions. The measurement data were evaluated using software provided by the manufacturer. DLS measurements were performed weekly for a period of 4 weeks to determine colloidal stability.

3.2.2. Atomic Force Microscopy (AFM)

For imaging PBNP complexes, two-fold diluted samples were applied onto poly-L-lysine (PLL)-coated surfaces. PLL-coated substrate surface was prepared by pipetting 100 µL of PLL (0.1% w/v) onto freshly cleaved mica, followed by incubation for 20 min, repeated rinsing with purified water, and drying with a stream of high-purity nitrogen gas. After a 10 min incubation time, mica surface was dried in N_2 stream. AFM images were collected in noncontact mode

3.3. Methods for in vivo particle characterization

3.3.1. In vivo MRI measurements

MRI measurements were performed in vitro with a nanoScan® PET/MR system (Mediso, Hungary), having a 1 T permanent magnetic field, 450 mT/m gradient system using a volume

transmit/receive coil with a diameter of 60 mm. MRI T1 relaxation rates and r1 relaxivity were calculated from inversion prepared snapshot gradient echo images. Experiments were performed in an adult male mouse under isoflurane anesthesia (5% for induction and 1.5–2% to maintain the appropriate level of anesthesia; Baxter, Arrane). Precisely, 300 µL of IR820-labelled PBNP solution containing 3 mg of Fe(Fe (III) in a 30 mg/mL concentration PBNP solution was administered intravenously into the tail. The T1-weighted MRI biodistribution images were collected at two different time points (pre- and post-injection) The MRI scans were performed with gradient echo sequence. Images were further analyzed with Fusion and VivoQuant dedicated image analysis software (88, 89).

3.3.2. In vitro and in vivo Fluorescence-labeled Organism Bioimaging Instrument (FOBI) measurements

The fluorescent labelled PBNPs were imaged using a two-dimensional epifluorescent optical imaging instrument. For in vitro scans, 0.5 mL of samples were tested with the following imaging parameters: excitation at 680 nm corresponding to the excitation maximum of the dye (excitation: 690 nm; emission: 820 nm), exposure time: 1000 msec and gain: 1. The emission spectrum of the dye was in the pass band of the used emission filter.

The in vivo experiments were performed in an adult male mouse under isoflurane anesthesia (5% for induction and 1.5–2% to maintain the appropriate level of anesthesia; Baxter, Arrane). Precisely, 300 µL of fluorescently labelled PBNP solution was administered intravenously into the tail vein. The biodistribution images were collected at two different time points (pre- and post the injection).

4. Results and Discussion

4.1. PEGylated, fluorescent Prussian blue nanoparticles

4.1.1. Preformulation

Preforulation studies include but are not limited to only physico-chemical and analytical investigations of a previously known material, but also the investigation of the need of possible excipients, biopharmaceutical and therapeutic availability studies.

In order to achieve this, we used our previous knowledge, for the temperature dependence of the PBNP formulation, and investigated the surface charge and size-distribution of each sample, in every formulation. Thus, not all samples were amenable to fluorescent imaging in vivo, furthermore, they lacked stability during the early inspections. The aggregation of the nanosystem along with fluorescent dye wash-off was noted.

Fluorescein labelling of PBNPs was not successful. Experimenting with other dyes we found that Rhodamine B, Eosine Y and Methylene blue sorption to PBPNs was feasible. However, Rhodamine B labelled PBNPs lacked stability: immediately after labelling, aggregation was visible. The long-term stability of Eosine Y and MB labelled PBNPs and their in vivo imaging signalling performance was thus further investigated.

4.1.2. In vitro results

Atomic Force Microscopy (AFM)

AFM images are shown in Figure 1. PBNPs appeared as objects with a flat rectangular surface protruding from a rounded halo (Figure 1a). The rectangular surface represents the real geometry of the particles (Figure 1c) while their halo is the consequence of tip convolution, i.e., the effect of imaging a rectangular prism by a tetrahedral AFM tip. Rectangularity of the particles (together with their halo) was found to be 0.7701 ± 0.1041 (mean ± SD), indicating that PBNPs indeed represent rectangular topography. The height of the particles showed monomodal distribution with a mean ± SD of 17.790 ± 8.922 nm (Figure 1).

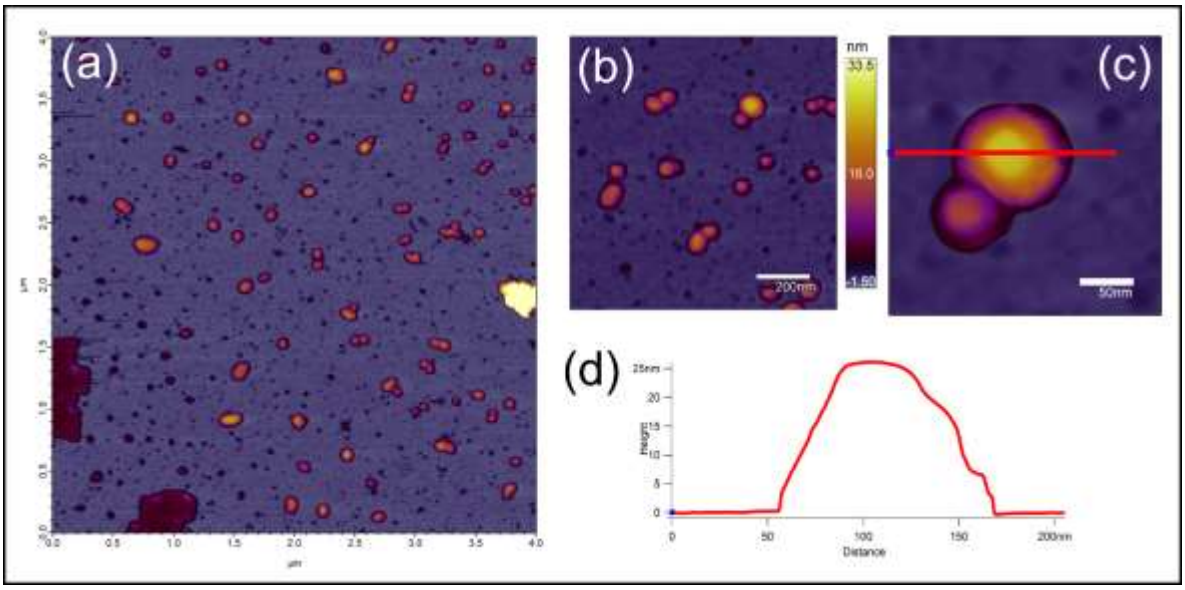

Figure 1. (a) Height-contrast AFM image of PBNPs on the mica surface (scale bar = 4 µm × 4 µm). (b) Modified PBNPs on the mica surface; height-contrast AFM image (scale bar = 200 nm). (c) A modified PBNP nanoparticle (scale bar = 50 nm). (d) The cross-section graph of (c) PBNP nanoparticle (abscissa = 0–200 nm; ordinate = 0–25 nm).

Measuring In Vitro Fluorescence

After the two main approaches of fluorescent labelling, PBNP-MB@PEGs were tested in vitro, to study whether the emitted signal is adequate for in vivo imaging. For that purpose, 20-fold diluted samples were produced and measured, along with the stock PBNP-MB@PEG solutions. The samples prepared according to method a showed lower intensity on the emitted light, on the other hand, method b provided a significantly better signal in emission, which made the sample a better choice for in vivo use (Figure 2 a,b).

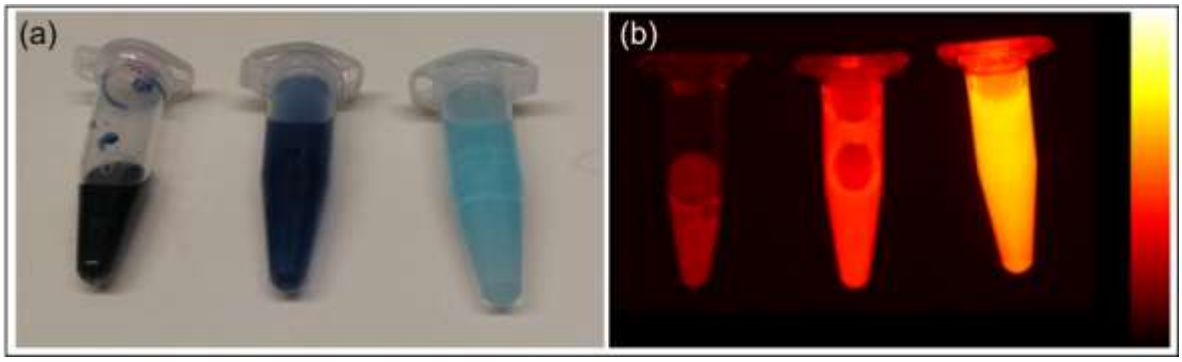

Figure 2. (a) PBNP-MB@PEG6000_100µL (stock solution, 5× and 20× dilution left to right). (b) Fluorescent signal of PBNP-MB@PEG6000_100µL dilutions in FOBI.

Stability Measurements of PBNP-MB@PEG Nanoparticles

The mean zeta potential of all the samples had a net negative potential of −25 mV or greater. During the four week stability test, the mean hydrodynamic diameter, non-modified PBNPs size showed a slight change in the diameter, i.e., after the third week, all PEGylated particles started to aggregate. PEG6000 containing nanoparticles were the most stable, along with the lowest PDI and diameter, keeping a solid size of 24.82 ± 5.83 nm (number-based particle size distribution). However, aggregation was detected in the majority of the samples after 4 weeks of investigation.

4.1.3. In Vivo and Ex Vivo Measurements

The fluorescent labelling and imaging: Methylene blue labelled Prussian blue nanoparticles

Due to the strong visible fluorescent signal of PBNP-MB@PEG6000s, 200 µL of the suspension of nanoparticles was injected into the lateral tail vein of C57BL/6 male mice (Figure 3 (A, B)). The semiquantitative distribution of the labelled PBNPs was determined based on their normalized mean fluorescent intensity. On the ex vivo images (Figure 3 (B), taken 3 h after the first injection, PBNP-MB@PEG6000 accumulation can be observed in the gastrointestinal tract, kidneys, spleen, liver, and heart.

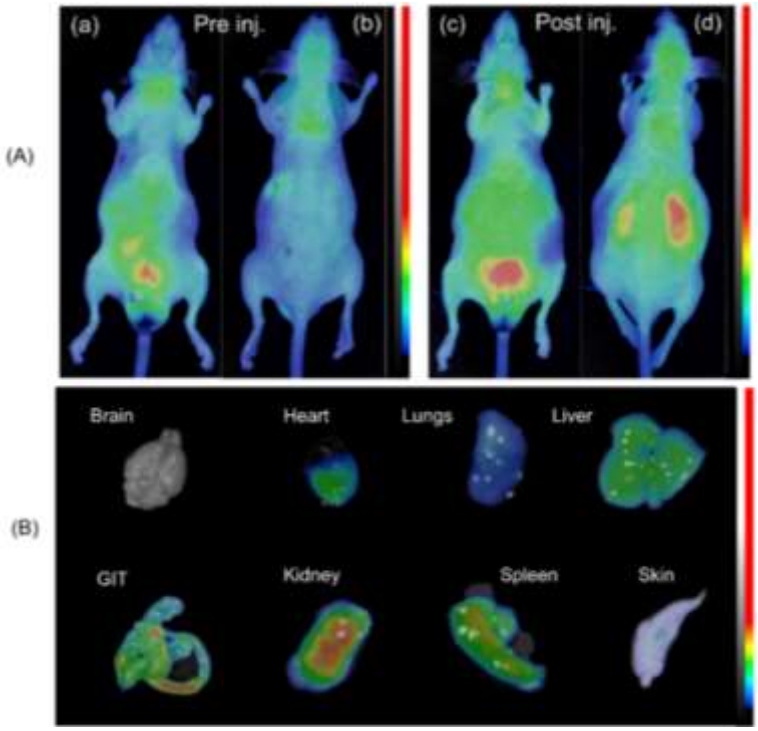

Figure 3. (A) (a,b) C57BL/6 mice before intravenous treatment from anterior and posterior planes. (c,d) C57BL/6 mice pre-PBNP treatment (anterior and posterior planes). (a) anterior, preinjection; (b) posterior, preinjection; (c) anterior, postinjection; and (d) posterior, postinjection. Color scale equals mean fluorescent intensity (MFI) in arbitrary units (A.U.) Figure 4 (B) ex vivo images, 3 h post injection

4.2. Prussian blue-based dual fluorescent and magnetic contrast agent

The final PBNP complex nano structure was prepared by the combination of Shokouhimehr's method and our previously published one-step citrate coated PBNP procedure. The synthesis of PBNPs with and without coating resulted in two different types of PBNP solutions, which were mixed. During the incubation and mixing period, the particles were able to connect to each other via carboxyl groups of citric acid and a form a bigger and iron-richer formula. The porous surface of the nanoparticles assured the conjugation points for the fluorescent IR820 dye (Figure 4).

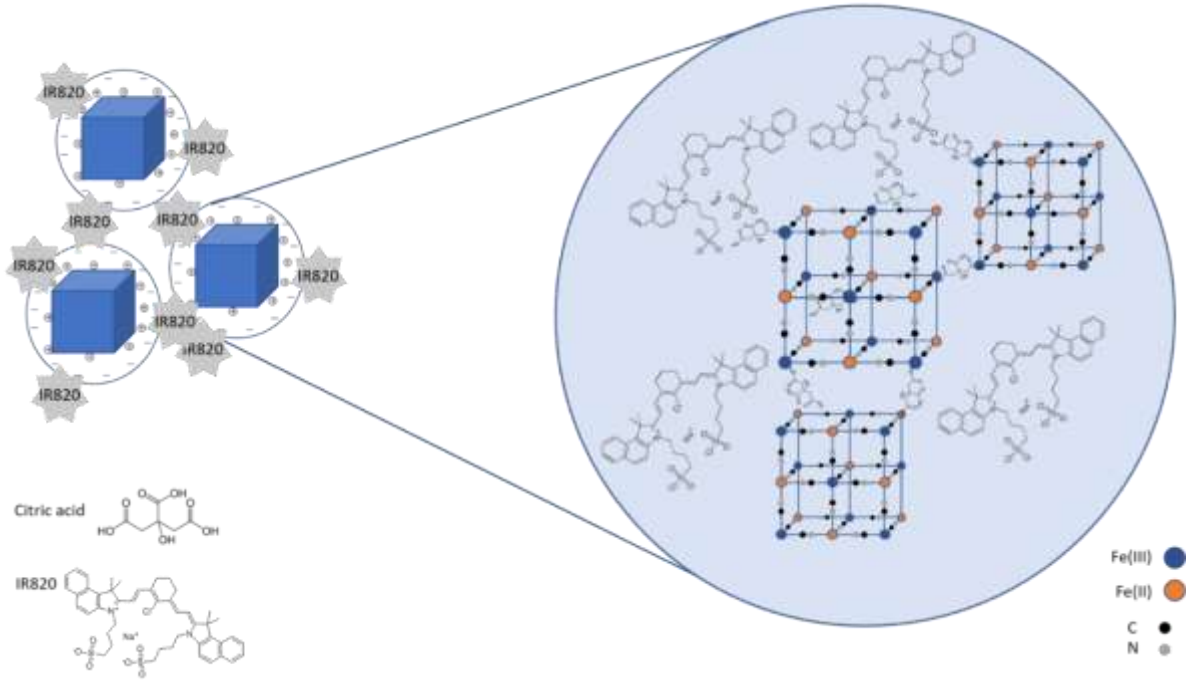

Figure 4. Schematic illustration of the presumed connection of Prussian blue nanoparticles (PBNPs) with and without coating and the particle conjugation by IR820. The blue halo around the PBNPs represent the non-biocompatible PBNP-species (PB-HCl) acting as a coating surface. The colors represent the following ions or atoms, respectively: blue: Fe(III)); orange: Fe(II); black: C; gray: N(88).

4.2.1. In vitro results

DLS and zeta potential

The applied citric acid as surface-capping agent controlled the size and the biocompatibility of the synthetized particles and seemed an appropriate agent to avoid aggregation. The created nanoparticles were a colloidally stable system. The mean hydrodynamic diameter (intensity-based harmonic average) of complex PBNPs was 82.91 ± 1.21 nm (average ± SD), as determined by DLS. There was no significant colloidal alteration during the 4-week duration of the study, as the

calculated 0.244 ± 0.014 polydispersity index (PDI) shows. The mean zeta potential of PBNPs at pH 7.4 was −33.3 ± 3.8 mV (n=3).

Atomic Force Microscopy

The measured width of the particles was influenced by tip convolution. Figure 7a shows PBNPs on AFM images as objects with a flat rectangular surface protruding from a rounded halo. The rectangular surface represents the real geometry of the particles while their halo is the consequence of tip convolution, i.e., the effect of imaging a rectangular prism by a tetrahedral AFM tip. Rectangularity of the particles (together with their halo) was found to be 0.774 ± 0.111 (mean ± SD), indicating that PBNPs indeed represent rectangular topography. The height of the particles was 36.457 ± 9.496 nm (mean ± SD) (Figure 5.).

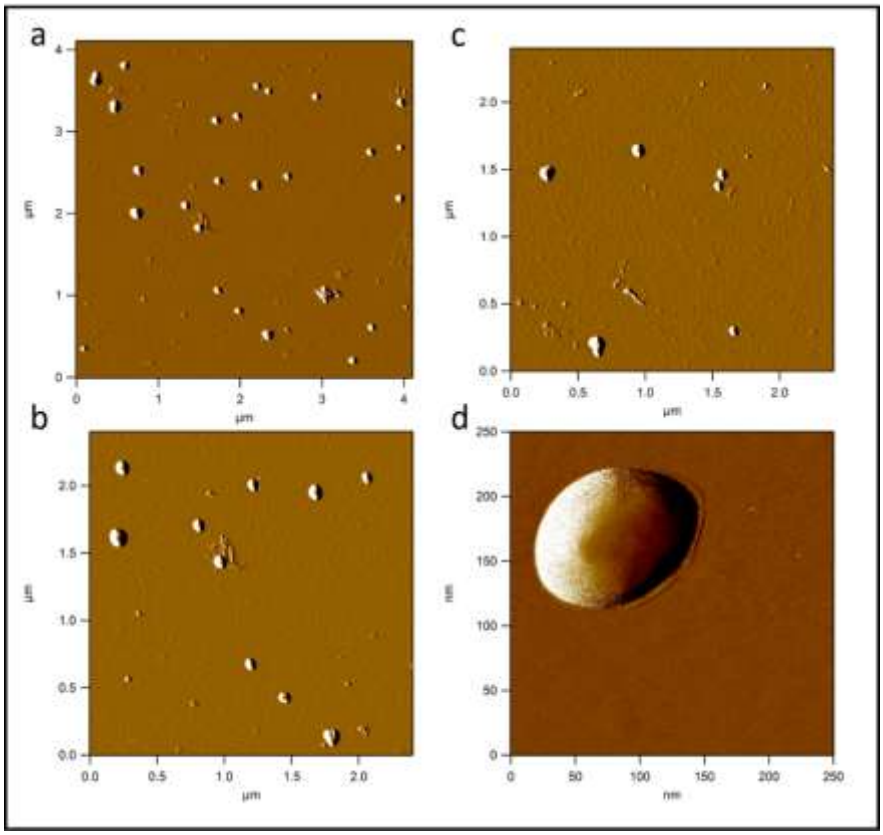

Figure 5. Atomic force microscopy (AFM) amplitude-contrast images with different magnification of PBNPs on mica surface. Rectangularity of the particles (together with their halo) was found to be 0.774 ± 0.111 (mean ± SD), indicating that PBNPs indeed represent rectangular topography. The height of the particles was 36.457 ± 9.496 nm (mean ± SD). The size of the images are 4.125 µm x 4.125 µm (Figure 5a.), 2.5 µm x 2.5 µm (Figure 5b. and c.) and 250 nm x 250 nm (Figure 5d.) respectively. Figure 5b. and 5c. are different areas of the measured sample.

4.2.2. In vivo results

Magnetic resonance imaging

Nanoparticles without any conjugated specific in vivo targeting agent are initially dispersed in the circulation system and started to accumulate mainly in the reticuloendothelial system (RES; e.g. liver, spleen). To investigate the PBNP uptake efficiency, especially in RES, the PBNP distribution was determined on T1-weighted MR images (Figure 6). In the case of in vivo MRI scans, we were able to register contrast changes between the pre- and post-injection scans immediately after the PBNP administration. Enhanced signal intensities were registered in the lungs, liver, kidneys, and abdominal vein (Figure 6.), which supports the results of previous publication.

Figure 6. Axial T1-weighted MR images of a mouse (A) before and (B) 30 minutes after intravenous administration of Prussian blue. Red arrows indicate that regions where signal intensity changes happened. Enhanced signal intensities were registered in the lungs, liver, kidneys, and abdominal vessel, which supports the results of a previous publication.

Optical imaging

Due to the strong visible fluorescent signal of IR820 conjugated PBNPs the semiquantitative distribution of the particles was determined based on their normalized mean fluorescent intensity. Figure 7A illustrates the autofluorescence signal from the animal at pre-injection condition at 690 nm, while Figure 7B shows the fluorescent signal after the PBNP administration. Enhanced dye concentration was registered in the head and thoracic region based on the high dye content of the circulation system, furthermore the images illustrated the liver of the animal. According to the studies of Zhang et. al. and Huang et. al., IR820 connected to different types of carrier systems shows great photo- and pH stability, as well as in aqueous media.

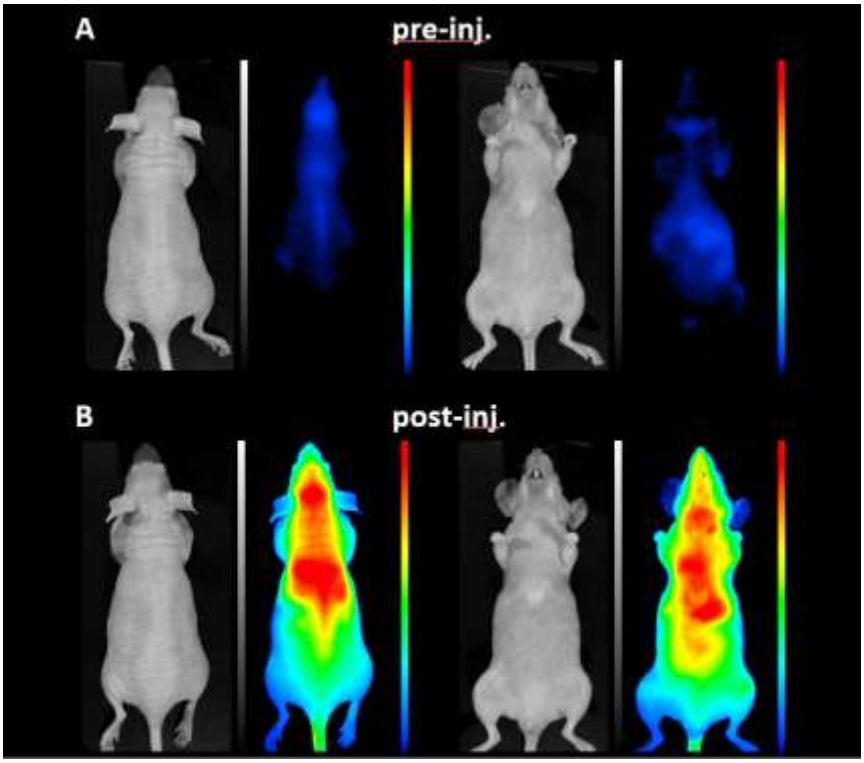

Figure 7. Non-excitated and fluorescent images of a mouse (A) before and (B) after intravenous administration of Prussian Blue (images from left to right: prone white, prone fluorescent, supine white, supine fluorescent images) at 690 nm. After the PBNP administration enhanced dye concentration was registered in the head and thoracic region based on the high dye content of the circulation system, furthermore the images illustrated the liver of the animal. The images are highlighted on the same dynamic color look-up table, which illustrates the signal intensity with different tone from cold to hot colors.

5. Conclusion

In this study the synthesis and modification of biocompatible, stealth, fluorescent and MRI contrast capability of PBNPs were demonstrated.

The PEGylated, MB labelled PBNPs offer a novel synthesis method to produce contrast material for the classical fluorescent measurement methodology. The MB-labelled PB nano-objects were investigated in vivo after intravenous administration, whereas the hepatobiliary and renal uptake and excretion were detected.

The synthesized NIR-820 conjugated PBNP nanoparticles appear to be an appropriate MRI and optical contrast material. By application of surface modification of citrate coated PBNPs with coatless nanoparticles, a slightly enlarged, iron rich complex nano system was produced. The obtained nanofiber was observed to possess an enhanced in vitro and in vivo T1-weighted MR contrast. The further conjugation with NIR-820 dye resulted in an optically active complex nano material for in vivo use. The nano system exhibited high colloidal stability and monodispersity after each modification step. The relaxivity constant in this investigation demonstrated that produced nano material is an appropriate candidate for further MRI and OI investigation.

With both PEGylation and fluorescent labelling PB nanoparticles are able to be conferred with advanced technological properties in order to offer a finely tuned platform for clinical application after further development. The optimal aim is implementing clinical translation as well as the application of MB and NIR wavelength. As fluorescent detection and imaging in a suboptimal in vivo system are more convincing if extended towards human clinical trials, the use of standard are more clinically relevant black mice might have been a preferred choice for validity and detection of our studies. However, the rather transparent nude mice are a less clinically translatable yet easily imageable.

Therefore, applying the concept of “QbD” during the laboratory synthesis of the nanoparticles, for the detection of the critical synthesis parameters, as well as measuring the nanosystem in two imaging modalities were an optimal choice for this study, resulting in further development and towards the clinical implementation.

5.1. Achievements

I. A stable nanoparticle platform was created, which held its stability for the 4 weeks period of examination.
    - The system emitted fluorescent signal, which was detected during in vivo imaging
    - The possible clearance routes were observed, clearance was facilitated with the addition of PEG

II. Prussian blue nanoparticles were successfully labelled with methylene blue

III. The connection of the fluorophore was facilitated by the fluorescent coating

IV. In vivo fluorescent imaging was executed and fluorescent images were captured over the examination time of 3 hours.

Based on the fluorescent labelling experience,

V. A stable Prussian blue-based T1 MRI contrast-capable nanoparticle was developed and it was successfully labelled with fluorescent dye,
    - Characterization was executed in vitro with DLS, AFM, TEM, and Zeta-potential measurements

VI. In vivo T1 MRI contrast was obtained,

VII. And NIR fluorescent contrast was measured in vivo.

6. Bibliography of the candidate's publications

6.1. Publications related to the topic of the Ph.D. thesis

Forgách, L., Hegedűs, N., Horváth, I., Kiss, B., Kovács, N., Varga, Z., ... & Máthé, D. (2020). Fluorescent, Prussian Blue-Based Biocompatible Nanoparticle System for Multimodal Imaging Contrast. *Nanomaterials*, *10*(9), 1732.

Hegedűs, N., Forgách, L., Kiss, B., Varga, Z., Jezsó, B., Horváth, I., ... & Máthé, D. (2022). Synthesis and preclinical application of a Prussian blue-based dual fluorescent and magnetic contrast agent (CA). *PloS one*, *17*(7), e0264554.

6.2. Other publications and abstracts related to the theme of the Ph.D. thesis.

Forgách, L., Kiss-Hegedus, N., Horváth, I., Szigeti, K., & Máthé, D. (2022). Prussian blue nanoparticles: an advanced platform for multimodal imaging. *Biophysical Journal*, *121*(3), 425a-426a.

6.3. Publications not related to the theme of the Ph.D. thesis

Máthé, D., Kiss, B., Pályi, B., Kis, Z., Forgách, L., Hegedűs, N., ... & Kellermayer, M. S. (2021). The 3M Concept: Biomedical Translational Imaging from Molecules to Mouse to Man. *The EuroBiotech Journal*, *5*(3), 155-160.

Omer, S., Forgách, L., Zelkó, R., & Sebe, I. (2021). Scale-up of electrospinning: Market overview of products and devices for pharmaceutical and biomedical purposes. *Pharmaceutics*, *13*(2), 286.