PEG-PAsp coated magnetite nanoparticles

● : magnetite nanoparticles

~ : PEG-PAsp block copolymer

Pancreatic cancer (TGF- β inhibitor(-))

Blood Vessel

Pancreatic cancer (TGF- β inhibitor(+))

● : PEG-PAsp coated magnetite nanoparticles

Title: Enhanced magnetic resonance imaging of experimental pancreatic tumor *in vivo* by block-copolymer-coated magnetite nanoparticles with TGF- β inhibitor

Running title: Magnetite nanoparticles for enhanced MRI

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Abstract

Early detection of solid tumors, particularly pancreatic cancer, is of substantial importance in clinics. Enhanced magnetic resonance imaging (MRI) with iron oxide nanoparticles is an available way to detect the cancer. The effective and selective accumulation of these nanoparticles in the tumor tissue is needed for improved imaging, and in this regard, their longevity in the blood circulation time is crucial. We developed here block copolymer-coated magnetite nanoparticles for pancreatic cancer imaging, by means of a chelation between the carboxylic acid groups in poly (ethylene glycol)-poly (aspartic acid) block copolymer (PEG-PAsp) and Fe on the surface of the iron oxide nanoparticles. These nanoparticles had considerably narrow distribution, even upon increased ionic strength or in the presence of fetal bovine serum. The PEG-PAsp-coated nanoparticles were further shown to be potent as a contrast agent for enhanced MRI for an experimental pancreatic cancer, xenografts of the human-derived BxPC3 cell line in BALB/c nude mice, with combined administration of TGF- β inhibitor. Iron

staining of tumor tissue confirmed the accumulation of the nanoparticles in tumor tissue. Use of the PEG-PAsp-coated magnetite nanoparticles, combined with the TGF- β inhibitor, is of promising clinical importance for the detection of intractable solid cancers, including pancreatic cancer.

Keyword: magnetic resonance imaging, pancreatic cancer, TGF- β , magnetite nanoparticles, poly(ethylene glycol)

1. Introduction

Pancreatic cancer, one of the intractable solid tumors, is the fourth leading cause of cancer-related deaths in the United States and the fifth in Japan [1]. The average survival period of patients suffering from advanced pancreatic adenocarcinoma is still extremely short, only 6 months, despite recent progress in the chemotherapies [2]. Although cancer detection and treatment have been greatly improved through the development of diagnostic imaging modalities, it is still difficult to detect pancreatic cancer [3]. Consequently, the development of diagnostic systems to detect these cancers is of great importance.

Recently, superparamagnetic iron oxide (SPIO) nanoparticles composed of either magnetite (Fe_3O_4) or maghemite ($\gamma\text{-Fe}_2\text{O}_3$) have been studied as contrast agents for magnetic resonance (MR) imaging [4]. Commercial application for human diagnosis based on SPIO particles is currently available. However, since cancer detection requires the systemic administration of iron oxide nanoparticles, the circulation time of the particles must be prolonged. Several studies have already reported that the behavior of magnetic nanoparticles in the bloodstream depends closely on their nanoscale morphology, including overall diameter, size distribution, or nature of the surface [5, 6]. Additionally, the surface modification of iron oxide nanoparticles has proved a versatile strategy for improving their biological performance, including the reduction of immunogenicity and enhancement of targeted delivery to specific tissues [7]. However, the overall correlation between the surface modification of nanoparticles and their *in vivo* behavior remains to be further elucidated.

Various methods of stabilization for SPIO nanoparticles have been reported to date [8]. One of the most feasible approaches could be the stabilization of SPIO by coated with biocompatible polymers [9]. Suitable polymers, including poly(ethylene glycol) (PEG) and its block copolymers, are promising for the development of SPIO systems with defined surface properties. This coating of particles with PEG, or PEGylation, to avoid their uptake by the reticuloendothelial system, is under intensive investigation. We also previously reported the accumulation of $\beta\text{-FeOOH}$ nanoparticles coated with PEG-poly(α,β -aspartic acid) block copolymer (PEG-PAsp) into experimental colon adenocarcinoma, which could be applicable for tumor-selective MR imaging [10]. The multivalent bonding of PEG-based block copolymer to magnetic nanoparticles may thus help to facilitate the accumulation of these nanoparticles into some solid tumors. However, magnetic nanoparticles of any design have not yet been successful in exhibiting sufficient accumulation in intractable solid cancers, including pancreatic

adenocarcinoma [1]. In addition to improving the performance of iron-based contrast agents (e.g. biocompatibility), the co-administration of adjuvant small molecules could increase the accumulation of these agents in target cancer tissue. In fact, we have recently shown that the administration of the small molecule TGF- β inhibitor (LY364947) at a low dose [11], which could minimize the potential side effects of the TGF- β inhibitor, can alter the tumor microenvironment and enhance the EPR effect in these cancers [12]. Therefore, the combined use of TGF- β inhibitor could be promising to diagnose intractable cancers with a long-circulating MRI contrast agent. Here, we demonstrated the successful MR imaging of experimental pancreatic cancer by the systemic administration of newly developed SPIO nanoparticles coated by PEG-PAsp in aid of TGF- β inhibitor.

2. Materials and Methods

2.1. Reagents

β -Benzyl L-aspartate and bis(trichloromethyl)carbonate (triphosgene) were purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA) and Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan), respectively. α -Methoxy- ω -amino-poly(ethylene glycol) ($\text{CH}_3\text{O-PEG-NH}_2$; $M_w=12000$) was purchased from NOF Corporation (Tokyo, Japan). Tetrahydrofuran (THF), *n*-hexane, *N,N*-dimethylformamide (DMF), CH_2Cl_2 were doubly-distilled according to the standard procedures. The magnetite nanoparticles were supplied by Toda Kogyo Corporation (Hiroshima, Japan; average particle size of magnetite: 10 nm). Resovist® was obtained from Bayer HealthCare Co., Ltd. (Osaka, Japan). TGF- β inhibitor was purchased from EMD Chemicals Inc. (San Diego, CA, USA) (LY364947; catalog no. 616451).

2.2. Synthesis of poly(ethylene glycol)-poly(α,β -aspartic acid) block copolymer (PEG-PAsp)

PEG-PAsp was synthesized by a previously reported procedure [13]. Briefly, poly(ethylene glycol)-*b*-poly(β -benzyl L-aspartate) block copolymer (PEG-PBLA) was prepared by ring-opening polymerization of N-carboxy anhydride of β -benzyl L-aspartate (BLA-NCA) from the ω - NH_2 group of PEG ($M_w = 1.2 \times 10^4$). Molecular weight distribution of PEG-PBLA was narrow as $M_w/M_n = 1.06$, which was determined by gel permeation chromatography [columns: TSK-gel G3000HHR, G4000HHR (Tosoh, Yamaguchi, Japan); eluent: DMF containing 10 mM LiCl; flow rate: 0.8 ml/min; detector: refractive index (RI); temperature: 40 °C]. The composition of these block copolymers was determined by ^1H NMR from peak intensity ratios of methylene protons of PEG (OCH_2CH_2 ; $\delta = 3.7$ ppm) and phenyl protons of the β -benzyl groups of PBLA ($-\text{CH}_2\text{C}_6\text{H}_5$; $\delta = 7.3$ ppm). The polymerization degree of BLA in block copolymer was calculated to be 38. The benzyl groups of PEG-PBLA were then removed by alkaline hydrolysis using 0.1 N NaOH to obtain PEG-PAsp as follows:

2.3. Preparation of PEG-PAsp-coated magnetite nanoparticles

PEG-PAsp-coated magnetite nanoparticles were prepared according to the previous method with slight modification [10]. Briefly, magnetite solution was quickly added to an aqueous solution of PEG-PAsp with varying feed molar ratios of aspartic acid residues to Fe ([Asp]/[Fe]) in the range of 0.01 to 1. The final concentration of magnetite was adjusted to 10 mmol/L. The mixed solutions were incubated at room temperature for 24 h to obtain magnetite nanoparticles coated with PEG-PAsp. Purification of the PEG-PAsp-coated magnetite nanoparticles was carried out by ultrafiltration (MWCO 200 000; polysulfone membrane, Toyo Roshi Co. Ltd., Tokyo, Japan).

2.4. Physicochemical characterization of the nanoparticles

The morphology and size distribution of the nanoparticles were examined by transmission electron microscopy (H-7000, Hitachi, Ltd., Tokyo, Japan) at an accelerating voltage of 75 kV. The TEM samples were prepared by mounting a drop of aqueous iron oxide nanoparticles suspension on carbon-coated 400 mesh Cu grids and allowing them to dry in air. Fourier transform infrared (FT-IR) spectra were obtained using a FT-IR spectrophotometer (FT/IR615, JASCO Corporation, Hachioji, Tokyo, Japan) with a resolution of 4 cm⁻¹. To characterize the interaction between block copolymer and magnetite nanoparticles, a small amount of nanoparticles powder was milled with KBr, and then pressed into a disc for analysis. Each spectrum was scanned 64 times to increase the signal-to-noise ratio. The Fe content in the nanoparticles was determined by ion coupled plasma-mass spectroscopy (ICP-MS, 4500, Hewlett Packard, Palo Alto, CA, USA). The amount of adsorbed block copolymer on magnetite nanoparticles was measured by thermogravimetric analysis (TGA) (EXSTAR6200 TG/DTA, Seiko Instruments Inc., Chiba, Japan) in nitrogen atmosphere with a heating rate of 10 °C/min in the temperature range of 25-1100 °C.

2.5. Light Scattering and ζ-Potential Measurements

The size distribution of the PEG-PAsp-coated magnetite nanoparticles was examined by dynamic light scattering (DLS) DLS-7000 (Otsuka Electronics Co., Ltd., Osaka, Japan).

Vertically polarized light with a wavelength of 488 nm from an Ar-ion laser (15 mW) was used as the incident beam. All measurements were conducted at 37 °C, and the data were analyzed by the cumulant method to determine the hydrodynamic diameters of the particles. The ζ -potential of PEG-PAsp-coated magnetite nanoparticles at 37 °C was measured by a Zetasizer NanoZS instrument equipped with a DTS5001 cell (Malvern Instruments Ltd., Worcestershire, UK).

2.6. Characterization of the r_2 relaxivities

The MR contrast effect of the magnetite nanoparticles was examined by measuring their proton relaxivities, r_2 , of which the definition is the slope of the concentration dependence given as:

$$1/T_2 = 1/T_2(0) + r_2[\text{Fe}]$$

Thus, a plot of $1/T_2$ versus concentration gives the relaxivity as the slope, where T_2 is the transversal relaxation time, $1/T_2$ is the transversal relaxation rate constant in the presence of a paramagnetic species, and $1/T_2(0)$ is the transversal relaxation rate constant in the absence of a paramagnetic species. The magnetite nanoparticles were dispersed into deionized water at concentrations of 0.5, 1.0, 1.5, 2.0, and 2.5 mM and the T_2 of these nanoparticle solutions was measured at 25 °C in water with a 0.47 T minispectrometer (Minispec, Bruker Optics Inc., Woodlands, TX, USA) using the Carr-Purcell-Meiboom-Gill (CPMG) method [14].

2.7. *In vivo* MR imaging

The BxPC3 human pancreatic adenocarcinoma cell line was obtained from the American Type Culture Collection (Manassas, VA, USA). The BxPC3 cells were grown in RPMI 1640 medium supplemented with 10% FBS. BALB/c nude mice (female, 5–6 weeks of age), obtained from Charles River Laboratories Japan Inc. (Tokyo, Japan), were inoculated subcutaneously with BxPC3 cells (1×10^7). After 3–4 weeks, MR imaging of the tumors was conducted with a 4.7 T scanner (INOVA200, Varian, Inc., Palo Alto, CA, USA). Twenty-four hours prior to the *in vivo* MR imaging, animals were treated with TGF- β inhibitor, 5 mg/ml in 4 μ l of DMSO and diluted by 100 μ l of PBS, at 1 mg/kg by intraperitoneal injection. Subsequently, the mice were injected at a dose of 0.1 mmolFe/kg, with Resovist[®] or PEG-PAsp-coated magnetite nanoparticles. A total of 4 conditions (n = 5 mice each) were investigated, i.e. with or without TGF- β inhibitor for both Resovist[®] or PEG-PAsp-coated magnetite nanoparticles. Imaging was performed at different temporal points (e.g., preinjection, 1 h postinjection, and 2 h postinjection). For the T_2 -weighted MR imaging of live mice, the following parameters were adopted: spin-echo method, point resolution = $234 \times 234 \mu\text{m}$, section thickness = 2.0 mm, TE = 60 ms, TR = 3000 ms, number of acquisitions = 5. All animals were treated in accordance with the guidelines of the Animal Ethics Committee of the University of Tokyo.

2.8. Histology

The excised samples were fixed overnight in 4% paraformaldehyde and then paraffin-embedded. Embedded samples were thin sliced at 10 μm thick and then stained using

an Iron Stain Kit (Muto Pure Chemicals Co., Ltd., Tokyo, Japan), based on McFadzean's protocol [15], with nuclear post-staining by 1% Safranin O. Iron staining was observed using an AX80 microscope (Olympus Corporation, Tokyo, Japan). The photographs were further quantified using Adobe Photoshop software (Adobe Systems Incorporated, San Jose, CA, USA), ImageJ software (National Institute of Health, MD, USA), and Microsoft Excel software (Microsoft Corporation, Redmond, WA, USA).

Figure 1. TEM image of the PEG-PAsp-coated magnetite nanoparticles. Bar: 100 nm.

3. Results and Discussion

3.1. The physicochemical properties of the PEG-PAsp-coated magnetite nanoparticle: Diameter and surface polymer density.

For solid tumor diagnosis, it is important to develop well-designed magnetite nanoparticles. The key physicochemical properties of magnetite nanoparticles are size, surface polymer density, and surface charge, since these characteristics can affect accumulation of magnetite nanoparticles to solid tumor. The PEG-PAsp-coated magnetite nanoparticles were prepared by mixing solutions of magnetite nanoparticles and PEG-PAsp with various molar ratios of the Asp residues to Fe (Asp/Fe); Asp/Fe ranged from 0.01 to 0.5, where $[Fe] = 10 \text{ mmol/L}$. As seen in Fig. 1, the transmission electron microscopy (TEM) image with 75 kV accelerating voltage of nanoparticles mounted on carbon grid from aqueous solution revealed that PEG-PAsp-coated magnetite nanoparticles take a cubic shape with a mean particle diameter of approximately 10 nm. The PEG-PAsp coating was observed as a layer with a thickness of approximately 5 nm, surrounding the magnetite nanoparticles. It was also observed in the TEM image that these PEG-PAsp-coated nanoparticles form clusters with a size range of 100 nm.

Figure 2. Physicochemical properties of the PEG-PAsp-coated magnetite nanoparticles. (A) Hydrodynamic diameter vs. relative concentration of aspartic acid residue in the medium normalized by $[Fe]$ (mol/mol-Fe) ($=[Asp]/[Fe]$), and (B) Change in the adsorbed density of PEG-PAsp on the magnetite surface estimated from TGA analysis with a bulk concentration of PEG-PAsp. Temperature = 37 °C; medium: distilled water.

The hydrodynamic diameter of these nanoparticles in aqueous medium was then measured with DLS and shown to be in the range of 100 to 120 nm with unimodal distribution, for Asp/Fe ratios ranging from 0.02–0.5. This DLS data is consistent with the cluster formation of nanoparticles indicated from TEM images. However, with a lowered Asp/Fe ratio as 0.01, the hydrodynamic diameter increased significantly (Fig. 2A). This result indicates that there is a critical surface concentration of PEG to effectively prevent the PEG-PAsp-coated magnetite nanoparticles from the agglomeration. The purified nanoparticles were stable in distilled water as 100 nm-scaled cluster at room temperature as well as at 37 °C, maintaining the initial photon count and distribution in DLS analysis for at least one month, even after the ultrafiltration to remove free PEG-PAsp possibly remained in the reactant.

The density of the PEG-PAsp block copolymer on the magnetite particle surface was estimated by TGA. Here, nanoparticles were heated in the nitrogen atmosphere to selectively vaporize the polymer fraction. Eventually, the amount of absorbed polymer on the surface of the nanoparticles was measured from the weight change by heating. The polymer density was then calculated from the TGA measurement for all the nanoparticles, assuming the cubic morphology as evidenced by microscopy and a density of 5.05 g/cm³ for magnetite. As seen in Fig. 2B, the number of polymer strands on the nanoparticle surface was as high as 100. This data suggests that the PEG density on the magnetite nanoparticles is a little lower than that of PEGylated gold nanoparticles prepared through the surface tethering of PEG-SH [16].

3.2. The mechanism of PEG-PAsp adsorption on the magnetite nanoparticles.

To confirm the formation of PEG-PAsp coating on the magnetite nanoparticles, the ζ -potential of bare and PEG-PAsp-coated magnetite nanoparticles was measured in 10 mM MOPS buffer as a function of pH (Figure 3). The isoelectric point (IEP) of the bare magnetite nanoparticles was estimated as approximately 6.6, which is consistent with the reported IEP value of iron oxide [17]. In a lower pH (below the IEP), the magnetite nanoparticle surface was protonated to result in a positive ζ -potential. Thus, in this pH range, electrostatic attraction between positively-charged magnetite nanoparticles and negatively charged PEG-PAsp is expected to occur, allowing the PEG-PAsp adsorption to the nanoparticle surface. Alternatively, the bare magnetite nanoparticles possess negative ζ -potential at physiological pH 7.4, whereas the ζ -potential shifted to the neutral value for PEG-PAsp

Figure 3. Change in the ζ -potential with pH for bare (●) and PEG-PAsp-coated (■) magnetite nanoparticles. Temperature = 37 °C; medium: 10 mM MOPS buffer.

modified nanoparticles in 10 mM MOPS buffer (pH 7.4), being consistent with the formation of a PEG shell layer. Also, these data suggest that there should be an adsorption mechanism other than simple electrostatic interaction, because magnetite has a negative ζ -potential value at pH 7.4 to induce electrostatic repulsive force against negatively-charged carboxylates in PEG-PAsp. The adsorption mechanism under physiological pH was suggested to be the monodentate chelation (I) (Figure 4) from the result of Fourier transform infrared spectroscopy [18], as explained in detail in Supplemental Text with Supplemental Figure 1 and Supplemental Table 1.

Figure 4. Modes of carboxylate-metal complexation: monodentate (I), bidentate chelating (II), and bidentate bridging (III).

3.3. Comparison study of the physicochemical characteristics of the PEG-PAsp- and dextran-coated magnetite nanoparticles.

The MRI detection limit was compared between the PEG-PAsp- and dextran-coated magnetite nanoparticles in the field of 0.47 T at 25 °C from the relaxivity r_2 , exhibiting the sensitivity of the T_2 MRI contrast agent. The dextran-coated magnetite used in this study was the one already in clinical use, Resovist[®]. Eventually, the relaxivity r_2 of the PEG-PAsp nanoparticle was calculated to be 138 mM⁻¹s⁻¹, the value similar to Resovist[®] [19].

The hydrodynamic diameter observed between the PEG-PAsp-coated and dextran-coated magnetite nanoparticles differed significantly in an NaCl-concentration-dependent manner (Fig. 5A). Although the hydrodynamic diameter of the PEG-PAsp-coated magnetite nanoparticles did not change significantly up to 3 M NaCl, indicating the appreciable stability of the PEG-PAsp-coating, that of Resovist[®] significantly increased even at NaCl = 0.15 M, and reached more than one micrometer at NaCl = 0.5 M, due to the drastic aggregation. Note that the PEG-PAsp-coated nanoparticles did not show any change in their size even after one month storage in 10 mM

Figure 5 NaCl concentration (A) and time (B) dependencies of the relative hydrodynamic diameter of magnetite nanoparticles, □: PEG-PAsp-coated nanoparticles, ○: dextran-coated nanoparticles (Resovist[®]). Fe concentration = 2 mmol/L; temperature = 37 °C; medium 10 mM Tris-HCl buffered saline (pH 7.4). 10% fetal bovine serum was contained in (B).

Tris-HCl buffered saline (pH 7.4, 37 °C) (data not shown).

The colloidal stability of the PEG-PAsp-coated magnetite nanoparticles in physiological conditions was also examined. We incubated them in 10 mM Tris-HCl buffered saline (pH 7.4) containing 10 % fetal bovine serum at 37 °C for 24 h and measured the change of the hydrodynamic diameter (Fig. 5B). The size of the PEG-PAsp-coated magnetite nanoparticles did not obviously change during the 24-hour storage time. On the other hand, the size of Resovist[®] increased by the formation of aggregates after 16 hours of storage time.

The stability of the PEG-PAsp-coated nanoparticles may come from the multivalent bonding between flanking carboxylic groups and the magnetite surface, as suggested by FT-IR study. These findings are consistent with a report showing that PEG-oligo(aspartic acid) block copolymer-coated iron oxide nanoparticles were stable at pH 2-11 and in 1 M NaCl, where the repeating number of aspartic acid units was 3 or more [20]. In contrast, instability of Resovist[®] against salt addition may be due to weak interaction between magnetite and hydroxyl groups of dextran [21].

3.4. MR imaging of experimental pancreatic cancer *in vivo*.

The MR imaging of tumor tissue *in vivo* was then conducted by comparing PEG-PAsp- and dextran-coated magnetite nanoparticles, Resovist[®]. Resovist[®] has already been approved for clinical use as a liver-specific MRI contrast agent, due to accumulation into the reticuloendothelial system (RES) of the normal liver. Most malignant liver tumors do not contain RES cells and therefore are contrasted positive by Resovist[®]. A xenografted BxPC3 human pancreatic adenocarcinoma cell line in nude mice, characterized histologically by fibrosis and hypovascularity, was used as a model of intractable cancer. Recently, we reported that the administration of TGF- β inhibitor to tumor model mice significantly enhanced the intratumoral accumulation of nanoparticles encapsulating anticancer drugs [11]. Thus, we tested the effect of the i.p. administration of TGF- β inhibitor with the i.v. administration of Resovist[®] or the PEG-PAsp-coated magnetite nanoparticles on their imaging capability in size-matched xenografts of the BxPC3 cell line. Fig. 6A shows the T₂-weighted MR images of the tumors at different time periods after the intravenous administration (preinjection, and 1 and 2 hrs postinjection) of PEG-PAsp-coated magnetite nanoparticles with and without TGF- β inhibitor.

Resovist[®] failed to image the tumor even with the co-administration of TGF- β inhibitor, presumably due to the non-specific accumulation into the reticuloendothelial system [8]. In contrast, the PEG-PAsp-coated magnetite nanoparticles exhibited significant negative enhancement of signal intensity in the tumor region of T₂-weighted images when combined with TGF- β inhibitor, suggesting the accumulation of detectable amounts of the PEG-PAsp-coated magnetite nanoparticles within 2 hours after injection. Therefore, the difference in behavior of these two types of magnetite nanoparticles *in vivo* had a crucial importance in achieving effective tumor accumulation for successful MR imaging.

Figure 6 MR imaging of experimental pancreatic cancer *in vivo* and the distribution of the nanoparticles in cancer and liver tissues. (A) T₂-weighted images of tumor-implanted mice (tumor sites are circled by red dotted line) at different temporal points after injection of PEG-PAsp-coated magnetite nanoparticles and TGF- β inhibitor. All images were obtained in a field strength of 4.7 T. (B) Histological sections of BxPC3 xenograft stained with Prussian blue. The distribution of Resovist[®] and PEG-PAsp-coated magnetite nanoparticles, at 5.5 mg/kg with and without TGF- β inhibitor at 1 mg/kg, were examined 24 h after the administration. (C) Histological sections of liver stained with Prussian blue. The distribution of Resovist[®] and PEG-PAsp-coated magnetite nanoparticles, at 5.5 mg/kg with TGF- β inhibitor at 1 mg/kg, were examined 24 h after the administration. (D and E) Areas of iron staining in the tumor and liver were quantified. PEG-PAsp, PEG-PAsp coated magnetite nanoparticles with (+) and without (-) inhibitor. Error bars in the graphs represent standard errors of the mean (n = 6), and P values were calculated by two-tailed Student's t test. n.s.: not significant.

To further verify the accumulation of iron oxide nanoparticles in the tumor, we performed Prussian blue staining of the tumor tissues to detect iron oxide, which stains blue. As shown in

Fig. 6B, positive staining of the tumor for iron oxide was only obvious in the condition with PEG-PAsp-coated nanoparticles combined with the TGF- β inhibitor. Areas of iron staining in the tumor was then quantified as seen in Fig. 6D, demonstrating a significant increase in the areas of positive staining by TGF- β inhibitor treatment. The presence of iron oxide was consistent with the MRI results. Iron oxide was observed in the area rich in fibrotic components, suggesting that the administration of TGF- β inhibitor transiently increases the permeability of the tumor capillary to promote the extravasation of the PEG-PAsp-coated magnetite nanoparticles, even though the BxPC3 tumor has the characteristic of hypovascularity [11].

Lastly, we examined liver tissues from the mice treated with Resovist[®] or the PEG-PAsp-coated magnetite nanoparticle, with or without TGF- β inhibitor, by iron staining (Fig. 6C). Although aggregates of Resovist[®] accumulated in the liver, particularly in cells with smaller nuclei (presumably Kupffer cells), far less PEG-PAsp-coated magnetite nanoparticles accumulated in the liver without aggregation. These results did not differ with or without TGF- β inhibitor, which was determined by the area of Prussian blue staining (Fig. 6E).

4. Conclusion

In conclusion, we here demonstrated the physicochemical properties of PEG-PAsp-coated magnetite nanoparticles and the feasibility of these nanoparticles as MR contrast agents for cancer diagnosis. Improving the stability of nanoparticles might be important for enabling a longer half-life in the bloodstream and a better accumulation in tumor tissue, leading to effective MR imaging with contrast agents. The neutral ζ -potential of the PEG-PAsp-coated nanoparticle may contribute to avoidance of reticuloendothelial system uptake. Formation of the stable and dense PEG layer on the magnetite surface through the anchoring of PEG-PAsp by the monodentate chelation of COO⁻ residues to iron atoms definitely plays a substantial role in the increased stability of the nanoparticles *in vivo*. The use of PEG-PAsp-coated magnetite nanoparticles combined with a TGF- β inhibitor could thus become a novel regime in the diagnosis of intractable cancers, including pancreatic adenocarcinoma.

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