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Targeted diagnostic magnetic nanoparticles for medical imaging of pancreatic cancer☆

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ABSTRACT

Highly aggressive cancer types such as pancreatic cancer possess a mortality rate of up to 80% within the first 6 months after diagnosis. To reduce this high mortality rate, more sensitive diagnostic tools allowing an early stage medical imaging of even very small tumours are needed. For this purpose, magnetic, biodegradable nanoparticles prepared using recombinant human serum albumin (rHSA) and incorporated iron oxide (maghemite, γ-Fe_2O_3) nanoparticles were developed. Galectin-1 has been chosen as target receptor as this protein is upregulated in pancreatic cancer and its precursor lesions but not in healthy pancreatic tissue nor in pancreatitis. Tissue plasminogen activator derived peptides (t-PA-ligands), that have a high affinity to galectin-1 have been chosen as target moieties and were covalently attached onto the nanoparticle surface. Improved targeting and imaging properties were shown in mice using single photon emission computed tomography–computer tomography (SPECT–CT), a handheld gamma camera, and magnetic resonance imaging (MRI).

1. Introduction

During the last decades the development of early diagnostic methods for various tumours enabled an improvement in the treatment of cancer patients. This achievement, however, has not been achieved for pancreatic ductal adenocarcinoma (PDAC). PDAC is currently the fourth leading cause of cancer death in the United States of America [1]. Furthermore it is anticipated to become the second leading cause of cancer-related deaths in the year 2030 [1,2]. PDAC possesses a mortality rate of up to 80% within the first 6 months after diagnosis [3], and the 5-year survival rate is only 6.7% [4,5].

The paramount problem with pancreatic adenocarcinoma is that in most cases this cancer is diagnosed only in late stages, after possible metastasis spread, especially into the liver. Only 10%–15% of patients are diagnosed in the early stages of the disease [6].

Magnetic resonance imaging (MRI), computed tomography (CT), and single photon emission computed tomography–computer tomography (SPECT–CT) are state of the art in the diagnosis of pancreatic tumours. However, these medical imaging methods often don't have the ability to distinguish pancreatitis from pancreatic cancer [7], which is even worse as pancreatitis is a risk factor for carcinogenesis. Even the addition of more sophisticated imaging techniques such as diffusion weighted imaging is of no help in this effort [8].

In preclinical MRI studies, such as s of tumour cell migration and regional tumour growth, contrast enhanced MRI is used for both, diagnostic

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purposes and therapeutic monitoring. (Super-)paramagnetic nanoparticles offer the advantage, that they can be visualized in the morphologic standard T1-weighted (T1w) and T2-weighted (T2w) sequences.

NPs represent a novel class of therapeutics and diagnostics for cancer therapy. Recent studies demonstrated that therapeutics bound to or encapsulated into NPs provide an enhanced efficacy as well as reduced side effects compared to the respective unbound therapeutic entities [9–11]. Due to the leaky vasculature within a variety of tumours combined with their poor lymphatic drainage, nanoparticles can selectively accumulate in the tumour tissue following intravenous injection [12]. In addition, the attachment of targeting ligands that can bind to receptors or other biochemical structures which are presented and overexpressed on the surface of cancer cells, enhances the interaction with these cells resulting in further increase of accumulation in the tumour. Moreover, the targeting ligands also promote internalization by receptor-mediated endocytosis [13,14], which is a prerequisite for advanced intracellular therapeutic approaches such as interference with the cells' metabolism via siRNA. Tumour-associated antigens that are already at earlier stages highly expressed on the cell surface of cancer cells but not in neighbouring tissue or only in negligible amounts are ideal for tumour targeting. Attachment or incorporation of diagnostic markers thus could allow the detection of the tumour by the targeted NPs using medical imaging. Since pancreatic adenocarcinomas are tumours with a very low perfusion (hypovascularized tumours) and hence radiologically low contrast agent uptake, a nanoparticle accumulation in these tumours, therefore, would be of great help for diagnosis as well as therapy.

Previous studies showed that these magnetic NPs (MNPs) consisting of recombinant human serum albumin (rHSA) and a magnetic core are non-toxic in vitro and in vivo and exhibited promising in vitro MRI-behaviour [15]. The main advantage of MNPs is their ability to covalently bind different molecules to their surface for presentation to cellular molecular units such as receptors. Loaded NPs were able to release the drug or diagnostic agent after internalization and procession of the particles in the targeted tissue, by using different binding techniques as described by Wartlick et al. and Weber et al. [11,16]. This fact underlines the versatility of these particles in the scope of modular nanosystem platforms.

Recently, it was shown that the galectin-family (Gal), especially galectin-1 (Gal-1), acts as functional receptors for tissue plasminogen activator (t-PA) [17]. The binding is specific with a strong affinity and, hence, may provide a promising strategy for pancreatic cancer targeting. The expression of Gal-1 was reported to be upregulated in pancreatic cancer cells but is not expressed in adjacent normal tissues or adjacent inflammatory pancreas [18,19].

The prime objective of the present study is to investigate different in vivo imaging procedures for their ability to improve the pancreatic cancer diagnosis using non-toxic human serum albumin nanoparticles that offer the possibility for attachment of a variety of targeting ligands and, as a further step, allow the binding of drugs (theranostics). As targeting moiety glycosylated peptides derived from t-PA, Gal-1's natural ligand, were bound covalently to previously developed [15] magnetic maghemite-containing nanoparticles (MNPs). The targeted MNPs were physico-chemically characterized, and their potential in vivo toxicity was determined by histology. The in vivo imaging methods included MRI as well as SPECT-CT and handheld gamma camera after radiolabelling the MNPs with ^{67}Ga.

2. Materials and methods

2.1. Nanoparticle preparation

MNPs were prepared following a method previously described by Rosenberger et al. [15]. For the incorporation of the magnetic γ-CAN maghemite NPs (CAN, $(NH_4)_2Ce(IV)(NO_3)_6$-γ-Fe_2O_3 NPs) into the rHSA nanoparticles, rHSA (Sigma-Aldrich, Steinheim, Germany) was dissolved to 100 mg/mL in 10 mM NaCl solution. 2000 μg or 4000 μg iron, respectively, were added to the protein solution and incubated for 1 h at 20 °C (Eppendorf thermomixer, 300 rpm, Hamburg, Germany). Afterwards desolvation took place by addition of 4 mL ethanol 96% (V/V) under constant stirring with a pump rate of 1 mL/min (ISMATEC IPN, Glattbrugg, Switzerland). This procedure allowed a defined nanoparticle formation process. By using 117.5 μL glutaraldehyde 8% (v/v) (Sigma-Aldrich, Steinheim, Germany) as a bifunctional crosslinker, the free available amino groups on the surface of the denaturized protein were inactivated. The amount of required glutaraldehyde was calculated on the basis of 60 amino groups per molecule of rHSA. This bifunctional aldehyde was then added in excess in a quantity that would be required to crosslink 200% of these groups. The nanoparticle suspension was stirred at room temperature over 24 h to ensure the crosslinking process to be quantitative.

To remove free glutaraldehyde, denaturized protein, and γ-CAN maghemite NPs in excess, three cycles of centrifugation (20 min, × 20,100 rcf) and resuspension of the centrifugation pellet in 1 mL ultrapurified water is necessary. Each resuspension step was performed in an ultrasonic bath (Bandelin, Sonorex, Berlin, Germany). Finally, the amount of MNPs in suspension was determined gravimetrically.

The particle size, size distribution (PDI), and surface charge (ZP) of the obtained MNPs were determined by dynamic light scattering (DLS) using a Zetasizer Malvern Nano ZS (Malvern Instruments Ltd., Malvern, UK). For this purpose, a 10 μL MNP suspension was diluted with ultrapure water to 1 mL.

1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic-NHS (DOTA-NHS) ester (CheMatech, Dijon, France) as chelator for ^{67}Ga was attached onto the surface of the MNPs using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) as crosslinking moiety. Briefly, 5 mg MNP was resuspended in 0.1 M 2-(N-morpholino)ethanesulfonic acid buffer (MES buffer) (Thermo Fisher Scientific Inc., Rockford, USA) and incubated with a 10-fold molar excess of EDC and 5 mg DOTA-NHS for 1 h at room temperature. Afterwards, the DOTA-MNPs were purified three times by centrifugation and resuspension in Milli-Q water as described above. The amount of unbound DOTA-NHS ester in the supernatant was determined using HPLC analysis.

As targeting moiety tPApep.1_{LAC} was selected (see SI, *t-PA peptides and galectins interaction studies*, SI Table S1). This sequence was synthesized by Peptide Specialty Laboratories GmbH, Heidelberg, Germany. 50 μg tPApep.1_{LAC} (Peptide Specialty Laboratories GmbH, Heidelberg, Germany) was attached onto the surface of DOTA-MNPs using the same binding protocol as described above. The amount of unbound protein in the supernatant was determined using a FLUOstar Galaxy (MTX Lab Systems, Inc., Virginia, U.S.A.). The attached number of tPApep.1_{LAC} per nanoparticle was calculated using a modified equation by Nobs et al. [20]:

$$n = a \cdot N \cdot \frac{4}{3}\pi r^3 \cdot d$$

Equation 1: n = number of targeting moieties per MNP, a = mol of targeting moiety per MNP [g], N = Avogadro number (6.022×10^{23}), r = mean radius of MNPs, d = density of MNPs [g/cm^3] (set as 1 g/cm^3).

The obtained DOTA-MNPs incubated with 2000 μg iron were used for MRI 11.7 T experiments, SPECT–CT and handheld gamma camera scans, whereas MNPs incubated with 4000 μg of iron were employed for MRI investigations at 1.5 T.

2.2. Cell culture

The human pancreatic tumour cell line PANC-1 was obtained from the European Collection of Cell Cultures (ECACC Cell Lines, Sigma Aldrich). PANC-1 cells were maintained as monolayer culture in RPMI-1640 (Lonza, Verviers, Belgium) with 10% FCS (Sigma-Aldrich, St. Louis USA), 1% streptomycin/penicillin (Invitrogen, USA) and 2 mM