Biochimica et Biophysica Acta xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen

Multifunctionalized iron oxide nanoparticles for selective targeting of pancreatic cancer cells☆

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ARTICLE INFO

Article history:
Received 27 October 2016
Received in revised form 26 December 2016
Accepted 28 January 2017
Available online xxxx

Keywords:
Pancreatic cancer
Magnetic nanoparticles
Multifunctionalization
Nanocarriers
CD47
Gemcitabine
Controlled drug release
Active targeting
Nanomedicine

ABSTRACT

Nanomedicine nowadays offers novel solutions in cancer therapy by introducing multimodal treatments in one single formulation. In addition, nanoparticles act as nanocarriers changing the solubility, biodistribution and efficiency of the therapeutic molecules, thus generating more efficient treatments and reducing their side effects. To apply these novel therapeutic approaches, efforts are focused on the multi-functionalization of the nanoparticles and will open up new avenues to advanced combinational therapies. Pancreatic ductal adenocarcinoma (PDAC) is a cancer with unmet medical needs. Abundant expression of the anti-phagocytosis signal CD47 has also been observed on pancreatic cancer cells, in particular a subset of cancer stem cells (CSCs) responsible for resistance to standard therapy and metastatic potential. CD47 receptor is found on pancreatic cancer and highly expressed on CSCs, but not on normal pancreas. Inhibiting CD47 using monoclonal antibodies has been shown as an effective strategy to treat PDAC *in vivo*. However, CD47 inhibition effectively slowed tumor growth only in combination with Gemcitabine or Abraxane. In this work, we present the generation of multifunctionalized iron oxide magnetic nanoparticles (MNPs) that include the anti-CD47 antibody and the chemotherapeutic drug Gemcitabine in a single formulation. We demonstrate the *in vitro* efficacy of the formulation against CD47-positive pancreatic cancer cells. This article is part of a Special Issue entitled "Recent Advances in Bionanomaterials" Guest Editor: Dr. Marie-Louise Saboungi and Dr. Samuel D. Bader.

1. Introduction

Magnetic nanoparticles (MNPs) have been explored during the last decades in several scientific fields based on their excellent physical and chemical properties, such as superparamagnetism, good colloidal stability, low toxicity and good biocompatibility [1–8]. In recent years MNPs have been widely investigated for their use in biomedical applications including diagnosis *via* magnetic biosensing or magnetic resonance imaging (MRI) [9–14], therapy [15–18] and magnetic separation techniques [19–22].

Even though therapeutic systems based on magnetic nanoparticles have received a lot of attention in nanomedicine, controlling the targeting of specific cells still remains a challenge. Passively targeted nanoparticles *via* the enhanced permeability and retention (EPR) effect fail to accomplish tumor specificity in many examples. Therefore, the conjugation of cell-specific targeting ligands on the surface of magnetic nanoparticles that enable receptor-mediated endocytosis can be advantageous, as it can provide specificity, increasing the efficiency of treatments and reducing their side effects [23–25]. A great variety of targeting ligands for cancer diagnosis and therapy have been developed [26]. Generally active targeting is accomplished by the attachment of a targeting ligand on the surface of the MNP, which specifically recognizes a receptor that is over-expressed on the target cells. The most common method for the preparation of actively targeted MNPs is the conjugation of antibodies, antibody fragments, peptides, sugars, or small molecule ligands [27].

Nanoparticles (NPs) are ideal platforms for developing novel combinational therapies, as they act as nanocarriers that can be functionalized with multiple components. NPs developed for biomedical applications require careful physicochemical and targeting design, but also require

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http://dx.doi.org/10.1016/j.bbagen.2017.01.035
0304-4165/

additional considerations paid to drug loading, transport, and release [25]. By developing advanced multifunctionalization strategies, NPs can be decorated with the desired loads of each active component [28], which include targeting agents, standard chemotherapeutic agents, and novel therapeutic agents (*e.g.* therapeutic antibodies). The multifunctionalization is performed in a way that first, the NPs are able to carry and protect a significant drug payload; second, multiple therapeutic agents can be properly loaded remaining functionally active; and third, the therapeutic cargo release mechanism and rate are modulated for optimal therapeutic efficacy. In this sense, we have previously shown how to generate multifunctional MNPs with large drug payloads per targeting agent (200:1) [29], which cannot be reached by antibody-based targeted drug delivery approaches [30–32].

Overall, nanomedicine has opened new avenues towards the development of selective, minimally-invasive and efficient therapeutic modalities against several diseases such as cancer. In particular, pancreatic cancer is currently the fifth cause of cancer-related deaths and is characterized by early metastasis, extensive desmoplasia and pronounced resistance to chemotherapy and radiation. Regardless of the extensive research efforts, no major progress has been made regarding improvement in clinical endpoints recently. Since the introduction of the antimetabolite Gemcitabine in the '90s [33], only the use of combination therapies such as Folfirinox [34], and more recently the combination of Gemcitabine with nab-paclitaxel (Abraxane) [35], has been able to moderately extend the median survival, but eventually the vast majority of patients still succumb to the disease. In particular, the studies using Abraxane, which is a colloidal suspension of 130 nm particles prepared by homogenization of paclitaxel with human serum albumin, highlight the importance of drug delivery and targeting [36] on improving the pharmacokinetics [37], the bioavailability [38] and the intra-tumoral accumulation of drugs [39] and therefore the impact that an appropriate delivery can have on the clinical outcome of a given drug. It is therefore of the utmost importance not only to develop new effective anti-PDAC treatments but to develop systems that are able to specifically deliver the combination of the different therapeutic agents to the tumor cells.

It has been shown for different types of cancers that distinct populations of cells with stem cell properties, namely cancer stem cells (CSCs), are responsible for resistance to standard therapy and bear metastatic potential. We and others have provided conclusive evidence for such a hierarchical organization in human pancreatic ductal adenocarcinoma (PDAC) [40–42], and the survival of such resistant CSCs during chemotherapy can justify the later relapse of the disease that still happens to most of the patients. We have recently shown that CD47 is expressed on primary PDAC cells and we have demonstrated that inhibiting CD47 function using monoclonal antibodies constitutes an effective strategy to treat PDAC [43]. In summary, we observed that treatment with monoclonal antibodies for CD47 in combination with Gemcitabine or Abraxane significantly reduced primary tumor growth, using PDX models of PDAC. Specifically, we found that anti-CD47 therapy alone had only marginal impact on the size and rate of tumor growth, while treatment in combination with Gemcitabine or Abraxane resulted in efficient growth control of tumors and prevented relapse after discontinuation of treatment [43]. These results suggest that anti-CD47 therapy could be used to treat primary PDAC tumors, but combination with other anti-cancer therapeutic agents, such as Abraxane or Gemcitabine, is needed [43]. Therefore, the development of novel nanotherapies, including actively targeted MNPs with the possibility of combining different therapeutic agents in a single vehicle seems highly attractive to overcome the difficulties of treating pancreatic cancer [44,45].

In this work we introduce a new strategy for pancreatic cancer treatment based on nanotechnology by combining the inhibition of CD47, the chemotherapeutic effect of Gemcitabine, and the efficient vehiculization of those components in a single formulation by using nanoparticles. For this purpose we developed multifunctional MNPs that target a receptor overexpressed in pancreatic cancer cells allowing at the same time the delivery of therapeutic agents with different mechanisms of action without compromising their biological activity. This approach resulted in an effective delivery of both Gemcitabine and anti-CD47 antibody to pancreatic cancer cells. Thus, MNPs build up a good platform that can be further improved for *in vivo* applications to administer other drug combinations currently evaluated to increase the efficiency of treatments, while reducing their side effects.

2. Materials and methods

2.1. Materials

Purified anti-human CD47 antibodies (B6H12 clone) were purchased from eBioscience (anti-CD47). Gemcitabine (Gem) was purchased from Fluorochem. Ultrapure reagent grade water (18.2 MΩ, Wasserlab) was used in all experiments. Dimercaptosuccinic acid (DMSA) coated MNPs were produced by means of the co-precipitation technique as described before [46] and have been provided by Liquid Research Ltd. (UK). The MNPs (MF66) have a zeta potential: −56 mV, an average core size of 12 ± 3 nm, and a hydrodynamic diameter (Zaverage): 97 nm (PDI: 0.18). These MNPs present good stability in PBS buffer and RPMI cell culture medium with and without 10% of bovine serum, showing a hydrodynamic size of 98 nm (PDI: 0.18) in PBS, 100 nm (PDI: 0.18) in RPMI without serum and 105 nm (PDI: 0.18) in RPMI 10% serum. **A** Gemcitabine derivative, Gem-S-S-Pyr was prepared according to described procedures [28].

2.2. Measurements

Ultraviolet-visible (UV–Vis) and fluorescence spectra were recorded on a Synergy H4 microplate reader (BioTek) using 96-well plates. Hydrodynamic diameter and zeta potential measurements were determined using a Zetasizer Nano-ZS device (Malvern Instruments). Hydrodynamic diameter and zeta potential were measured from dilute sample suspensions (0.1 mg Fe ml^{-1}) in water at pH 7.4 using a zeta potential cell. High-performance liquid chromatography (HPLC) was performed using a 1260 Infinity HPLC (Agilent Technologies) with a ZORBAX 300SB-C18 column 5 μm, 9.4 × 250 mm.

2.3. Multifunctionalization of MNPs

2.3.1. MNP activation

6 ml of MNPs at 2 mg Fe ml^{-1} were incubated overnight at 37 °C with 50 μmol of 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide (EDC)/g Fe, 25 μmol of *n*-hydroxy succinimide (NHS)/g Fe and 25 μmol of cysteamine hydrochloride/g Fe, previously neutralized by 1 M equivalent of sodium hydroxide (NaOH). After 16 h, the sample was washed by cycles of centrifugation and redispersion in Milli-Q water 3 times. The presence of sulfhydryl groups introduced onto the MNPs was quantitatively measured by reaction with 2,4-dinitrothiocyanatebenzene (DNTB) [47].

2.3.2. Covalent attachment of anti-CD47 antibodies on MNPs

A solution of the anti-CD47 (1 mg ml^{-1}) in 0.01 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 0.15 M sodium chloride (NaCl), pH 8.2, was incubated for 1 h at room temperature with a 5 M equivalent solution of 2-iminothiolane. This strategy, as has been previously described, allows the oriented immobilization of the antibodies on the nanoparticles [29,48]. After that, the modified antibody was purified by gel filtration through a desalting resin (Sephadex G-25) using PBS, 0.002 M (EDTA), pH 7.4. The sulfhydryl groups of MNPs were activated as follows: 2 ml of aqueous suspension of pre-activated MNPs at 2 mg Fe ml^{-1} was mixed with 25 μmol/g Fe of 2-aldrithiol solution during 2 h at 40 °C. After reaction, 200 μl of brine was added and the sample centrifuged 10 min at 10,000 ×*g* and

Please cite this article as: S. Trabulo, et al., Multifunctionalized iron oxide nanoparticles for selective targeting of pancreatic cancer cells, Biochim. Biophys. Acta (2017), http://dx.doi.org/10.1016/j.bbagen.2017.01.035