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A transferrin variant as the targeting ligand for polymeric nanoparticles incorporated in 3-D PLGA porous scaffolds

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ABSTRACT

We have developed doxorubicin (DOX)-loaded poly(lactide-*co*-glycolide) (PLGA) nanoparticles (DP) conjugated with polyethylene glycol (PEG) and transferrin (Tf) to form Tf-PEG-DPs (TPDPs), and incorporated these TPDPs into three-dimensional (3-D) PLGA porous scaffolds to form a controlled delivery system. To our knowledge, this represents the first use of a Tf variant (oxalate Tf) to improve the targeted delivery of drug-encapsulated nanoparticles (NPs) in PLGA scaffolds to PC3 prostate cancer cells. The PLGA scaffolds with TPDPs incorporated have been shown to release drugs for sustained delivery and provided a continuous release of DOX. The MTS assay was also performed to determine the potency of native and oxalate TPDPs, and a 3.0-fold decrease in IC_{50} values were observed between the native and oxalate TPDPs. The lower IC_{50} value for the oxalate version signifies greater potency compared to the native version, since a lower concentration of drug was required to achieve the same therapeutic effect. These results suggest that this technology has potential to become a new implantable polymeric device to improve the controlled and targeted drug delivery of Tf-conjugated NPs for cancer therapy.

1. Introduction

Systemically administered chemotherapy can be curative for some tumors, but it is not particularly effective in treating many solid malignant tumors, such as tumors in lung, brain, breast, colon and prostate [1,2,3]. Generally, these tumors do not respond well to conventional systemic chemotherapy or radiotherapy, especially when the tumors are large or poorly vascularized. If the tumor is operable, surgical removal is the preferred therapy, but many tumors, such as solid malignant gliomas, can recur within 2 cm of the original lesion [4,5]. Therefore, one approach to address this challenge is to directly introduce chemotherapeutic agents in the region *via* their controlled release from polymeric scaffolds and wafers placed in the tumor resection cavity. Scaffolds and wafers have the ability to deliver high local drug concentrations at the tumor site for extended periods of time while maintaining low systemic drug exposure. One successful example is the use of GLIADEL wafers. These wafers are composed of poly(carboxyphenoxy-propane/sebacic acid) anhydride wafers containing carmustine (BCNU), and represent an effective approach for delivering chemotherapeutic agents for the treatment of malignant gliomas. This technology has demonstrated that local chemotherapy with BCNU wafers is well tolerated and offers a survival benefit to patients with newly diagnosed malignant gliomas [6]. This not only results in higher therapeutic efficacy, lower toxicity, and lower acquired drug resistance, but also reduces the need for repeat chemotherapeutic administrations, increasing patience compliance and improving quality of life [7,8,9,10].

Several *in vitro* and *in vivo* assays have been conducted regarding the incorporation of therapeutic drugs (free or loaded in nanoparticles) into three-dimensional (3-D) poly(lactic-*co*-glycolic acid) (PLGA) scaffolds [11,12,13,14,15,16]. PLGA has several advantageous features, such as being biocompatible, bioresorbable, and biodegradable, and has been approved by the Food and Drug Administration (FDA) for several human clinical uses. Moreover, in the field of drug delivery, PLGA has been extensively investigated as a matrix for long-term delivery of drugs [17]. Although loading free drugs in PLGA scaffolds has led to some improvements, their effectiveness has still been limited due to increased side effects associated with the high toxicity of the free drugs [9,18]. Loading the drugs in nanoparticles (NPs) that were subsequently incorporated in PLGA scaffolds improved nonspecific toxicity, but they still exhibited limited therapeutic efficacy due to the low specificity of the NPs that resulted in only a fraction of the drugs reaching tumor cells [9,18]. Fortunately, this low specificity between the NPs and the target tumor cells can be improved using the efficient cellular mechanism of uptake of the human serum transferrin (Tf). This protein has been used as a cancer-targeting agent in multiple delivery systems since the transferrin receptor (TfR) is overexpressed in many types of cancer cells [19].

Although the conjugation of native Tf to therapeutics improves tumor-specific cellular uptake, significant limitations in Tf's efficacy as

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a drug carrier still exist since an iron-bound Tf molecule (holo-Tf) is restricted to a single 5–10 min passage through a cell [20]. Once holo-Tf binds TfR, it becomes internalized *via* receptor-mediated endocytosis, and releases iron. Iron-free Tf (apo-Tf) is then recycled to the cell surface, where it quickly dissociates from TfR. Since rebinding of iron by Tf is an inefficient process, recycled Tf is often assumed to not be able to rebind iron [21,22], and therefore TfR, thus limiting the amount of payload delivered.

Our research group wanted to therefore increase the time Tf remained associated with a cell, *i.e.*, increase its cellular association, and developed and analyzed a mathematical model for the Tf/TfR cellular trafficking pathway to identify inhibiting the iron release from Tf in the endosome as a means for increasing the cellular association of Tf [23]. To achieve this molecular design criterion, we generated a variant of Tf by replacing the synergistic carbonate anion of Tf with oxalate, and demonstrated increased cellular association compared to native Tf, which led to enhanced delivery of a conjugated drug. We then followed up this work by generating oxalate Tf-conjugated NPs that exhibited greater cellular association relative to their native Tf counterparts. Subsequently, we developed doxorubicin (DOX)-loaded poly(lactide-*co*-glycolide) (PLGA) nanoparticles (DP) conjugated to polyethylene glycol (PEG) and Tf, which we refer to as Tf-PEG-DPs or TPDPs, that demonstrated greater drug carrier efficacy when using oxalate Tf instead of native Tf [24].

Here, we extended this work to determine if TPDPs incorporated in three-dimensional (3-D) PLGA scaffolds would exhibit increased effectiveness of the NPs due to increased cellular association when using oxalate Tf instead of native Tf. To our knowledge, this represents the first use of oxalate Tf to improve the targeted delivery of drug-encapsulated NPs in 3-D PLGA scaffolds to tumor cells. The release of NPs from the scaffolds was investigated, and the therapeutic efficacy of our system was studied *in vitro* using the MTS assay performed on PC3 prostate cancer cells line. Our results demonstrated that oxalate TPDP released from the scaffolds suppressed tumor growth much more effectively than native TPDP. Moreover, the scaffolds released the drug in a controlled manner (around 1 month) in comparison to the burst release observed when free DOX was incorporated in the scaffolds (14 days). Both the novel targeting feature and the incorporation of TPDPs within scaffolds have the potential for protecting the patient from toxic side effects commonly associated with highly concentrated boluses.

2. Materials and methods

2.1. Preparation of DOX-loaded nanoparticles

Doxorubicin hydrochloride (DOX)-loaded nanoparticles (DP) were prepared by modifying the nanoprecipitation method described by Mu and Feng [25]. Briefly, 50 mg of poly(D,L-lactide-*co*-glycolide) (PLGA, 50:50, MW 7000–17000, acid terminated) were dissolved in 1.8 mL of dimethyl sulfoxide (DMSO). This solution was then mixed with 200 μL of DOX (5 mg/mL) and 2.56 μL of triethylamine (TEA, diluted 1:10). The polymer solution was then added dropwise to an aqueous phase (10 mL ddH_2O containing 0.02% D-α-tocopherol polyethylene glycol 1000 succinate (TPGS)) with mixing using a magnetic stir bar. The NP dispersion was stirred overnight. Subsequently, the dispersion was filtered through a 0.22 μm pore-size membrane (Millipore, Bedford, MA). The NPs were then washed with ddH_2O three times by centrifugation at 8600*g* for 30 min at 20 °C. The washed DP was stored at 4 °C. All reagents and materials were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise specified.

2.2. Conjugation of the novel targeting ligand and PEG to the nanoparticles

The 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) molecule and N-hydroxysuccinimide (NHS) were used to activate surface carboxyl groups on DP. Briefly, 3 mg of DP were suspended in 0.2 mL of 2-(*N*-morpholino) ethanesulfonic acid (MES) buffer (100 mM, pH 5.5). To activate the carboxyl groups on the DP, 3.2 μL of NHS (1 mg/mL) and 4.5 μL of EDC (1 mg/mL) were mixed with the above DP suspension for 15 min. The reaction was quenched by raising the suspension pH to 7.4 by adding 0.5 M of phosphate buffer. To PEGylate DP, heterobifunctional PEG maleimide-PEG10000-NH_2 (Nanocs Inc., New York, NY) with a 5000:1 PEG:DP molar ratio was added to the activated DP suspension. The solution was then allowed to react for 2 h at room temperature to allow the amine groups on heterobifunctional PEG to form a permanent bond with the activated DP. Free EDC/NHS and PEG were removed by centrifugation at 8600*g*. To couple Tf to the PEGylated DP, Tf was first thiolated with IT as described previously [24]. Free IT was removed by centrifugation through Zeba desalting columns in phosphate buffer. The thiolated Tf (5000:1 Tf:DP molar ratio) was subsequently added to the PEGylated DP, enabling the thiolated Tf to form a permanent linkage with the maleimide group on the PEGylated DP. After overnight incubation at room temperature, free Tf molecules were removed by centrifugation at 8600*g*. The Tf-PEG-DPs (TPDPs) were collected and stored with 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES buffer) at 4 °C.

2.3. Iron loading of Tf and generation of oxalate Tf

The native and oxalate versions of Tf were generated by varying the iron loading procedure. Briefly, 20 μL of 250 mM of the iron chelating agent nitrilotriacetate (NTA) were mixed with 10 μL of 250 mM iron (III) chloride. To generate native Tf samples, the mixture of NTA and iron (III) chloride was mixed with 20 mM HEPES buffer containing 20 mM bicarbonate. To generate oxalate Tf samples, the mixture of NTA and iron (III) chloride was instead mixed with 20 mM HEPES buffer containing 20 mM oxalate. This iron mixture with bicarbonate or oxalate was subsequently added to Tf samples in a 10:1 Fe:Tf molar ratio and incubated at room temperature for 2 h to ensure all Tf were iron loaded. Excess free iron from iron-loaded samples was removed by centrifugation at 8600*g*.

2.4. FITC-Tf conjugation in nanoparticles

First, fluorescein isothiocyanate (FITC) was conjugated to transferrin. A 10 mg/mL stock solution of FITC was dissolved in DMSO, and then 80 μL of this solution was added to a stock solution of Tf previously prepared in 0.1 M $NaHCO_3$ buffer at pH 9.0 (5 mg/mL Tf concentration). The reaction mixture was allowed to react for 1 h at room temperature in the dark. To remove free FITC from the FITC-conjugated Tf, the solution was dialyzed (cutoff value of 6000–8000 kDa) for 2 days against three changes of 0.1 M phosphate buffer (pH 7.4) [26]. Subsequently, FITC-Tf was conjugated to NPs (FITC-Tf-NPs) with a similar procedure as the one mentioned above (Section 2.2.), except that the NPs were not DOX loaded. The FITC-Tf-NPs were collected and stored in the dark with 0.1 M of phosphate buffer (pH 7.4) at 4 °C.

2.5. Characterization of the nanoparticles

The size and zeta potential of the NP samples were quantified using a Malvern Zetasizer Nano ZS model Zen 3600 (Malvern Instruments Inc., Westborough, MA). The NP concentration [*NP*] was measured within

Table 1
Characterization of the doxorubicin-loaded PLGA nanoparticles.

Parameters	DP	TPDP
Diameter (nm)	98 ± 0.5 nm	123 ± 2 nm
Zeta potential (mV)	−23.8 ± 0.6 mV	−30.5 ± 1.4 mV
Polydispersity index	0.081 ± 0.012	0.115 ± 0.04