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Synthesis and characterization of fluorinated conjugates of albumin

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ABSTRACT

A small fluorocarbon dendron that contains nine chemically identical fluorine atoms was covalently conjugated to albumin via a flexible linker. Two versions were made, which differ by 10% in the linker length. Both versions display split ^{19}F signal and much shorter ^{19}F longitudinal relaxation time than their small molecule counterparts. 10% difference in the flexible linker length has negligible impact on the ^{19}F signal.

1. Introduction

Albumin is the most abundant protein in plasma with a concentration around 35–50 g/L in human sera [1]. It is a monomeric, multi-domain globular protein with a molecular weight of 66.5 kDa. Human serum albumin has 585 amino acid residues. Albumin has many medical [2,3] and pharmaceutical applications [1,4], one of which is as macromolecular carrier of imaging agents to prolong circulation time and to enhance tumor accumulation. There are two classes of imaging agents that use albumin as the carrier: ^{99m}Tc-aggregated albumins for single-photon emission computed tomography (SPECT), such as Pulmolite® and Nanocoll®; and albumin-bound gadolinium chelates for magnetic resonance imaging (MRI), such as gadofosveset (Ablavar®) [5].

SPECT has lower spatial resolution than MRI and involves ionizing radiation. In fact, the use of ^{99m}Tc-aggregated albumins in disease diagnosis has been in decline [6]. However, albumin-bound gadolinium chelates have their own problems. For example, for non-covalently albumin-bound gadolinium chelates, such as gadofosveset, the release of gadofosveset from albumin diminishes the correlation between MRI-determined tumor microvascular parameters and histopathological data [7]. In the case of covalently albumin-bound gadolinium chelates, such as albumin-(Gd-DTPA)$_{30}$, dissociation of Gd-DTPA from albumin is prevented. As a result, MRI-determined tumor microvascular parameters correlate much better with histopathological data [7]. However, the long circulation time of covalently-bound Gd-DTPA increases the possibility that the highly toxic Gd^{3+} is released from the complex into blood circulation. This risk factor limits albumin-(Gd-DTPA)$_{30}$ and its analogs to preclinical studies [7].

An alternative class of albumin-based imaging agents is fluorinated albumins for ^{19}F MRI. ^{19}F is a stable isotope with a spin quantum number of 1/2 and 100% natural abundance. ^{19}F is the second most sensitive stable nucleus for MRI (83% as sensitive as ^{1}H). Compared with ^{1}H MRI, ^{19}F MRI is free of endogenous background signal. ^{19}F imaging agents are directly MRI visible and the ^{19}F signal intensity is proportional to imaging agent concentration. In comparison, gadolinium-based contrast agents are indirectly visible through the ^{1}H signal whose intensity has complex dependency on gadolinium concentration. Hence ^{19}F MRI is much more suited for quantitative imaging than ^{1}H MRI.

To form albumin-based ^{19}F imaging agents, fluorocarbon molecules need to bind to albumin, either non-covalently or covalently, analogous to albumin-bound gadolinium chelates. Both types have been explored before, but not in a site-specific manner. Non-covalently fluorinated albumins can be formed simply by mixing a fluorocarbon molecule (e.g., tetrafluorosuccinic acid) with albumin [8]. However, non-covalently fluorinated albumins would suffer the same problems of non-covalent albumin-gadolinium chelates in that the imaging agent can dissociate readily from albumin. This problem is avoided when fluorocarbon

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molecules are covalently linked to albumin. One prior example is conjugating fluorinated anhydrides to the ε-amino group of lysine [9]. However, albumin has a large number of lysine residues (58 for human serum albumin [10]). Therefore it is very difficult to obtain a single product through the fluorination of lysine residues. As imaging agents are pharmaceuticals that require regulatory approval, it is highly desirable to have a single molecular entity with well-defined chemical structure. The goal of this project is to explore site-specific fluorination of albumin.

One approach to site-specific protein fluorination is to introduce fluorinated amino acids such as L-4-trifluorophenylalanine through genetic engineering using expanded genetic codon [11]. However, the ^{19}F signal from a single $-CF_3$ group in an amino acid is too weak for ^{19}F MRI. Another approach to site-specific protein fluorination is through chemical conjugation to the –SH group of cysteine. Albumin has 35 cysteine residues, 34 of which are paired to form 17 disulfide bonds [12]. This leaves a single cysteine, Cys-34, for site-specific conjugation.

Our group has developed fluorinated dendrimers as ^{19}F imaging agents [13,14]. Due to the spherical symmetry of the dendritic structure, ^{19}F signals from multiple fluorine atoms coalesce into a single ^{19}F signal for imaging. We have made a family of fluorocarbon dendrons with each dendron containing 9–243 chemically identical fluorine atoms [15].

To explore the chemistry of site-specific labeling of albumin with these fluorocarbon dendrons, we successfully conjugated the smallest dendron of this family, a perfluoro-*tert*-butanol derivative, to Cys-34 of bovine serum albumin (BSA) in this work.

2. Results and discussion

2.1. Design of fluorinated albumins

Our conjugation of the small fluorocarbon dendron to albumin utilizes the prosthetic group maleimide, which is commonly used for albumin conjugation through Cys-34 [1]. Fig. 1 shows the structure outline of the fluorinated albumin. Between the fluorocarbon dendron and the prosthetic group is a 3-segment flexible linker, made of one lysine flanked by two tetraethylene glycol units. This 3-segment linker serves several purposes: to avoid steric clash between the fluorocarbon dendron and albumin; to provide motion freedom to the ^{19}F signal emitter to prevent signal broadening; and to facilitate albumin conjugation through electrostatic attraction because lysine and albumin are oppositely charged at neutral pH.

Fig. 2 shows detailed structures of two fluorinated albumins. The two versions differ in the way lysine is incorporated into the flexible linker. As a result, one linker is four methylene units longer than the other. The intention is to explore whether the length of the flexible linker has any effect on the resulting ^{19}F signal.

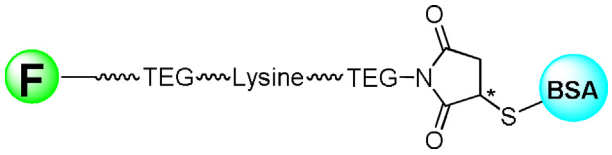

Fig. 1. Structure of fluorinated albumin. **F** represents the fluorocarbon dendron; TEG refers to tetraethylene glycol; BSA is bovine serum albumin. The asterisk indicates a chiral center generated during conjugation.

2.2. Synthesis of fluorinated albumins

The synthesis of fluorinated albumins started with connecting the fluorocarbon dendron to the flexible linker. As shown in Scheme 1, protection of one of the hydroxyl groups in tetraethylene glycol **1** with 4-toluenesulfonyl chloride afforded compound **2** [16]. The resulting tosylate **2** was subjected to S_N2 substitution with sodium azide to give compound **3** [16]. Conversion of the remaining hydroxyl group in compound **3** to tosylate afforded precursor **4** [17], which then reacted with the small fluorocarbon dendron, sodium perfluoro-*tert*-butanoate, to give the azide compound **5**. Reduction of the azide group in **5** with Ph_3P followed by hydrolysis gave free amine **6**. The coupling between amine **6** and Fmoc-Lys(Boc)-OH proceeded smoothly to give compound **7** with good yield. It should be noted that the reaction temperature and the molar ratio of the reactants are both important; the coupling reaction was conducted at 0 °C and Fmoc-Lys(Boc)-OH was slightly in excess of **6** at a molar ratio of 1.2:1. Removal of the Fmoc group in compound **7** with piperidine in DMF afforded the secondary amine **8** with high yield.

Next we turned our attention to introducing the prosthetic group maleimide to the fluorocarbon dendron via the flexible linker (Scheme 2). Reaction of the azide compound **3** with bromo *tert*-butyl acetate by a modified procedure afforded a new azide compound **9** [18]. This reaction extends the flexible linker by two carbon atoms and at the same time introduces a protected carboxylic group for further condensation with the amino group in compound **8**. Reduction of the azide group in compound **9** with Ph_3P afforded the free amine compound **10** [18]. Reaction of **10** with *N*-methoxycarbonyl-maleimide **12**, which was prepared from maleimide **11** according to literature procedure [19], afforded compound **13**, an ester. Slight excess of compound **12** (the **12**:**10** molar ratio was 1.25:1) and moderate reaction time (1 h) at 0 °C were required to achieve good yield for this reaction. Deprotection of the *tert*-butyl group in compound **13** with TFA, followed by coupling with amine **8**, afforded compound **14** at 61% yield. Exposing **14** to TFA removed the Boc protection group of the ε-amino group of lysine, resulting in compound **15**, which was purified by preparative HPLC with high yield. Compound **15** has excellent aqueous solubility at neutral pH.

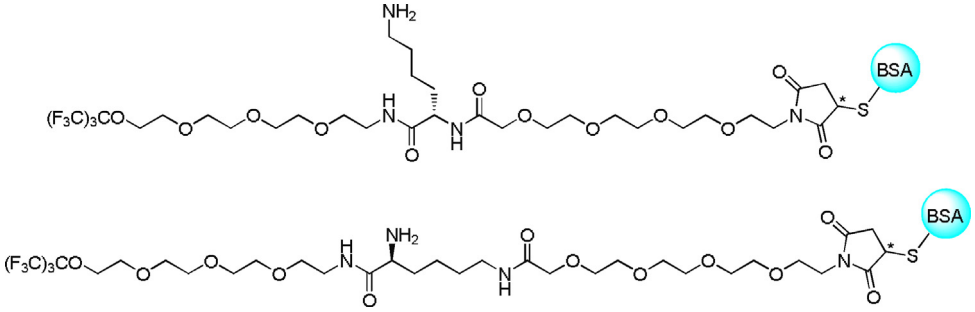

Fig. 2. Fluorinated albumins with shorter (top) and longer (bottom) flexible linker between the fluorocarbon dendron and albumin.