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Biodistribution and excretion of monosaccharides-albumin conjugates measured with *in vivo* near infrared fluorescence imaging

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Abstract

Target specific small molecules as modulators of drug delivery may play a significant role in the future development of therapeutics. Small molecules can alter the *in vivo* pharmacokinetics of therapeutic macromolecules leading to more efficient drug delivery with less systemic toxicity. The potential of creating a more effective drug delivery system through glycosylation has led, for instance, to the addition of galactose to increase drug delivery to the liver. However, there are many other monosaccharides with potentially useful targeting properties that require further characterization. Here, we investigate the potential of glycosylation to guide molecular therapies using 5 different monosaccharides conjugated to human serum albumin (HSA). Additionally, we investigate how the amount of glycosylation may alter the pharmacokinetic profile of HSA. We introduce the use of *in vivo* near infrared (NIR) optical imaging to characterize the effect of differential glycosylation on the pharmacokinetics of macromolecules.

Keywords

pharmacokinetics; excretion; near infrared fluorescence; *in vivo* imaging; monosaccharide

Introduction

Target- organ or cell specific drug delivery can alter the pharmacokinetics of macromolecules including proteins or nano-particles thus, changing drug catabolism and excretion with possible improvements in the therapeutic index (i.e. improved target delivery with decreased off-target toxicity). Conjugation of drugs with small molecules such as sugars, short peptides, or folic acid can dramatically alter the pharmacokinetics (1). Glycosylation, in particular, has been investigated for targeting hepatocytes, macrophages, vascular endothelial cells, and various cancer cells via the so-called “lectin” family of sugar receptors, commonly over-expressed on cancer cells (2–5). For example, radiolabeled galactose conjugated to albumin has been used to assess hepatocyte function. Fluorescence-labeled galactosamine conjugated albumin has recently been found to

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Supporting Information Available. Analytical mass spectroscopy conditions and data of all conjugates are shown in the Supporting information. This material is available free of charge via the Internet at <http://pubs.acs.org>.

specifically bind, internalize, and visualize the peritoneal dissemination of various ovarian cancer nodules with high specificity and sensitivity (6). Through these studies, it becomes clear that galactose conjugation dramatically alters the biodistribution of the molecule to which it is bound. Other monosaccharides may lead to similar alterations in catabolism and excretion.

The effect of a variety of different types and amounts of monosaccharides has not been systematically studied. Here, we conjugate low (ranging 3 to 5) and high (ranging 23 to 25) numbers of various mono-saccharides (galactose- β , glucose- α , mannose- α , fucose α and β) to human serum albumin, which is also labeled with a NIR fluorescent probe. These conjugates were then used to evaluate the change in PK and excretion using NIR fluorescence imaging.

Experimental Procedures

Synthesis and QC of monosaccharide conjugated albumin

Linker-modified monosaccharides (Fig. 1) were prepared as reported previously, and conjugation to human serum albumin was carried out as reported previously for bovine serum albumin conjugates (7–8). Briefly, linker-modified monosaccharides were dissolved in a 50/50 mixture of DMF/H₂O at a final concentration of 150 mM. N-(3-dimethylaminopropyl)-N'-ethyl-carbodiimide hydrochloride (EDC, Sigma-Aldrich, St. Louis, MO) was dissolved in a 50/50 mixture of DMF/H₂O at a final concentration of 300 mM. N-hydroxysuccinimide (NHS, Sigma-Aldrich) was dissolved in DMF at a final concentration of 300 mM. The NHS ester was preformed by combining the carbohydrate, EDC, and NHS in an Eppendorf tube at a ratio of 2:1:1 and allowing the reaction to stand at room temperature with occasional gentle mixing. After 60 minutes, each reaction mixture was added to a solution of HSA (4 mg/mL in 10 mM sodium borate, 90 mM NaCl, pH 8.0, Sigma-Aldrich) that had been pre-cooled to 4°C to give a final ratio of carbohydrate to HSA of approximately 5–8:1 to produce low density conjugates or 37–72:1 to produce high density conjugates. After 15 minutes, the solution was warmed to room temperature and allowed to stand for 1 h (Fig. 1). HSA-conjugates were then dialyzed extensively against water containing 6 mM NaCl (SpectraPor 7, MWCO = 10,000). The extent of conjugation (Fig. 2) was evaluated by measuring the average mass of each conjugate by MALDI-MS (Applied Biosystems Voyager-DE Pro time-of-flight mass spectrometer, Life Technologies Co., Carlsbad, CA), and then determining the mass shift relative to unmodified HSA (Table 1, also see Supplementary figure 1).

Synthesis of fluorophore conjugated glycosylated HSA probes

The NHS ester of IRDye 800CW (IR800) was purchased from LI-COR Co. (Lincoln, NE). Glycosylated HSA and non-glycosylated samples (6 nmol) were incubated with NHS ester of IR800 (40 nmol for low sugar and 60 nmol high sugar) in 0.1M Na₂HPO₄ (pH 8.5) at room temperature for 15 min. Each mixture was purified with a Sephadex G25 column (PD-10; GE Healthcare, Piscataway, NJ)(Fig. 1). The protein concentration was determined by measuring the absorbance at 280 nm with a UV-Vis system (8453 Value UV-Visible Value System; Agilent Technologies, Santa Clara, CA). The concentration of IR800 was measured by absorbance with the UV-Vis system to confirm the number of fluorophore molecules conjugated to each glycosylated HSA (9). The number of fluorophore molecules per all HSA conjugates was ~1.7–2.3.

In Vivo Imaging

All procedures were carried out in compliance with the Guide for the Care and Use of Laboratory Animal Resources (1996), National Research Council, and approved by the local Animal Care and Use Committee.

Forty micrograms of either high or low IR800-HSA-glucose- α (Glc- α), IR800-HSA-mannose- α (Man- α), IR800-HSA-galactose- β (Gal- β), IR800-HSA-fucose- α (Fuc- α), IR800-HSA-fucose- β (Fuc- β), or IR800-HSA (control) was injected intravenously into the tail vein of female nude mice (National Cancer Institute Animal Production Facility, Frederick, MD) ($n=3-4$ in each group). Mice were anaesthetized with isoflurane, and serial images of the dorsal and ventral surfaces were obtained using the Pearl Imager (LI-COR Biosciences, Lincoln, NE). The instrument has a light-tight chamber equipped with a cooled charge-coupled device camera. Illumination is with diode lasers, and the excitation and emission filters are specifically tuned for IR800. Images were acquired and processed using Pearl Cam Software v1.0 (LI-COR Biosciences, Lincoln, NE). Longitudinal images were acquired at 10 minutes, 6 hours, 24 hours, and 48 hours after injection of the imaging probe; and mice were anesthetized with vaporized isoflurane via nose cone for all imaging. For performing the quantification of signals, regions of interest (ROIs) of equal sizes were drawn and placed over areas representative of background, neck, liver, abdomen, and bladder. Total intensity for each ROI was normalized to the sum of the total intensity of all the ROIs drawn per animal, and the normalized values were used for statistical analysis.

Ex Vivo Organ Imaging

After imaging at the 48 hour time point, the mice were euthanized with carbon dioxide inhalation and liver, spleen, kidneys, blood, bone, colon, stomach, and pancreas were removed. Organs were placed on a non-fluorescent black plate and imaged with the Pearl Imager. However, for all conditions except with the IR800-HSA probe, only the liver could be depicted. So, we then compared uptake of each glycosylated conjugate on an organ by organ basis.

For performing the quantitative analysis, identical sized ROIs were drawn on comparable parts of each corresponding organ. Total intensity for each ROI was used for statistical analysis. Sugar to sugar comparison was performed with a one-way ANOVA with Tukey's multiple comparisons post-test. High vs. low sugar comparison was performed with an unpaired, two-tailed t test. All statistical analysis was performed using GraphPad Prism version 4.0c for Macintosh (GraphPad Software, San Diego California USA, www.graphpad.com).

Results

In vivo Biodistribution study using NIR fluorescence imaging

Findings with HSA-IR800 (control)—To assess the influence of IR800 conjugation on HSA biodistribution, we carried out serial *in vivo* imaging studies using an HSA-IR800 probe without glycosylation (Fig. 3). At 10 minutes, diffuse distribution was observed throughout the body of the mouse. The primary location of the HSA at 10 minutes was in the vasculature as demonstrated by clear visualization of the blood vessels (Fig. 3). The liver was also identified, most likely due to the high blood volume in the liver. At 6 hours, we observed a diffuse signal with loss of the vessel delineation, likely representing leakage of IR800-HSA into the extracellular space and capillary beds near the skin. At 24 and 48 hours a similar diffuse pattern was observed that differed only in intensity. By 48 hours, signal was barely visible.

Low Sugar -HSA-IR800 Probes—The time course of fluorescence intensity was obtained after injection of IR800-HSA labeled with low sugar numbers (3–5/HSA) of Glc- α , Man- α , Gal- β , Fuc- α , or Fuc- β (Fig. 4a). Rapid accumulation of fluorescence was noted in the liver just 10 minutes after injection for all of the low sugar probes. Also at 10 minutes, the IR800-HSA low sugar probe was seen in the vessels with a minimal bladder signal. The vasculature disappeared by 6 hours. At 6 hours, in addition to intense liver signal, Glc- α and Gal- β labeled probes demonstrated high bowel (arrows) and some bladder signal while Fuc- α , Fuc- β , and Man- α showed only signal in the liver and bladder (not bowel) at 6 hours. Another observation is that for all of the low sugar conjugates, the intensity of signal around the liver and abdomen was brighter at 6 hours than at 10 minutes. At 24 hours, all low sugar conjugates showed only liver signal with slight bladder signal associated with an overall decrease in fluorescence intensity. At 48 hours all sugar conjugates demonstrated minimal liver signal although Fuc- α and Fuc- β displayed higher liver signal than Glc- α , Gal- β , and Man- α .

High Sugar-HSA-IR800 Probes—The high sugar probes (high sugar HSA-IR800) demonstrated rapid accumulation in the liver at 10 minutes (Fig. 5a). However, unlike the low sugar probes, the blood vessels were not clearly seen 10 minutes after injection. Further, all high sugar probes showed some bladder signal at 10 minutes. At 6 hours, Glc- α , Gal- β , and Fuc- β demonstrated high liver, bowel (arrow), and bladder signal. In contrast, Man- α and Fuc- α did not demonstrate bowel signal. By 24 hours, the high sugar probes show only faint liver and bladder signal with Gal- β demonstrating the lowest liver signal intensity. At 48 hours post injection, the high sugar probes were no longer visible.

In the quantitative comparison of both the low (Fig. 4b) and high (Fig. 5b) sugar probes, the only significant difference was observed in the signal within the abdomen at 6 hours. Similar results were obtained between high and low sugar probes, with Glc- α and Gal- β demonstrating the highest relative percentage of total intensity at 6 hours. In both the high and low sugar groups, Gal- β abdomen intensity was statistically significantly higher compared to Man- α , Fuc- α , and Fuc- β ($p < 0.05$).

Ex Vivo Organ Analysis

Only the liver and spleen showed a difference between the probes (Fig. 6,7). The low Gal- β probe demonstrated the lowest relative intensity of the low sugar probes at 48 hours (Fig. 6b). However, this difference is only statistically significant in comparison to low Glc- α ($p < 0.05$) (Fig. 6d). The rest of the low sugar probes were similar in intensity. The high Gal- β probe also had the lowest relative intensity when compared to the other high sugar probes (Fig. 6c). This is statistically significant in comparison to high Glc- α , Man- α , and Fuc β ($p < 0.05$) (Fig. 6e). The other organ that showed a difference was the spleen. In the low sugar probes, low Man- α had the highest total intensity (Fig. 7b). This increase was statistically significant relative to low Glc- α , Gal- β , and Fuc- β ($p < 0.05$) (Fig. 7d). Low Fuc- α is relatively greater in intensity than low Glc- α , Gal- β , and Fuc- β , but that difference was not statistically significant. The high sugar probes have similar intensities between each other in the spleen (Figs. 7c and e).

When comparing liver signal between low and high sugar conjugates using the same sugar in *ex vivo* organs at 48hrs, all low sugar conjugates had higher total intensity than the high sugar conjugates (Fig. 6f). Low Glc- α , Man- α , Gal- β , Fuc- α , and Fuc- β all had significantly higher total intensity than the high conjugates with P values of .02, .03, .01, .009, and .01; respectively. When comparing splenic signal in *ex vivo* organs at 48hrs, all low sugar conjugates had a relatively higher total intensity than the high sugar conjugates (Fig. 7f). Low Glc- α is the only sugar conjugate that did not show statistical significance. Low Man- α ,

Gal- β , Fuc- α , and Fuc- β all have significantly higher total intensity than the high conjugates with P values of .0011, .0079, .014, and .0042; respectively.

Discussion

Here we used optical imaging to observe the biodistribution and excretion patterns associated with molecular probes composed of HSA conjugated to high and low numbers of various monosaccharides: Glc- α , Gal- β , Man- α , Fuc- α , and Fuc- β , all conjugated with identical conjugation chemistry. HSA is a representative soluble protein that has high bioavailability, is nonimmunogenic, and demonstrates good *in vivo* stability with an average half-life of 19 days (10). The conjugation of IR800 to HSA demonstrated distribution throughout the body with little specificity suggesting that the addition of the NIR probe minimally changed the biodistribution of HSA. However, the conjugation of either low numbers or high numbers of sugar molecules led to rapid and specific changes in biodistribution, particularly to the liver and spleen. Similar observations have been made using radiolabeled BSA conjugated to galactose, fucose, and mannose (11–13). The effect of the amount of sugar residue conjugated to BSA on binding properties has been studied, and reveals that mannosylated BSA demonstrated a higher rate of uptake as the number of mannose residues per BSA molecule increased with a plateau effect (13). Similarly, we found that conjugation of HSA to a low number of sugar molecules leads to slower clearance of the probe from the circulation compared to HSA conjugated to a high number of sugar molecules. This is demonstrated by the observation that at 10 minutes, the blood vessels can still be observed in all low sugar number HSA conjugates but not in high sugar number HSA conjugates.

With this conjugation and labeling methods, both reactions can use amino groups at the side chain of lysine or at the N-terminal. There are 60 lysines in the human serum albumin, therefore, theoretically 61 available conjugation sites exist in a single albumin molecule. In the previous literatures, maximum 35 molecules of diethylenetriamine pentaacetic acid (DTPA) were conjugated with a single albumin molecule via the same amine groups for the use of macromolecular MRI contrast agents (14). In our experience, maximum 28 galactosamine and glucosamine molecules were conjugated with a single albumin molecule among 63 theoretically available carboxyl groups of glutamate using a similar direct amidation method using EDC (15). Therefore, although numbers of available conjugation sites are varied depending on molecules to be conjugated and conjugation chemistry, about a half of theoretically available functional groups might be practically conjugatable probably because of steric hindrance.

The current study suggests that different organs have different overall receptor specificity for different glycosylated probes. The hepatocytes, for instance, demonstrate an affinity for galactose, and this is mainly through the asialoglycoprotein receptor (16). Non-parenchymal cells in the liver include sinusoidal endothelial cells and Kupffer cells. Mannose receptors are expressed on both of these non-parenchymal cell types (17). However, there is a lectin only expressed by Kupffer cells that demonstrates high affinity for fucose and galactose but not mannose (18). Cultured rat sinusoidal endothelial cells and Kupffer cells were used to distinguish between the binding characteristics of BSA conjugated mannose and fucose (17). It was demonstrated that both mannosylated and fucosylated BSA was taken up by sinusoidal endothelial cells, and that this uptake was inhibited by excess mannose or fucose but not by galactose. They further demonstrated that Kupffer cells could take up mannosylated and fucosylated BSA, but the uptake of fucosylated BSA could be inhibited by galactose (17).

The specific binding of glycosylated HSA molecules to cell surface receptors is supported by the excretion profiles exhibited by the different sugar conjugates. Glc- α and Gal- β demonstrate rapid liver uptake and excretion into the gut. This supports the concept that Glc- α and Gal- β may be preferentially taken up by hepatocytes eventually culminating in hepatobiliary excretion. Man- α and Fuc- α , however do not demonstrate gut excretion observed by Glc- α and Gal- β suggesting that these sugars may lead to specific uptake into the liver via a different receptor. It would make sense that uptake of Man- α and Fuc- α by non-parenchymal cells via the mannose receptor leads to excretion by routes other than bile.

Additionally, presence of the mannose receptor on macrophages in the spleen explains the increased signal observed by low Man- α and low Fuc- α on *ex vivo* organ studies.

An additional challenge to using optical imaging probes is their inability to accurately quantitate signal compared with radionuclide imaging including single photon emission tomography (SPECT) and positron emission tomography (PET). This limitation is minimized with near infrared probes which have much better light transmission properties in tissue than do visible range probes. Nonetheless, absolute molecule quantitation, similar to what can be done with radioisotopes, cannot be achieved using the current model. However, our semi-quantitative results correlate with previous studies using radiolabeling. Quantitation with radiolabeling of albumin conjugated to sugars has some limitations, especially since catabolism in the liver or the RES may lead to separation of the radiolabel and the albumin. Moreover, this effect varies with the radioisotope employed. For instance, the difference in the biodistribution of galactosylated BSA between labeling with ^{125}I ^{131}I , and ^{111}In (12) demonstrated that the choice of radioisotope alters the accuracy of quantitative pharmacokinetics. Regardless of the catabolism in hepatocytes or macrophages, radio-iodines are dehalogenated immediately after the internalization and excreted into the urine, in contrast, ^{111}In is trans-chelated and stays in the bound cells (19).

Unlike to indocyanine green (ICG), IR800 is mostly excreted into the urine (20). Therefore, once macrophages catabolize the albumin-conjugate, catabolized IR800 re-enters the circulation and is excreted into the urine. However, once hepatocytes catabolize the albumin-conjugate, catabolized IR800 can be excreted into the bile. Therefore, we observed the intestinal activity only with Man- α and Fuc- α conjugates.

In conclusion, we demonstrate that labeling with different sugars and altering the number of sugars per molecule dramatically changes the pharmacokinetics and excretion of albumin labeled with IR800 and detected with near infrared fluorescence imaging. Gal- β and Glc- α conjugates were excreted into the intestine suggesting uptake and catabolism by hepatocytes. In contrast, Man- α and Fuc- α conjugates were excreted into the urine suggesting the catabolism occurred mainly in macrophages. The differences of the splenic accumulation in the *ex vivo* organ study supported this hypothesis. The sugar conjugation might be useful for changing the catabolism and excretion route of a native compound and might lead to optimization of the dose to maximize efficacy while minimizing toxicity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 2.
Chemical structure of linkage between sugar and albumin.

Figure 3. Time course of non-glycosylated HSA labeled with the NIR fluorophore, IR800, at 10 minutes, 6 hours, 24 hours, and 48 hours after injection demonstrating a lack of targeted HSA delivery.

b. **Figure 4.**

Time course mouse images of HSA glycosylated with a low sugar number at 10 minutes, 6 hours, 24 hours, and 48 hours after injection are shown in (a). Rapid liver uptake can be appreciated as early as 10 minutes after injection in all sugar conjugated albumin compared with unconjugated albumin shown in Figure 2. At 6 hours, low Glc- α and Gal- β demonstrate activity present in the bowel (arrows). This is different when compared to the other sugars, and this difference is quantified and graphically represented in (b).

b.

Figure 5.

(a) Time course of HSA glycosylated with a high sugar number at 10 minutes, 6 hours, 24 hours, and 48 hours after injection demonstrating rapid liver accumulation after just 10 minutes. 6 hours after injection, high Glc- α , Gal- β , and Fuc- β have activity present in the bowel, (arrows). (b) Graphical representation quantifying the differences in intensity of the bowel signal. Also, note that fluorescence signal is not detected at 48 hours.

Figure 6.

Ex vivo quantitation of liver intensity 48 hours after injection. (a) a schematic of where each (b) low or (c) high sugar conjugated HSA distributes. (d) Low and (e) high sugar quantitation is also represented graphically. (f) At 48 hours, low sugar conjugates are brighter than high sugar conjugates in the liver.

Figure 7.

Ex vivo quantitation of spleen intensity 48 hours after injection. (a) schematic to illustrate where each (b) low and (c) high sugar conjugated HSA is located. (d) Low and (e) high sugar quantitation is represented graphically. (f) Comparison at 48hours illustrates that low sugar conjugates are brighter than high sugar conjugates in the spleen.

Table 1

Summary of the characterization data for the conjugates

Name	Ave Mass (by MALDI)	Change	Each saccharide adds	Ratio of saccharide to HSA	Density
Glc- α 23-HSA	74100	7624	335	23	high
Glc- α 05-HSA	68088	1612	335	5	low
Man- α 22-HSA	73844	7368	335	22	high
Man- α 05-HSA	67941	1465	335	5	low
Gal- β 25-HSA	74869	8393	335	25	high
Gal- β 05-HSA	68123	1647	335	5	low
Fuc- α 23-HSA	73841	7365	319	23	high
Fuc- α 03-HSA	67333	857	319	3	low
Fuc- β 24-HSA	74051	7575	319	24	high
Fuc- β 04-HSA	67767	1291	319	4	low