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In vivo kinetics and biodistribution analysis of neoglycoproteins: effects of chemically introduced glycans on proteins

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Abstract Biodistribution and *in vivo* kinetics analysis of chemically prepared neoglycoproteins are reviewed. Various mono- and oligosaccharides were conjugated onto the protein surface by use of chemical methods. Their kinetic and organ-specific accumulation have extensively been studied after intravenous injection and analyzed by conventional dissection studies, as well as noninvasive methods, such as SPECT, PET, or fluorescence imaging. These studies clearly show the glycan-structure dependency on protein kinetics, which will provide promising possibilities for pharmacological and diagnostic applications.

Keywords Neoglycoprotein · Bioconjugation · Chemical reaction · *N*-Glycans · Albumin · *In vivo* imaging · Tumor targeting · Biodistribution

Introduction

It has long been known that the glycans are involved in a variety of important physiological events, including cell-cell recognition, adhesion, signal transduction, and quality control [1]. For instance, the asparagine-linked glycans (*N*-glycans) containing sialic acid residues on soluble proteins or peptides enhance circulatory residence [2]. For examples, *N*-glycan-engineered erythropoietin (EPO) [3] or insulin [4] exhibits a remarkably higher stability in serum, which effects the prolonged bioactivity. Antibody-dependent cellular cytotoxicity (ADCC) and/or complement-dependent cytotoxicity (CDC) has also been proposed to be modulated by the sialic acids of *N*-glycans in immunoglobulin (IgG) through Siglec interactions by glycosylating or removing the sialic acids [5]. Recently, interest has shifted to the kinetics of these glycans in live animal. While dissection-based biodistribution experiments following intravenous injection of the labeled glycoconjugates into small animal models is the conventional method of choice, molecular imaging is the most promising tool to visualize the "on-time" *N*-glycan kinetics *in vivo*. In addition to fluorescence imaging, which could be conveniently performed at the small animal levels, magnetic resonance (MR) and the positron emission tomography (PET) imaging, which have technologically improved sensitivity, are well-suited for diagnostic applications [6]. Nevertheless, the challenge in efficient glycan imaging and biodistribution studies in living animals is to obtain the structurally pure oligosaccharides either from nature or by synthetic methods. In addition, the bioactivity of oligosaccharides might be derived from the multivalency and/or heterogeneous environment, *i.e.*, on cell surfaces that are composed of oligosaccharide clusters along with other biomolecules. Therefore, efficiently mimicking a glycan-involved bio-environment, *e.g.*, by conjugating the glycans to the proteins, to the cluster templates, or even to the surface of the cells, may provide information on the *in vivo* kinetics of glycans; a single molecule of the glycan, either obtained from a natural or synthetic source, is readily excreted from the body [7, 8]. The recent successful noninvasive imaging, biodistribution, and targeting study of glycans and/or glycoconjugates dealt with natural- and neoglycoproteins, liposomes, dendrimers, and nanoparticles [9].

In this mini-review, we would like to review the recent studies on glycan-based *in vivo* kinetics and tumor targeting using the neoglycoprotein probes, which were prepared by chemically modifying the protein templates by glycan structures. These biodistribution and imaging studies clearly visualize the remarkable dependence of the glycan structures on

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in vivo kinetics and organ-specific accumulation. For most of the biodistribution studies cited in this mini-review, the stability and the degradation analysis of the neo-glycoproteins was not performed. We therefore used the term "serum stability", when either the labeled neo-glycoproteins and/or their metabolites (which should be large enough for not being excreted through the biofiltration in the kidney) are stably circulated in the bloodstream. On the other hand, when the neo-glycoproteins, for instance, were trapped by scavenger cells in the serum and digested by proteases, the smaller peptide fragments could smoothly be excreted through the urinary bladder (and not taken up by the specific organs); we defined this phenomenon here as "serum clearance". We also used the term "organ accumulation", which defined here as the uptake of either the labeled neo-glycoproteins and/or their metabolites by the specific organs.

Neoglycoproteins modified by monosaccharides

Neoglycoproteins modified with simple monosaccharides have extensively been studied for decades, *e.g.*, for improving the pharmacokinetics of the proteins [10]. Glycan conjugation chemistry is generally performed by reacting with activated pyranoside donors [11], or frequently by reductive amination at the reducing end of the saccharides [12]. The general trend of protein modification by monosaccharides were provided by Gabius and co-workers: they have prepared the bovine serum albumin (BSA) modified by various monosaccharides, and their biodistribution and *in vivo* kinetics were throughly investigated in mice by labeling with ^{125}I [13]. They pointed out the importance of sugar density on protein serum stability; the high sugar loading on the protein led to decreased serum stability. The following section deals with the notable effects of protein modification by galactose, mannose, and fucose, the three important monosaccharides.

Dissection-based kinetics and biodistribution studies: effects of protein modification by galactose, mannose, and fucose

Galactose-modified proteins are recognized by asialoglycoprotein receptors on the liver parenchymal cells, and subsequently taken up by endocytosis [14]. The biodistribution of the galactose-modified ^{111}In-proteins in mice was evaluated by Hashida and co-workers [15, 16]. The protein size, galactose loading (density), and the injection dose were important factors for protein kinetics and organ-selective accumulation. While the glycoconjugates of small proteins, such as lysozymes, were rapidly accumulated in the liver, those of relatively large proteins, *e.g.*, γ-globulins, stayed longer in plasma [15]. Higher galactose density on protein surfaces on the other hand led to enhanced recognition by the asialoglycoprotein receptor, and hence the higher accumulation in the liver. This effect, however, became obscure when the neoglycoproteins were injected at higher dose due to the saturation of the receptor [16]. Overexpression of the asialoglycoprotein receptor on some tumor cells provides the promising opportunity to use the galactose-modified proteins as the tumor-targeting tracers. Kobayashi and co-workers recently examined the tumor accumulation of the galactosamine- or galactose-modified BSA (labeled by rhodamine green) in human adenocarcinoma bearing mice [17–19]; visualizing the submillimeter-sized tumor even after the dissection. ^{99m}Tc-Galactose-modified human serum albumin (HSA) has also been applied for clinical use in Japan and Europe to estimate hepatocyte mass and functions [20].

Conjugation with mannose, on the other hand, appends the neoglycoprotein's affinity to non-parenchymal cells, *e.g.*, Kupffer (the sinusoidal macrophage) or the endothelial cells that express the C-type lectins (mannose receptors) [21], as first described by Shen and co-workers using the ^{125}I-labeled human placental β-glucocerebrosidase [22]. ^{99m}Tc-labeled mannosylated HSA were developed by Arano [23] and Jeong groups [24], and they were very effective in detecting the sentinel lymph node. Mannose/protein conjugation approach is especially useful for the therapeutic applications of the enzymes. For instances, the trafficking pattern of ^{111}In-labeled catalase, as reported by Hashida and co-workers, were notably altered from parenchymal to non-parenchymal cells, thus preventing the injury caused by reactive oxygen species in a hepatic ischemia/reperfusion model [25]. They also achieved the targeting of mannosylated catalase to the liver, which could significantly reduce the damage by hydrogen peroxide [26].

Alternatively, the fucose-modified neoglycoprotein labeled by ^{111}In was reported to accumulate in the liver, especially in the Kupffer cells [27]. Das and co-workers also examined in detail the distribution of the fucose-conjugated HSA at the subcellular level; they clarified that most of the neoglycoprotein are localized in the lysosome-rich fractions [28].

Noninvasive imaging of *in vivo* kinetics and organ-specific accumulation of the proteins modified by monosaccharides

Besides the conventional dissection-based biodistribution studies discussed above, the recent efforts are devoted to the noninvasive imaging of the glycoconjugates. These methods can visualize the time-dependent kinetics and/or the organ-specific accumulation in a whole animal level based on radioactivity (single photon emission computed tomography (SPECT) and PET) or near-infrared fluorescence detection. Jeong and co-workers have successfully imaged the ^{99m}Tc-labeled monosaccharide/protein conjugates in mice by gamma camera [24, 29]. They found that the mannose-modified proteins were catabolized in non-parenchymal cells and excreted into the urine, whereas the galactose congeners were captured by the hepatocytes (intestinal excretion pathway) [29]. They

also succeeded in visualizing the monosaccharide conjugates trafficking to the lymph node, the liver, and the spleen by PET [30].

Kobayashi and co-workers compared the effects of the monsaccharide structures on protein kinetics by noninvasive fluorescence imaging; they have imaged the HSA conjugates with glucose, galactose, mannose, and fucose in nude mice based on the near-infrared fluorescence detection, IR800 [31]. They reported that glucose- and galactose-modified albumins were captured by the hepatocyte, while the mannose- and fucose-conjugates were catabolized in the macrophage (excreted into the urine), which well agreed with the Jeong's observation [24]. Kobayashi and co-workers further improved their fluorescence-based imaging strategy by applying a green light-activatable near-infrared fluorescence as the label [32].

Neoglycoproteins modified by oligosaccharides

Although the preparation and chemical conjugation of the larger and more complex oligosaccharide structures, *e.g.*, natural *N*-glycans, are in fact not trivial, the elucidation of their *in vivo* kinetics and organ-specific accumulation is one of the recent topics in the chemical glycobiology field. These investigations could come close to answering the "meaning of glycan structure complexity" in natural glycoproteins. They also lead to the general strategies in developing the neoglycoproteins for pharmacological applications. Namely, the protein kinetics and biodistribution could be precisely controlled when the suitable glycan structures are introduced.

Following the preliminary research dealings with the disaccharide conjugates [13, 33], Gabius and Unverzagt have extensively studied the structure-dependent biodistribution of the complex-type *N*-glycans [34–40]. In order to prepare the various *N*-glycan structures, a chemoenzymatic method was elegantly applied; namely, the chemically prepared biantennary heptasaccharide was further elongated by using a set of glycosyltransferases. The various *N*-glycan structures were subsequently attached to BSA through the isothiocyanate linkage (a few glycans loaded per BSA) before labeling with ^{125}I. The synthesized glycan structures and their biodistribution & serum stability properties in mice (after dissection) are summarized in Fig. 1. Notably, the substitution at the non-reducing end of galactose by *N*Ac-neuraminic acid increased serum stability [34], and the α(2–3)-sialylated neoglycoprotein especially exhibited higher stability than the corresponding α(2–6)-congener. Core-fucosylation at the reducing end of glucosamine, which would affect the conformation of the α(1–6)-arm structure based on the computational analysis, generally accelerated serum clearance, also depending on the sialoside linkage at the non-reducing end (α(2–6)-sialoside is cleared faster than the α(2–3)-sialoside) [35]. The introduction of the β(1–4)-bisecting glucosamine at the branching mannose resulted in increased accumulation in the liver and the spleen [36]. On the other hand, another bisecting substitution, *i.e.*, the β(1–2)-lactosamination of the branching mannose, enhanced the serum clearance [37]. It is noted that the complex-type *N*-glycans with triantennary structures had interesting effects; the elongation of mannose branching at the α(1–6)-arm direction accelerated the serum clearance, but branching at the α(1–3)-arm direction contributed to the accumulation in the liver, spleen, heart, and lung [38]. The accumulation in the heart and the lung could be in part mediated by the glycan receptors, but the original papers did not deal with the detailed mechanisms. Furthermore, the simultaneous substitution by the core fucose and the β(1–4)-bisecting glucosamine residues resulted in the accumulation in the liver, kidney, and spleen [39]. Although Gabius and Unverzagt have not examined which cells and/or glycan receptors are responsible for the glycan-dependent *in vivo* kinetics summarized in Fig. 1, the serum stabilization effects by the presence of the sialic acid residues are consistent with the well-known asialoglycoprotein receptor-mediated excretion mechanism of the glycoproteins in the liver [2–4]. Furthermore, the rapid serum clearance by the β(1–2)-lactosamination of the branching mannose could be proposed by considering the interaction with the C-type lectin on the parenchymal cells. These *N*-glycoconjugates were investigated extensively in tumor-bearing mice, but significant tumor accumulation was not observed. Nevertheless, these studies constitute a pioneering research field of glycoconjugate kinetics, in that they clearly show that the biodistribution and serum stability of the neoglycoprotein are precisely regulated by *N*-glycan structures.

Miscellaneous

Thanks to the recent advances in the bioconjugate chemistry, the various complex glycan structures could efficiently be introduced at the specific sites of the proteins and even those on the cell surface under mild conditions. In this section, the chemical mimicry of the glycan post-translational modification and the chemical glycan engineering of the lymphocyte surface, which efficiently recognizes the inflammation or the tumors in live animals, will be discussed as the representative examples.

Chemistry-based site-selective modification of proteins by oligosaccharides (chemical post-translational mimicry (PTM)) for inflammation targeting

Davis and co-workers recently succeeded in mimicking the glycan-post translational modifications (PTMs) of proteins by chemical methods, which was used in targeting the mouse and rat brain inflammation and disease [41]. They genetically

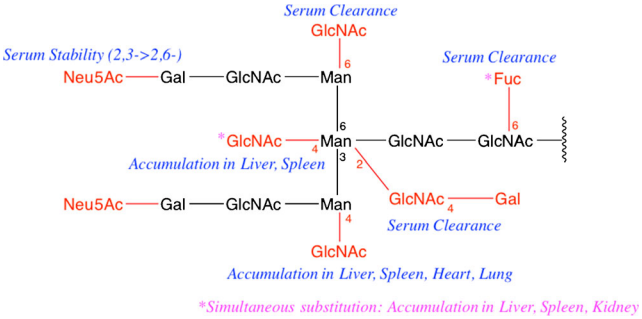

Fig. 1 Complex-type *N*-glycans synthesized by chemoenzymatic method and glycan-dependent kinetics and biodistribution of their BSA conjugates in mice (Data extracted from the original papers were independently summarized by the authors of this mini-review)

engineered a target protein in *E-coli* by exchanging methionine to artificial amino acids containing the azide and/or the acetylene moieties, and subsequently, the Cu(I)-mediated Huisgen cycloaddition reaction (Sharpless/Meldal click reaction) was performed to introduce glycans and/or other small functional molecules on the specific sites of the protein. Thus, by using SSβG with *Lac*Z-type galactosidase activity as a protein template, the sialyl Lewis X and the acidic *p*-toluenesulfonyl group were doubly introduced to specific sites of the protein, which were expected by computational modeling to interact with the P-selectin. This PTM mimic was then introduced to the interleukin-1β-induced rat models of cerebral inflammation, and after the dissection, the P-selectin expression in the brain tissues was analyzed by the treatment with the widely used probe X-Gal. Five-times more sensitive detection than the conventional antibody-based methods could be achieved. The overexpressed cerebral P-selectin could also be detected in a Malaria-infected mouse model (*Plasmodium*), by using the same chemistry-based PTM mimicry. It is noteworthy that the detection sensitivity of P-selectin could significantly be decreased by altering and/or removing the sialyl Lewis X structure or acid. Thus, the results show the importance of PTMs in acquiring the functional diversification by the glycans and/or the other small molecules.

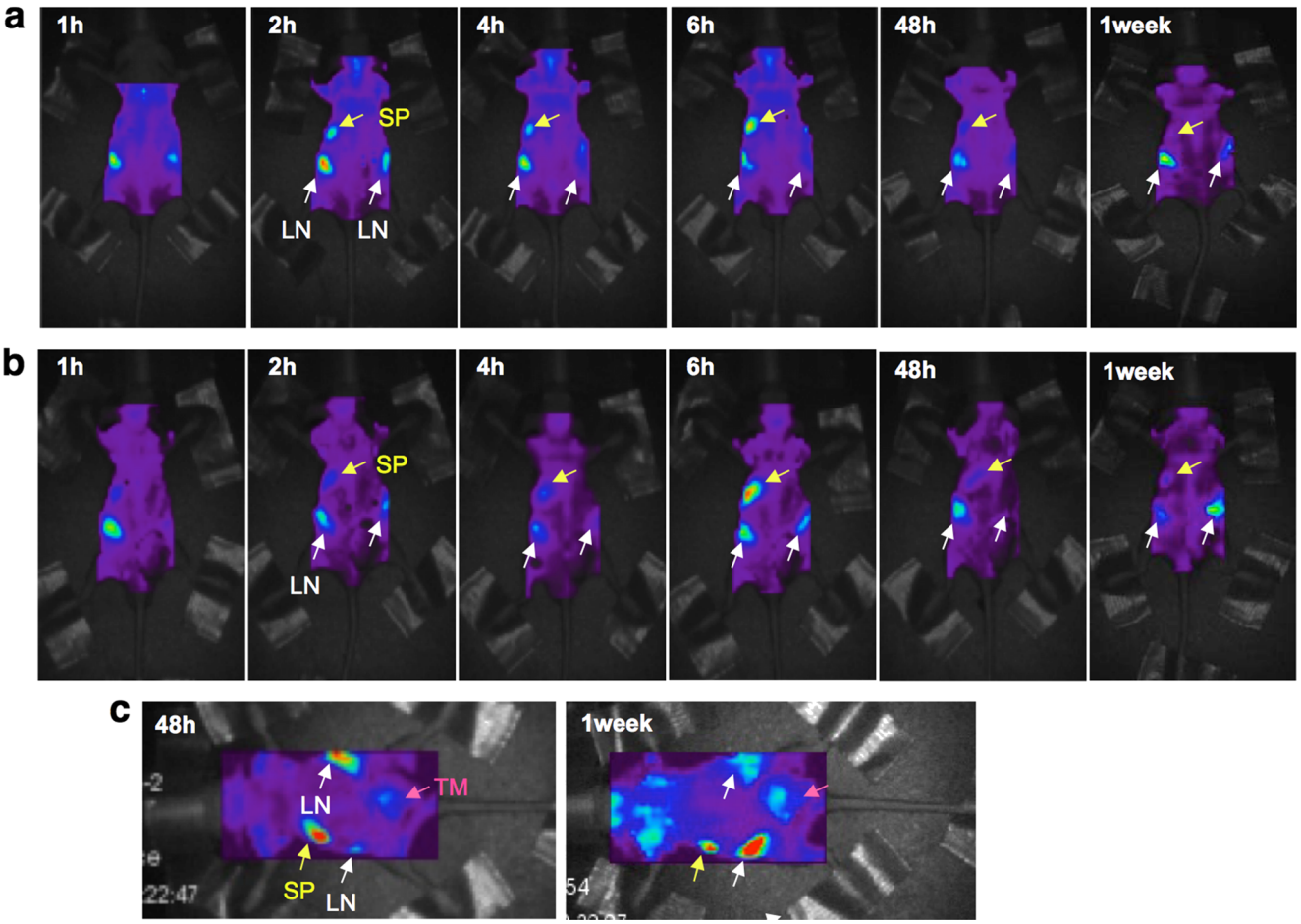

Fig. 2 Fluorescence imaging of lymphocytes in mice. Labeled and/or engineered cells were administrated intravenously (n=3, 100 μL/mouse, 10^4 cells) and whole body was scanned from the back side by eXplore Optix, GE Healthcare, Bioscience (excitation at 646 nm, emission 663 nm), 1 h, 2 h, 4 h, 6 h, 48 h, 1 week after injection. Data were normalized. *SP* spleen, *LN* lymph node of epidermal intestinal tract, *TM* DLD-1 human colon carcinoma. **a** Cy5-labeled cells to the nude mice. **b** Cy5-labeled cells to the DLD-1 implanted nude mice at dorsal division. **c** Both Cy5-labeled and *N*-glycan-engineered lymphocytes into the tumor model

New strategy for tumor targeting by *N*-sialoglycan engineered lymphocytes

Tanaka and co-workers on the other hand have succeed in introducing the various fluorophores and complex-type *N*-glycans selectively on the cell surfaces through their 6π-azaelectrocyclization technique, which treats cells with the probes at 37 °C for 10 min under the probe concentration of 10^{-8} M [42–46]. The reaction proceeds selectively on the lysine and/or phosphatidylethanolamine, being overexpressed on the surface of the cells. The labeling and/or engineering probes do not internalize the cells, and therefore, the reaction does not interfere with cell functions. Based on their method, the lymphocytes extracted from wild-type mouse, were labeled with Cy5 at near-infrared absorbance, and at the same time, modified by the complex-type sialo-*N*-glycan [47]. The artificial lymphocytes were injected intravenously to the mouse tumor model in which DLD-1 human colon carcinoma was implanted to the dorsal division, and the noninvasive imaging was performed (Fig. 2); the artificial cells not only trafficked to the spleen and the intestinal lymph nodes, but also accumulated in the tumor region. They have found that the *N*-glycan and its clusters [48], as well as the native lymphocytes [43] did not target the tumor *in vivo*, and therefore, the data shows that both functions of the *N*-glycan and the surfaces of the lymphocytes work synergistically to interact with the tumor cell. As a result of this work, new strategy for cancer targeting was proposed from the fields of synthetic organic chemistry & glycobiology. These results show the potential of the current glycan-modification method to tune the functions of the living cells very rapidly and conveniently, if said, in tailor-made fashion, in adjusting to *in vivo* kinetics response.

Prospective

The biodistribution and the imaging results cited in this mini-review give quite the promising possibility in the future for developing neoglycoprotein-based imaging tracers, which shows the preferable pharmacokinetics or selective targeting of specific organs and tumors. As discussed in the "Introduction", the natural glycans on proteins and cell surface are often constituted of several kinds of glycans (termed as glycoform), thus highly diversifying the glycobioenvironment. We therefore need to synthesize and mimic such heterogeneous glycoclusters for the efficient glycan-based imaging in the future. The various heterogeneous glycocluster environments could be constructed in a combinatorial fashion, leading to the promising strategy in developing not only the neoglycoprotein-based diagnostic tracers, but also vaccine adjuvants or virus-neutralizing pharmaceuticals. In order to evaluate in detail the heterogeneity in natural glycoclusters and then mimic them to develop useful glycan-based imaging tracers, synthetic chemists should seriously consider how structurally defined heterogeneous glycoclusters could be prepared efficiently and rapidly, *i.e.*, by the combinatorial approach. In other words, these issues depend on how many organic reactions, which are truly orthogonal to the functional groups, could be developed.

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References

1. Kamerling, J.P., Boons, G.-J., Lee, Y.C., Suzuki, A., Taniguchi, N., Voragen, A.G.J. (eds.): Analysis of Glycans, Polysaccharide Functional Properties & Biochemistry of Glycoconjugate Glycans, Carbohydrate-mediated Interactions. In: Comprehensive Glycoscience, From Chemistry to Systems Biology, Vol II & III. Elsevier, UK (2007)
2. Morell, A.G., Irvine, R.A., Sternlieb, I., Scheinberg, I.H., Ashwell, G.: Physical and chemical studies on ceruloplasmin. V. Metabolic studies on sialic acid-free ceruloplasmin in vivo. J. Biol. Chem. **243**, 155–159 (1968)
3. Elliott, S., Lorenzini, T., Asher, S., Aoki, K., Brankow, D., Buck, L., Busse, L., Chang, D., Fuller, J., Grant, J., Hernday, N., Hokum, M., Hu, S., Knudten, A., Levin, N., Komorowski, R., Martin, F., Navarro, R., Osslund, T., Rogers, G., Rogers, N., Trail, G., Egrie, J.: Enhancement of therapeutic protein in vivo activities through glycoengineering. Nat. Biotechnol. **21**, 414–421 (2003)
4. Sato, M., Furuike, T., Sadamoto, R., Fujitani, N., Nakahara, T., Niikura, K., Monde, K., Kondo, H., Nishimura, S.I.: Glycoinsulins: dendritic sialyloligosaccharide-displaying insulins showing a prolonged blood-sugar-lowering activity. J. Am. Chem. Soc. **126**, 14013–14022 (2004)
5. Kaneko, Y., Nimmerjahn, F., Ravetch, J.V.: Anti-inflammatory activity of immunoglobulin G resulting from Fc sialylation. Science **313**, 670–673 (2006)
6. Tanaka, K., Fukase, K.: PET (positron emission tomography) imaging of biomolecules using metal/DOTA complexes: a new collaborative challenge by chemists, biologists, and physicians for future diagnostics and exploration of in vivo dynamics. Org. Biomol. Chem. **6**, 815–828 (2008)
7. Vyas, S.P., Singh, A., Sihorkar, V.: Ligand-receptor-mediated drug delivery: an emerging paradigm in cellular drug targeting. Crit. Rev. Ther. Drug Carrier Syst. **18**, 1–76 (2001)
8. Willis, M., Forssen, E.: Ligand-targeted liposomes. Adv. Drug Deliv. Rev. **29**, 249–271 (1998)
9. Fukase, K., Tanaka, K.: Bio-imaging and cancer targeting with glycoproteins and *N*-glycans. Curr. Opin. Chem. Biol. **16**, 614–621 (2012). and references cited therein
10. Jain, K., Kesharwani, P., Gupta, U., Jain, N.K.: A review of glycosylated carriers for drug delivery. Biomaterials **33**, 4166–4186 (2012)
11. Lee, Y.C., Stowell, C.P., Krantz, M.J.: 2-Imino-2-methoxyethyl 1-thioglycosides: new reagents for attaching sugars to proteins. Biochemistry **15**, 3956–3963 (1976)

12. Gray, G.R.: The direct coupling of oligosaccharides to proteins and derivatized gels. Arch. Biochem. Biophys. **163**, 426–428 (1974)
13. Kojima, S., Ishido, M., Kubota, K., Kubodera, A., Hellmann, T., Kohnke-Godt, B., Wosgien, B., Gabius, H.-J.: Tissue distribution of radioiodinated neoglycoproteins and mammalian lectins. Biol. Chem. Hoppe-Seyler **371**, 331–338 (1990)
14. Ashwell, G., Harford, J.: Carbohydrate-specific receptors of the liver. Annu. Rev. Biochem. **51**, 531–554 (1982)
15. Nishikawa, M., Hirabayashi, H., Takakura, Y., Hashida, M.: Design for cell-specific targeting of proteins utilizing sugar-recognition mechanism: effect of molecular weight of proteins on targeting efficiency. Pharm. Res. **12**, 209–214 (1995)
16. Nishikawa, M., Miyazaki, C., Yamashita, F., Takakura, Y., Hashida, M.: Galactosylated proteins are recognized by the liver according to the surface density of galactose moieties. Am. J. Physiol. **268**, G849–G856 (1995)
17. Gunn, A.J., Hama, Y., Koyama, Y., Kohn, E.C., Choyke, P.L., Kobayashi, H.: Targeted optical fluorescence imaging of human ovarian adenocarcinoma using a galactosyl serum albumin-conjugated fluorophore. Cancer Sci. **98**, 1727–1733 (2007)
18. Hama, Y., Urano, Y., Koyama, Y., Choyke, P.L., Kobayashi, H.: D-galactose receptor-targated in vivo spectral fluorescence imaging of peritoneal metastasis using galactosamin-conjugated serum albumin-rhodamine green. J. Biomed. Opt. **12**, 051501 (2007)
19. Regino, C.A.S., Ogawa, M., Alford, R., Wong, K.J., Kosaka, N., Williams, M., Feild, B.J., Takahashi, M., Choyke, P.L., Kobayashi, H.: Two-step synthesis of galactosylated human serum albumin as a targeted optical imaging agent for peritoneal carcinomatosis. J. Med. Chem. **53**, 1579–1586 (2010)
20. Chopra, A.: [99mTc]-diethylenetriaminepentaacetic acid-galactosyl human serum albumin. 2007 Apr 24 [Updated 2007 Jun 12]. In: Molecular Imaging and Contrast Agent Database (MICAD) [Internet]. Bethesda (MD): National Center for Biotechnology Information (US); 2004–2013. Available from: http://www.ncbi.nlm.nih.gov/books/NBK23514/
21. Stahl, P.D., Wileman, T.E., Diment, S., Shepherd, V.L.: Mannose-specific oligosaccharide recognition by mononuclear phagocytes. Biol. Cell. **51**, 215–218 (1984)
22. Doebber, T.W., Wu, M.S., Bugianesi, R.L., Ponpipom, M.M., Furbish, F.S., Barranger, J.A., Brady, R.O., Shen, T.Y.: Enhanced macrophage uptake of synthetically glycosylated human placental β-glucocerebrosidase. J. Biol. Chem. **257**, 2193–2199 (1982)
23. Takagi, K., Uehara, T., Kaneko, E., Nakayama, M., Koizumi, M., Endo, K., Arano, Y.: 99mTc-labeled mannosyl-neoglycoalbumin for sentinel lymph node identification. Nucl. Med. Biol. **31**, 893–900 (2004)
24. Jeong, J.M., Hong, M.K., Kim, Y.J., Lee, J., Kang, J.H., Lee, D.S., Chung, J.-K., Lee, M.C.: Development of 99mTc-neomannosyl human serum albumin (99mTc-MSA) as a novel receptor binding agent for sentinel lymph node imaging. Nucl. Med. Commun. **25**, 1211–1217 (2004)
25. Yabe, Y., Nishikawa, M., Tamada, A., Takakura, Y., Hashida, M.: Targeted delivery and improved therapeutic potential of catalase by chemical modification: combination with superoxide dismutase derivatives. J. Pharmacol. Exper. Ther. **289**, 1176–1184 (1999)
26. Yabe, Y., Kobayashi, N., Nishikawa, M., Mihara, K., Yamashita, F., Takakura, Y., Hashida, M.: Pharmacokinetics and preventive effects of targeted catalase derivatives on hydrogen peroxide-induced injury in perfused rat liver. Pharm. Res. **19**, 1815–1821 (2002)
27. Opanasopit, P., Nishikawa, M., Yamashita, F., Takakura, Y., Hashida, M.: Pharmacokinetic analysis of lectin-dependent biodistribution of fucosylated bovine serum albumin: a possible carrier for Kupffer cells. J. Drug Target. **9**, 341–351 (2001)
28. Sarkar, K., Sarkar, H.S., Kole, L., Das, P.K.: Receptor-mediated endocytosis of fucosylated neoglycoprotein by macrophages. Mol. Cell. Biochem. **156**, 109–116 (1996)
29. Kim, S., Jeong, J.M., Hong, M.K., Jang, J.-J., Lee, J., Lee, D.S., Chung, J.-K., Lee, M.C.: Differential receptor targeting of liver cells using 99mTc-neoglycosylated human serum albumins. Arch. Pharm. Res. **31**, 60–66 (2008)
30. Choi, J.Y., Jeong, J.M., Yoo, B.C., Kim, K., Kim, Y., Yang, B.Y., Lee, Y.-S., Lee, D.S., Chung, J.-K., Lee, M.C.: Development of 68Ga-labeled mannosylated human serum albumin (MSA) as a lymph node imaging agent for positron emission tomography. Nucl. Med. Biol. **38**, 371–379 (2011)
31. McCann, T.E., Kosaka, N., Mitsunaga, M., Choyke, P.L., Gildersleeve, J.C., Kobayashi, H.: Biodistribution and excretion of monosaccharides-albumin conjugates measured with in vivo near infrared fluorescence imaging. Bioconjug. Chem. **21**, 1925–1932 (2010)
32. Alexander, V.M., Sano, K., Yu, Z., Nakajima, T., Choyke, P.L., Ptaszek, M., Kobayashi, H.: Galactosyl human serum albumin-NMP1 conjugate: a near infrared (NIR)-activatable fluorescence imaging agent to detect peritoneal ovarian cancer metastases. Bioconjug. Chem. **23**, 1671–1679 (2012)
33. Lehrman, M.A., Pizzo, S.V., Imber, M.J., Hill, R.L.: The binding of fucose-containing glycoproteins by hepatic lectins. J. Biol. Chem. **261**, 7412–7418 (1986)
34. André, S., Unverzagt, C., Kojima, S., Dong, X., Fink, C., Kayser, K., Gabius, H.-J.: Neoglycoproteins with the synthetic complex biantennary nonasaccharide or its α2,3/α2,6-sialylated derivatives: their preparation, assessment of their ligand properties for purified lectins, for tumor cells in vitro, and in tissue sections, and their biodistribution in tumor-bearing mice. Bioconjug. Chem. **8**, 845–855 (1997)
35. Unverzagt, C., André, S., Seifert, J., Kojima, S., Fink, C., Srikrishna, G., Freeze, H., Kayser, K., Gabius, H.-J.: Structure-activity profiles of complex biantennary glycans with core fucosylation and with/without additional α2,3/α2,6 sialylation: synthesis of neoglycoproteins and their properties in lectin assays, cell binding, and organ uptake. J. Med. Chem. **45**, 478–491 (2002)
36. André, S., Unverzagt, C., Kojima, S., Frank, M., Seifert, J., Fink, C., Kayser, K., von der Lieth, C.-W., Gabius, H.-J.: Determination of modulation of ligand properties of synthetic complex-type biantennary N-glycans by introduction of bisecting GlcNAc in silico, in vitro and in vivo. Eur. J. Biochem. **271**, 118–134 (2004)
37. André, S., Kojima, S., Prahl, I., Lensch, M., Unverzagt, C., Gabius, H.-J.: Introduction of extended LEC14-type branching into core-fucosylated biantennary N-glycan: glycoengineering for enhanced cell binding and serum clearance of the neoglycoprotein. FEBS J. **272**, 1986–1998 (2005)
38. André, S., Kojima, S., Gundel, G., Russwurm, R., Schratt, X., Unverzagt, C., Gabius, H.-J.: Branching mode in complex-type triantennary N-glycans as regulatory element of their ligand properties. Biochim. Biophys. Acta **1760**, 768–782 (2006)
39. André, S., Kožár, T., Schuberth, R., Unverzagt, C., Kojima, S., Gabius, H.-J.: Substitutions in the N-Glycan core as regulators of biorecognition: the case of core-fucose and bisecting GlcNAc moieties. Biochemistry **46**, 6984–6995 (2007)
40. Gabius, H.-J., André, S., Jiménez-Barbero, J., Romero, A., Solís, D.: From lectin structure to functional glycomics: principles of the sugar code. Trends Biochem. Sci. **36**, 298–313 (2011)
41. van Kasteren, S.I., Kramer, H.B., Jensen, H.H., Campbell, S.J., Kirkpatrick, J., Oldham, N.J., Anthony, D.C., Davis, B.G.: Expanding the diversity of chemical protein modification allows post-translational mimicry. Nature **446**, 1105–1109 (2007)
42. Tanaka, K., Masuyama, T., Hasegawa, K., Tahara, T., Mizuma, H., Wada, Y., Watanabe, Y., Fukase, K.: A submicrogram-scale protocol for biomolecule-based PET imaging via rapid 6π-azaelectrocyclization: first visualization of sialic acid-dependent circulatory residence of glycoproteins. Angew. Chem. Int. Ed. **47**, 102–105 (2008)

43. Tanaka, K., Minami, K., Tahara, T., Fujii, Y., Siwu, E.R.O., Nozaki, S., Onoe, H., Yokoi, S., Koyama, K., Watanabe, Y., Fukase, K.: Electrocyclization-based labeling allows efficient in vivo imaging of cellular trafficking. ChemMedChem **5**, 841–845 (2010)
44. Tanaka, K., Yokoi, S., Morimoto, K., Iwata, T., Nakamoto, Y., Nakayama, K., Koyama, K., Fujiwara, T., Fukase, K.: Cell surface biotinylation by azaelectrocyclization: easy-handling and versatile approach for living cell labeling. Bioorg. Med. Chem. **20**, 1865–1868 (2012)
45. Tanaka, K., Moriwaki, K., Yokoi, S., Koyama, K., Miyoshi, E., Fukase, K.: Whole-body imaging of tumor cells by azaelectrocyclization: visualization of metastasis dependence on glycan structure. Bioorg. Med. Chem. **21**, 1074–1077 (2013)
46. Tanaka, K., Nakamoto, Y., Siwu, E.R.O., Pradipta, A.R., Morimoto, K., Fujiwara, T., Yoshida, S., Hosoya, T., Tamura, Y., Hirai, G., Sodeoka, M., Fukase, K.: Development of bis-unsaturated ester aldehydes as amino-glue probes: sequential double azaelectrocyclization as promising strategy for bioconjugation. Org. Biomol. Chem. **11**, 7326–7333 (2013)
47. Tanaka, K., Minami, K., Tahara, T., Siwu, E.R.O., Koyama, K., Nozaki, S., Onoe, H., Watanabe, Y., Fukase, K.: A combined 6π-azaelectrocyclization/Staudinger approach to protein & cell engineering: non-invasive tumor targeting by N-glycan-engineered lymphocytes. J. Carbohydr. Chem. **29**, 118–132 (2010)
48. Tanaka, K., Siwu, E.R.O., Minami, K., Hasegawa, K., Nozaki, S., Kanayama, Y., Koyama, K., Chen, W.C., Paulson, J.C., Watanabe, Y., Fukase, K.: Noninvasive imaging of dendrimer-type N-glycan clusters: in vivo dinamics dependence on oligosaccharides structure. Angew. Chem. Int. Ed. **49**, 8195–8200 (2010)