Functionalization of AFM tips with hydrazide for coupling of glycoproteins (e.g. periodate-treated antibodies)

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AFM tips with hydrazide functions for coupling of glycoproteins

short version

for risks and details see full length procedure

1. Aminofunctionalization of the cantilever(s) (see *AFM_tip_aminofunctionalization*).
2. Coupling of Maleimide-PEG-NHS (see *AFM_tip_with_maleimide*.)
3. Periodate treatment of the antibody (or other glycoprotein)
   - Dilute antibody or other glycoprotein (2-10 µg) to 45 µL total volume with buffer 5.5 (100 mM sodium acetate, adjusted to pH 5.5 with HCl).
   - Dialyze in slide-a-lyzer tube (MWCO 10kDa for antibodies) against buffer 5.5 for 2 h.
   - Place slide-a-lyzer with foam pad on top of a small beaker filled with water (~25°C), without bringing the dialysis membrane into contact with the liquid. Add 5 µL 100 mM sodium periodate (21.4 mg/ml), close the tube and incubate 1 h at room temperature in the dark. Much longer times and/or higher periodate concentrations may be required for glycoproteins which do not contain sialic acid residues.
   - Dialyze for 2 h against buffer 5.5. Then dialyze against fresh buffer 5.5 overnight.
4. Place cantilever(s) on Parafilm in a polystyrene Petri dish.
5. Pre-mix the following components in a reaction vial:
   - 50 µL water
   - 2 µL EDTA (50 mM, pH 7.5)
   - 5 µL Hepes (500 mM, pH 7.5)
   - 4 µL PDPH (25 mM) in DMSO, add slowly under stirring with the pipette tip.
   - 4 µL TCEP hydrochloride (50 mM), mix shortly.
   - 4 µL Hepes (500 mM, pH 9.6), mix shortly to avoid oxidation.
6. Pipet the mixture onto the cantilever(s), cover with lid, incubate for 2-4 h.
7. Wash with water (3 × 5 min) and dry with a gentle stream of nitrogen.
8. Place cantilever(s) on Parafilm in a polystyrene Petri dish.
9. Pipet the periodate-treated and dialyzed antibody (or other glycoprotein) onto the cantilever(s); cover Petri dish with lid, and incubate for 2 h.
10. Wash in any kind of buffer which is to be used in the subsequent experiments (3 × 5 min).
11. Mount cantilever in AFM setup.

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AFM tips with hydrazide functions for coupling of glycoproteins

Please, read the preceding manual *general_overview* for the basic concept of AFM tip functionalization with long flexible PEG linkers.

Please, read the manual *AFM_tip_aminofunctionalization* for the details on how amino groups are generated on the AFM tip surface.

Please, read the manual *AFM_tip_with_maleimide* for coupling of Maleimide-PEG-NHS to the amino-functionalized tip surface.

Our standard method for linking of proteins to the AFM tip uses random coupling of one lysine residue of the protein to the PEG linker on the AFM tip (see manual *AFM_tip_with_acetal*). In case of an antibody, the linker might be attached to one of the Fab arms (**Figure 1A**) or to the Fc arm (**Figure 1B**). For obvious reasons, linking via the Fc arm is more desirable for force microscopy. Fortunately it is also more likely because the Fc arm is richer in lysine residues than the Fab arms.

For sophisticated AFM experiments, it may be necessary to completely avoid the asymmetric situation in **Figure 1A** and to restrict linker attachment to the Fc arm of the antibody, as shown in **Figure 1B**. The best method for this purpose is linker attachment to one of the two small oligosaccharide chains on the Fc arm of the antibody (see **Figure 1B**).

The same coupling chemistry can be used for most other glycoproteins and also for large polysaccharides. Interestingly, this method can also be used to couple the 3'-end of RNA or of ribonucleotides to the AFM tip (see **Notes***, below).*

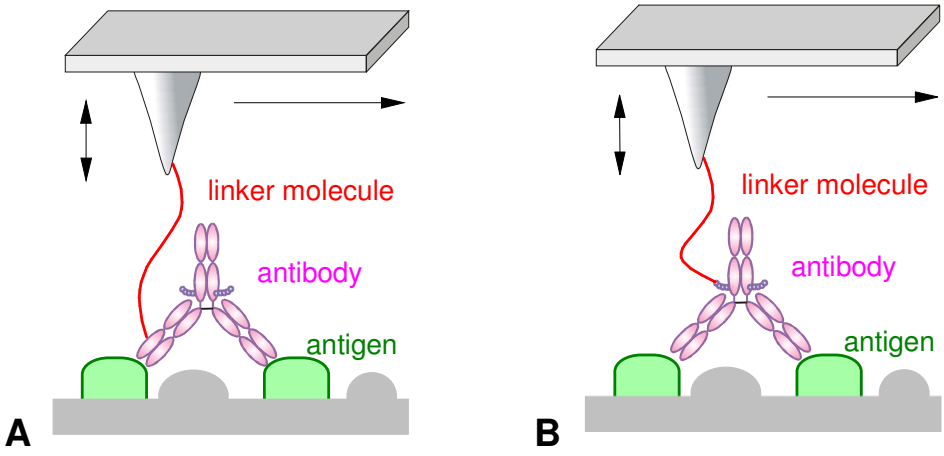

Figure 1: Recognition force spectroscopy/microscopy with antibody-functionalized AFM tips. (**A**) Random attachment of the linker on the antibody can result in a high asymmetry between the two antigen-binding sites. (**B**) High symmetry is achieved by attaching the linker to one of the two carbohydrate chains.

Per se, the oligosaccharide chain on the antibody does not contain a chemical group which is sufficiently reactive for coupling to the AFM tip in aqueous solution. However, some of the

sugar residues (the sialic acids) contain a side chain with vicinal OH groups, as shown in **Figure 2A**. Treatment with sodium periodate ($NaIO_4$) cleaves the C-C bond between the two OH groups. This results in an aldehyde group on the antibody molecule, plus the release of a formaldehyde molecule (**Figure 2B**).

The released formaldehyde must be removed by dialysis (**Figure 2C**) before the oxidized antibody can be coupled to the AFM tip. If free formaldehyde is not removed, it will block the coupling sites on the AFM tip and no antibody can be coupled.

Further details about the oligosaccharides of antibodies and about the periodate reaction are found in the **Appendix** at the end of this manual.

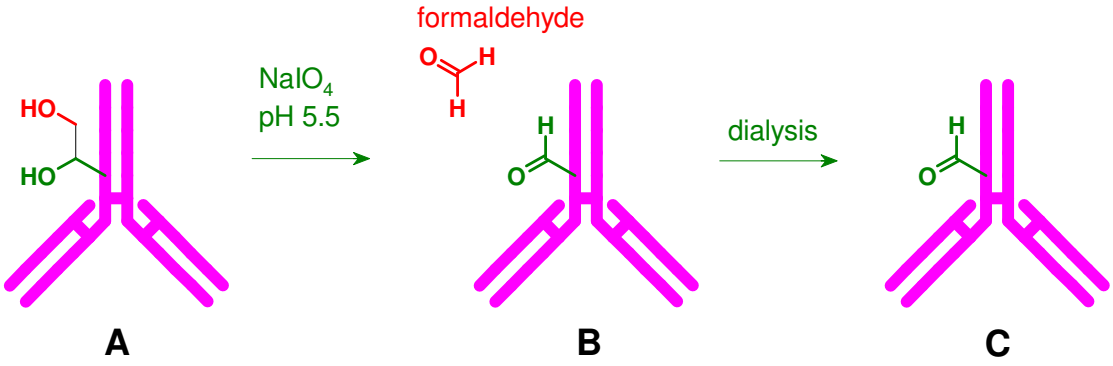

Figure 2: Periodate treatment of antibodies (or other glycoproteins) generates aldehyde functions on the central carbohydrate. These can be reacted with hydrazide groups to give stable hydrazone linkages (see **Figure 3**).

Figure 3 depicts the four-step protocol of tip functionalization with periodate-treated antibodies.

- The first step is the introduction of amino groups, as described in the manual *AFM_tip_aminofunctionalization*. The tip can be stored under argon in this state for a few days.
- The second step is coupling of Maleimide-PEG27-NHS, resulting in tip-linked PEG chains with terminal maleimide groups. The procedure is described in the manual *AFM_tip_with_maleimide*. After washing and drying, the tip can be stored under argon for a few days.
- In the third step, the maleimide group is reacted with a small molecule which contains both an SH-group (for coupling to the maleimide) and a hydrazide function (for coupling of an aldehyde). This molecule is not commercially available but it can be generated *in situ* from commercial PDPH. For this purpose, PDPH is dissolved in EDTA-containing buffer and TCEP is added for generation of the free SH-group. The EDTA is very important to prevent re-oxidation of the free SH group into a symmetric disulfide by the ambient atmosphere. The procedure is easy and well described in this manual. After washing and drying, the tip can be stored under argon for a few days.
- In the fourth step, the hydrazide on the AFM tip is reacted with the aldehyde group on a periodate-treated antibody. This results in the formation of a so-called hydrazone bond. The reaction is spontaneous and does not require an activator.

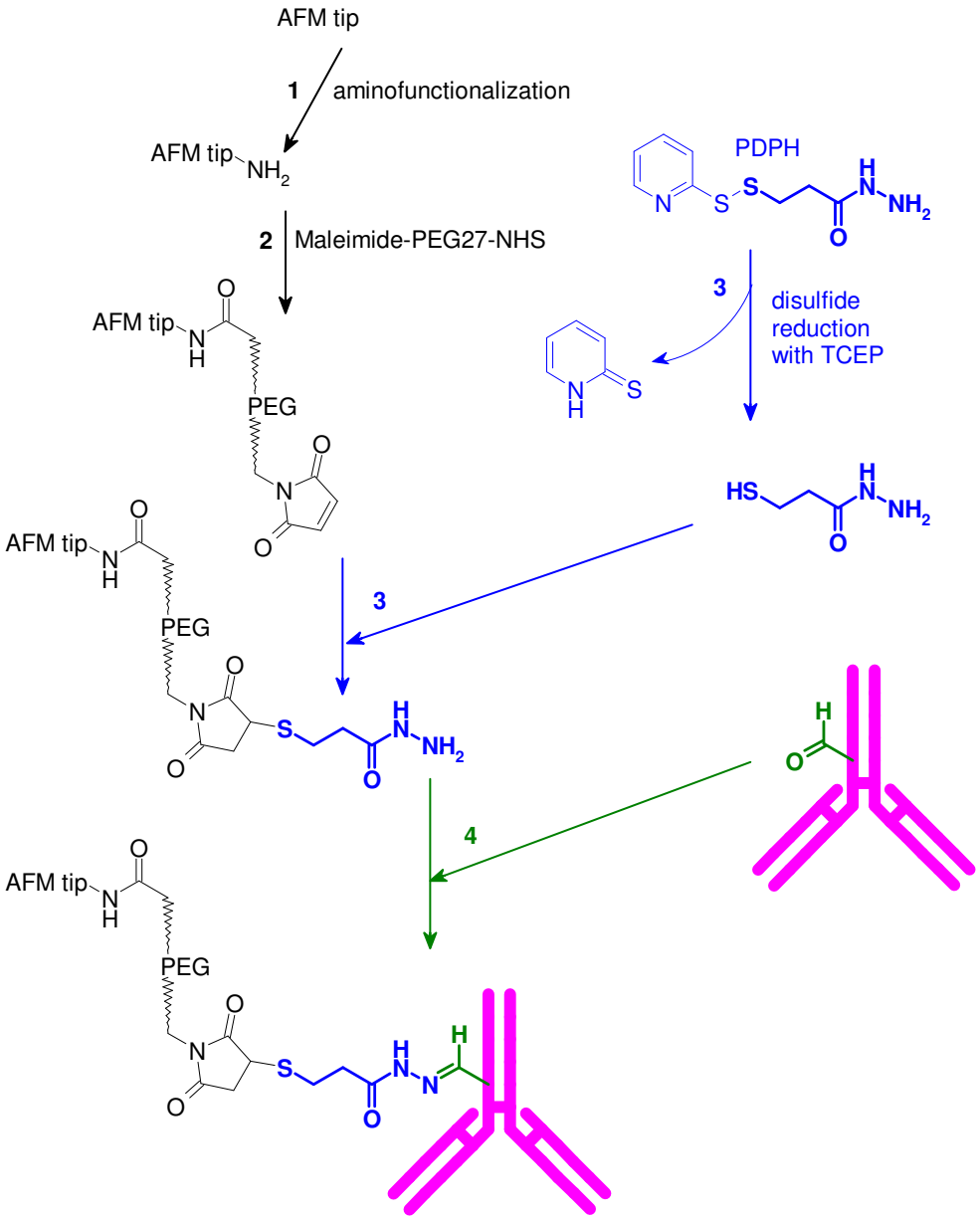

Figure 3: Four-step protocol for AFM tip functionalization with periodate-treated antibodies. (1) Aminofunctionalization with APTES or ethanolamine hydrochloride. (2) Coupling of Maleimide-PEG27-NHS. (3) The one-pot reaction with PDPH and TCEP extends the maleimide group with a hydrazide function. (4) Coupling of periodate-treated and dialyzed antibody via stable hydrazone linkage.

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Procedure

1. Aminofunctionalization of the cantilever(s) (see *AFM_tip_aminofunctionalization*).
2. Coupling of Maleimide-PEG-NHS (see *AFM_tip_with_maleimide*.)
3. Periodate treatment of the antibody (or other glycoprotein)
    - Take one aliquot of antibody (2-10 µg protein; e.g. 2-10 µL of a solution with 1 mg/mL) and dilute to 45 µL with buffer 5.5 (100 mM sodium acetate, = 8.2 g/L, adjusted to pH 5.5 with HCl)
    - Dialyze against buffer 5.5 for 2 h. For this purpose, a Slide-A-Lyzer MINI Dialysis Device from Thermofisher (e.g. product 69570 for MWCO 10 kDa) is inserted in the hole of a foam pad (product 69588) which floats on the stirred dialysis buffer. The Slide-A-Lyzer looks like a reaction vial ("Eppi"), the bottom of which has been removed by a horizontal cut. The bottom is flat and consists of a dialysis membrane. When inserting the Slide-A-Lyzer tube in a foam pad and placing the foam pad on top of buffer, the foam pad floats and the lower end of the tube reaches into the buffer, thereby immersing the dialysis membrane.
    - After dialysis, the foam pad is placed on top of a 50 mL beaker which should contain ~40 ml water (~25°C). The foam pad is large enough to cover the beaker completely and to sit on the rim of the beaker. The dialysis tube reaches few millimeters into the beaker but should be well above the water level. The purpose of this arrangement is to saturate the atmosphere under the dialysis membrane with water vapor, preventing drying of the protein sample.
    - Add 5 µL from a freshly prepared 100 mM periodate solution (21.4 mg/mL $NaIO_4$ in water) to the dialyzed antibody and mix the solution with the pipette tip. Close the Slide-S-Lyzer MINI Dialysis Device with its lid to prevent evaporation and cover the whole beaker plus the foam pad with aluminum foil to protect the mixture from light. Incubate for 1 h at room temperature in the dark. Much longer times and/or higher periodate concentrations may be required for those glycoproteins which do not contain sialic acid residues.
    - Transfer the foam pad into a beaker with buffer 5.5, dialyze for 2 h while slowly stirring of the buffer, then dialyze against fresh buffer overnight for rigorous removal of free formaldehyde. Free formaldehyde would consume, and thereby block, the hydrazide groups on the AFM tip. The antibody is now ready for coupling to a hydrazide-functionalized AFM tip. The periodate-treated antibody should be stored at 4°C until the tip has been functionalized with hydrazide groups (step No. 7, see below). The storage time should be kept to a minimum. In practice, step No. 3 and steps No. 4-7 can be begun in parallel on the same day. After coupling of Maleimide-PEG27-NHS and introduction of hydrazide functions, the AFM tips can be stored under argon atmosphere while the periodate-treated antibody is dialyzing overnight.
4. Place cantilever(s) on Parafilm in a polystyrene Petri dish.
5. Pre-mix the following components in a reaction vial:
    - 50 µL water
    - 2 µL EDTA (50 mM, pH 7.5)

- 5 µL Hepes (500 mM, pH 7.5)
- 4 µL PDPH (25 mM) in DMSO is slowly added from the submersed pipette tip which is constantly moved through the solution to prevent precipitation of PDPH upon contact with water.
- 4 µL TCEP hydrochloride (50 mM), mix shortly.
- 4 µL Hepes (500 mM, pH 9.6), mix shortly to avoid oxidation.

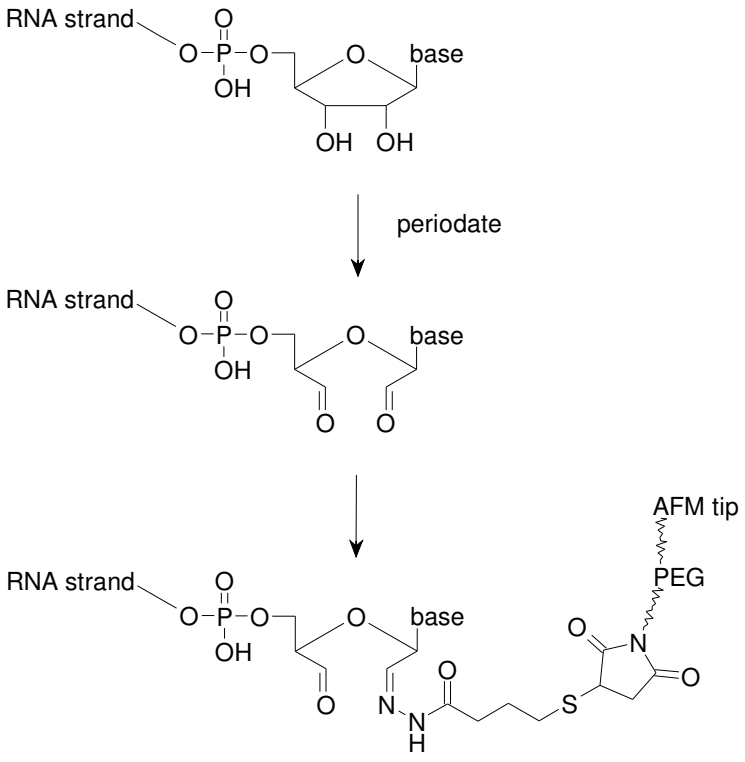
6. Pipet the mixture onto the cantilever(s), cover with lid, incubate for 2-4 h.
7. Wash with water (3 × 5 min) and dry with a gentle stream of nitrogen.
8. Place cantilever(s) on Parafilm in a polystyrene Petri dish.
9. Pipet the periodate-treated and dialyzed antibody (or other glycoprotein) onto the cantilever(s); cover Petri dish with lid, and incubate for 2 h.
10. Wash in any kind of buffer which is to be used in the subsequent experiments (3 × 5 min).
11. Mount cantilever in AFM setup.

Notes:

- Some people might ask why the attachment of hydrazide is so complicated and requires so many steps. Why is it not possible to synthesize a linker termed "Hydrazide-PEG-NHS"? The answer is that a hydrazide function will quickly react with the NHS ester group, resulting in tandem connection of many PEG linkers into extremely long chains. Theoretically, we could prepare Boc-hydrazide-PEG-NHS where the hydrazide function is temporarily blocked with the Boc group. Boc-hydrazide-PEG-NHS could be reacted with aminofunctionalized tips, resulting in Boc-hydrazide-PEG-tip. Then, the Boc group would have to be removed by trifluoroacetic acid and this would destroy the surface chemistry of the tip, as well as the magnetic coating of MAC levers. No such problem would occur with Fmoc-hydrazide-PEG-NHS but the Fmoc group has limited stability. As a consequence, some Fmoc groups would already get lost during coupling to the AFM tip, resulting in hydrazide-PEG-tip which would couple a second Fmoc-hydrazide-PEG-NHS, and so on. Moreover, toxic piperidine is needed for Fmoc group removal and piperidine rapidly gets degraded upon storage, so the procedure would not be very reproducible in the hands of non-experts.
- The method proposed in this manual can easily be applied by non-chemists. (1) It is convenient to use the same crosslinker (Maleimide-PEG27-NHS) which is applied for thiol coupling in general. (2) The attachment of hydrazide groups with the help of PDPH and TCEP requires only a few pipetting steps. (3) All reagents are commercially available. (4) PDPH, TCEP, and all other ingredients of this step can be stored as ready-to-use aliquots at -20°C for a long time.
- Sodium periodate ($NaIO_4$) should not be exposed to light more than is necessary during the manipulations. The solid should be stored in the dark in a desiccator (over blue gel or orange gel). The aqueous stock solution must always be freshly prepared

and the reaction of antibody (glycoprotein) with periodate should be carried out in a dark environment, e.g. under aluminum foil or in a dark cabinet.

- There would be little point in including $NaCNBH_3$ during hydrazone formation. In contrast to Schiff bases (which are formed from aldehydes plus primary amines), hydrazone linkages cannot easily be reduced. This is particularly true if the hydrazone is formed from a hydrazide (see **Figure 3**), rather than a hydrazine group. The general rule is that the double bonds formed between N-bases (amines, hydrazines, aminooxy groups) can be reduced by $NaCNBH_3$ only if the adduct with the aldehyde (>C=N-) is still weakly basic. If so, then at least a fraction of the adduct can be protonated around pH 5.5 (>C=NH^+-) and only in this protonated species the double bond can be reduced to a stable single bond (>CH-NH-) which can no longer be hydrozyded.
- Fortunately the hydrazone linkage between antibody and PEG linker is thermodynamically stable in aqueous solution, i.e., it does not hydrolyze. Consequently, it is not a problem that the hydrazone linkage cannot be fixed by reduction with $NaCNBH_3$.
- The procedure of this manual can be adapted for coupling of oligosaccharides and polysaccharides if these contain vicinal diol groups that can be oxidized with sodium periodate.
- The procedure of this manual can also be used for site-specific coupling of the PEG linker to the 3'-terminal ribose of RNA. The 3' end of RNA (but not of DNA) has a pair of vicinal OH groups which are easily oxidized with periodate, resulting in two aldehyde functions. One of the two aldehydes can be coupled to the hydrazide function on the AFM tip.



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Materials:

Buffer 5.5 contains 100 mM sodium acetate (8.2 g/L) at pH 5.5. For preparation of 2 liters, 16.4 g sodium acetate is dissolved in about 1.8 L water and the pH is adjusted to 5.5 with concentrated HCl. Then water is added to give a final volume of 2 L and the solution is mixed carefully.

100 mM periodate (21.4 mg/mL $NaIO_4$) is prepared by weighing few milligrams of $NaIO_4$ into a small glass vial. The actual weight in milligrams is divided by 21.4 mg/mL, yielding the volume of water (in mL) which must be used to dissolve the periodate at the desired concentration (21.4 mg/mL). For instance, 4.5 mg periodate must be dissolved in 4.5 / 21.4 = 0.210 mL water.

50 mM EDTA is prepared by weighing 931 mg of disodium EDTA dihydrate (EDTA-$Na_2.2H_2O$) into a beaker (50 mL) and adding water to a final volume of ~40 mL. A stir bar is added and the suspension is stirred with dropwise addition of NaOH (initially 20% NaOH, towards the end 2% NaOH) to adjust a pH of 7.4. The solution is transferred into a volumetric flask (50 mL) or a graduated cylinder and water is added to give a final volume of 50 mL. Aliquots of different size (2 mL, 200 µL, 20 µL) are stored at -20°C for up to several years.

500 mM Hepes (pH 7.5) is prepared by dissolving Hepes (free acid) in water at a concentration of 119.2 g/L. As an example, 11.915 g Hepes is weighed into a 100 mL graduated cylinder and water is added to give a final volume of 80 mL. The suspension is mixed until the solid is completely dissolved. Then water is added to give a total volume of 100 mL and the solution is mixed again. Alternatively, a 1 M stock solution of Hepes acid can be purchased, e.g., for Sigma-Aldrich, and diluted with an equal volume of water to give 500 mM Hepes acid. Subsequently, the 500 mM stock solution is transferred to a beaker and stirred while slowly adding 20% NaOH (5 M) until the final pH reaches the value 7.5. At this point, the true Hepes concentration will be lower than 500 mM but this fact is ignored. Half of the total solution is immediately used for preparation of 500 mM Hepes (pH 9.6, see next paragraph) while afterwards the remainder of 500 mM Hepes (pH 7.5) is divided into aliquots of different size (2 mL, 200 µL, and 20 µL) and stored at -20°C for up to several years.

500 mM Hepes (pH 9.6) is prepared from 500 mM Hepes (pH 7.5, see previous paragraph) by continuing the addition of 20% NaOH until pH 9.6 has been reached. Aliquots of different size (2 mL, 200 µL, and 20 µL) are stored at -20°C for up to several years.

50 mM TCEP hydrochloride is prepared by weighing about 50 mg into a beaker and adding water to give a final concentration of exactly 14.3 mg/mL. For instance, if the weight of the solid is exactly 50.0 mg then 50/14.3 = 3.49 mL of water is added. Aliquots of different size are prepared (e.g. 1 mL, 100 µL, and 10 µL) and stored at -20°C for up to several years. The high stability of TCEP in this stock solution is due to its low pH (~2) which prevents oxidation of TCEP in presence of air.

25 mM **PDPH** (5.73 mg/mL, MW = 229.32 g/mol) is prepared by weighing few milligrams of PDPH (Thermofisher product No. 22301, or Sigma-Aldrich product No. 803480, full name: 3-[2-pyridyldithio]propionyl hydrazide, old name: **SPDP hydrazide**) in a small glass vial. The actual weight in milligrams is divided by (5.73 mg/ml) to calculate the

volume of DMSO (in mL) which is required to dissolve PDPH at the desired concentration. For instance, 3.8 mg PDPH should be dissolved in 3.8 / 5.73 = 0.663 mL DMSO. Divide into aliquots (e.g. 10 µL) in reaction vials ("Eppis") and store at -20°C for up to several years.

References:

Hage, D. S., Wolfe, C. A. C., and Oates, M. R. (1997) Developement of a Kinetic Model to Describe the Effective Rate of Antibody Oxidation by Periodate. Bioconjugate Chem. 8, 914-920.

Kondejewski, L. H., Kralovec, J. A., Blair, A. H., and Ghose, T. (1994) Synthesis and Characterization of Carbohydrate-Linked Murine Monoclonal Antibody K20-Human Serum Albumin Conjugates. Bioconjugate Chem. 5, 602-611.

Wolfe C. A. C., and Hage, D. S. (1995) Studies on the Rate and Control of Antibody Oxidation by Periodate. Anal. Biochem. 231, 123-130.

Zara, J. J., Wood, R. D., Boon, P., Kim, C.-H., Pomato, N., Bredehorst, R., and Vogel, C.-W. (1991) A Carbohydrate-Directed Heterobifunctional Cross-Linking Reagent for the Synthesis of Immunoconjugates. Anal. Biochem. 194, 156-162. This is the only article describing simultaneous application of periodate and pyridyldithiopropionyl hydrazide!!!

Thermofisher Instructions for product No. 22301 (PDPH).

Risk and Safety

Please, consult the Risk and Safety tables in the manuals for the first and second step of tip functionalization (*AFM_tip_aminofunctionalization* and *AFM_tip_with_maleimide*).

	**EDTA disodium salt**, toxic if ingested or when the dust would be inhaled, not dangerous under normal working conditions
-	**Hepes, free acid,** irritant if ingested or when the dust would be inhaled, not dangerous under normal working conditions
	**SPDP hydrazide**, highly irritant if ingested or when the dust would be inhaled. Solutions will cause severe skin irritation. The toxicity has not been investigated but the chances are high that the material is toxic. Use proper protection against inhalation, ingestion, and skin contact!

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	**Tris(carboxyethyl)phosphine hydrochloride** (TCEP hydrochloride): H314, P280-P305 + P351 + P338-P310, R34 (Europe), S26-27-36/37/39 (Europe)

Be careful when using Pasteur pipettes which are connected to a nitrogen gas tank via silicon tubing. Make sure that the needle valve is closed when you open the main valve of the gas tank! Slowly open the needle valve! Always hold (or fix) the Pasteur pipette and not the silicon tubing. In this way, only the soft tubing may jump off the pipette if the nitrogen flow is opened too quickly. In the opposite case, the Pasteur pipette may become a dangerous weapon hurting yourself or your colleague.

See **Appendix** about antibody-linked oligosaccharides ➔

Appendix: Oxidation of Oligosaccharide side chains on IgG molecules

IgG molecules carry two oligosaccharide side chains, one on each heavy chain. The oligosaccharides are located on the Fc region, next to the central hinge (**Figures 4-7**).

The structure of the oligosaccharide can vary within one species (**Figures 4 and 5**) and from species to species. Typical oligosaccharide structures are shown in **Figures 4 – 7**.

The size of the oligosaccharide is much smaller than that of an antibody arm. A more realistic sketch is shown on the lower right of **Figure 5**.

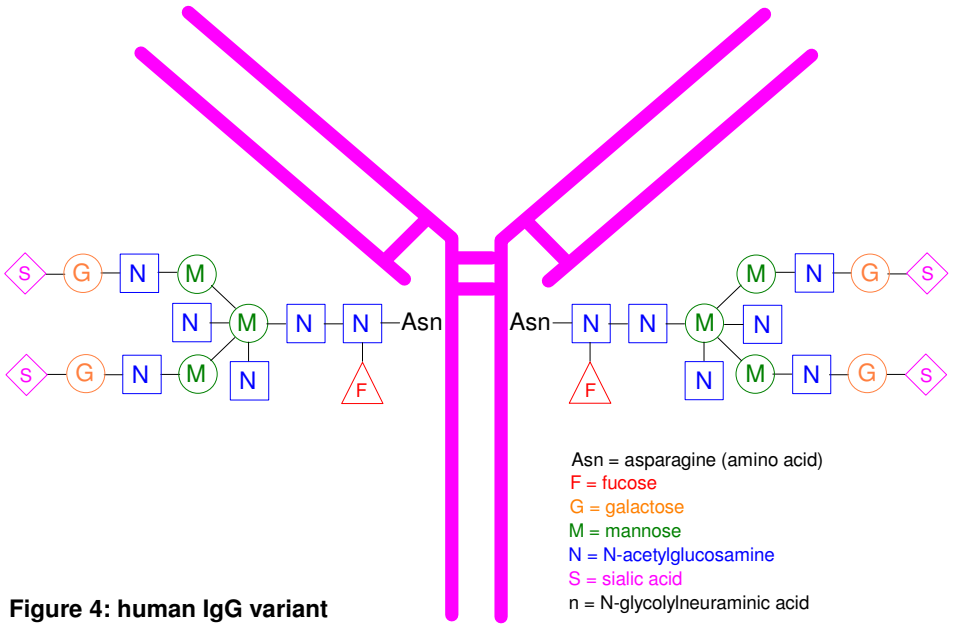

Figure 4: human IgG variant

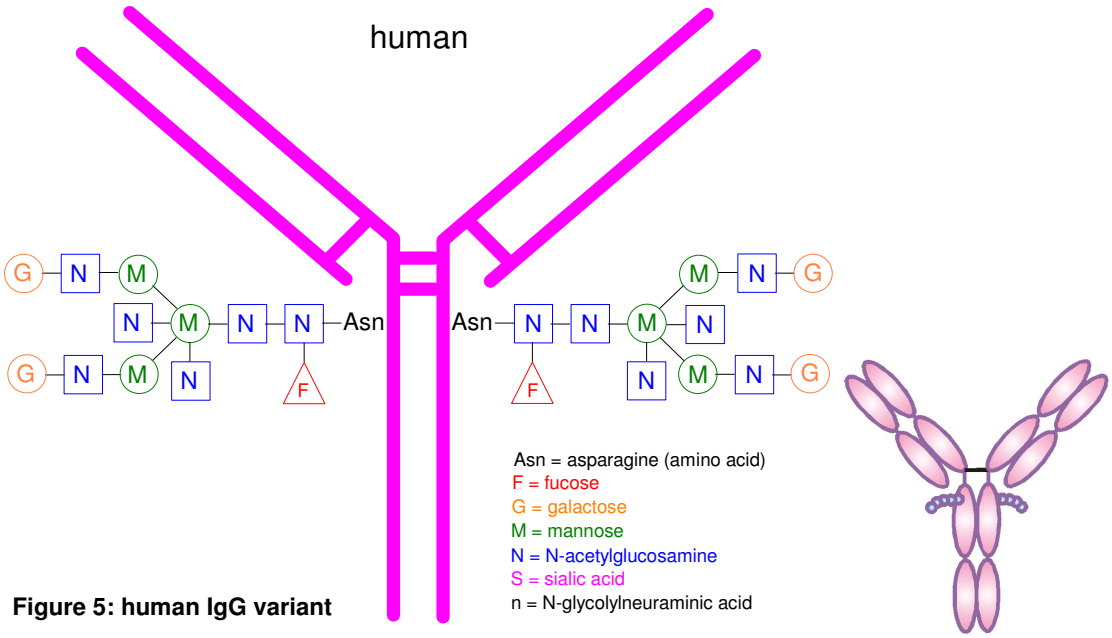

Figure 5: human IgG variant

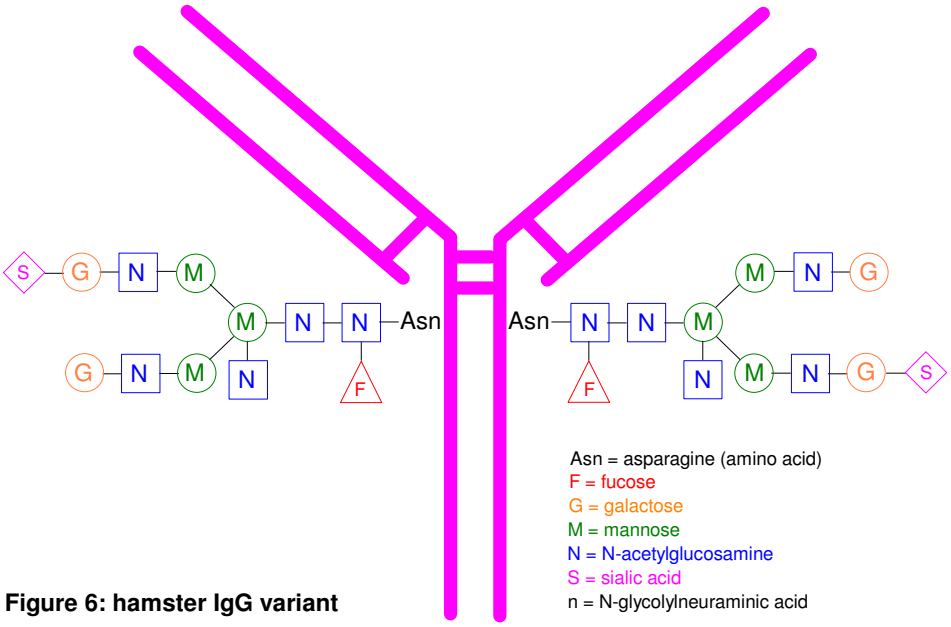

Figure 6: hamster IgG variant

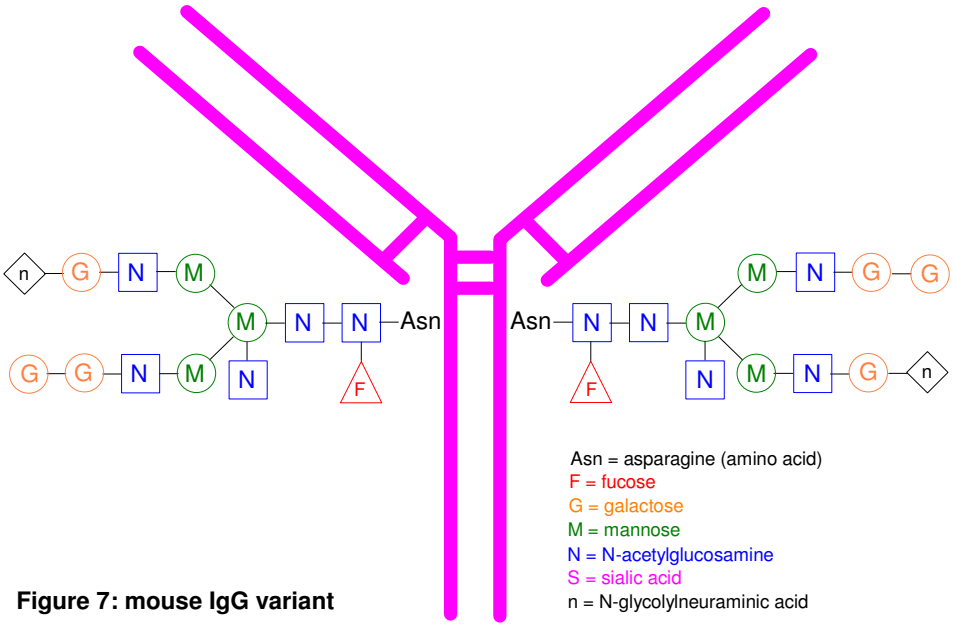

Figure 7: mouse IgG variant

A common structural feature of IgG oligosaccharides is the biantennary structure which contains mostly sialic acid residues at the terminal positions (**Figure 4**). If the sialic acid residues are missing, then the terminal sugars are usually galactose residues (**Figure 5**). Sometimes the galactoses can also be missing; then the oligosaccharide ends with several mannose residues (not shown).

The modern chemical name for "sialic acid" is *N*-acetyl-neuraminic acid (abbreviated as NANA). Its structure is shown in **Figure 8**.

In mouse antibodies (**Figure 7**) the NANA (*N*-acetyl-neuraminic acid) is replaced by NGNA (*N*-glycolyl-neuraminic acid). The chemical difference between NANA (**Figure 8**) and NGNA (**Figure 9**) is minimal (see blue circle in **Figure 9**).

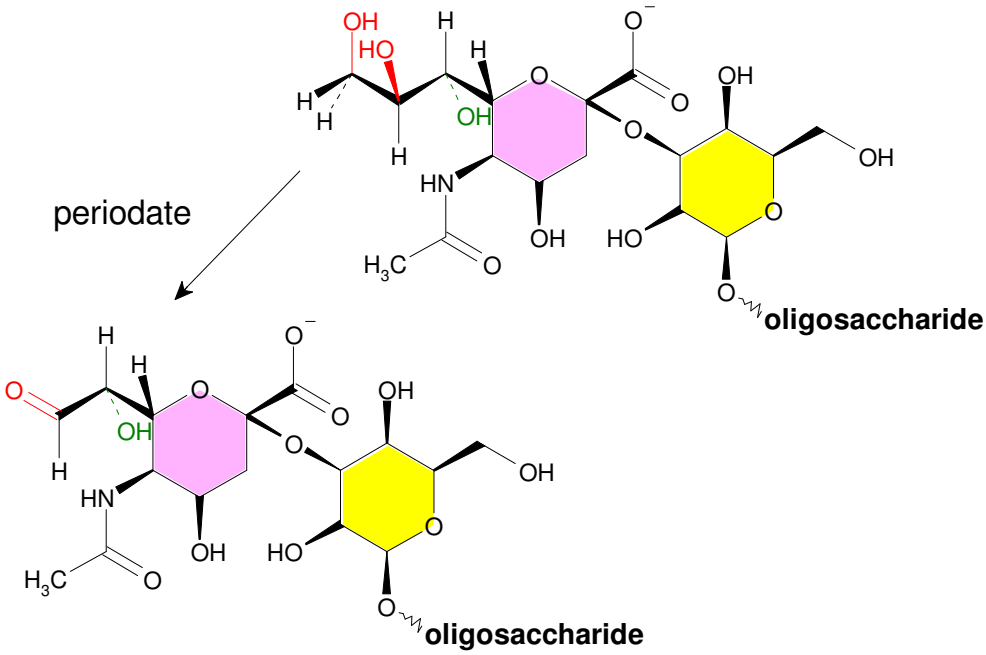

Figure 8: Periodate oxidation of a terminal sialic acid (*N*-acetyl-neuraminic acid, NANA) which is linked to a galactose residue.

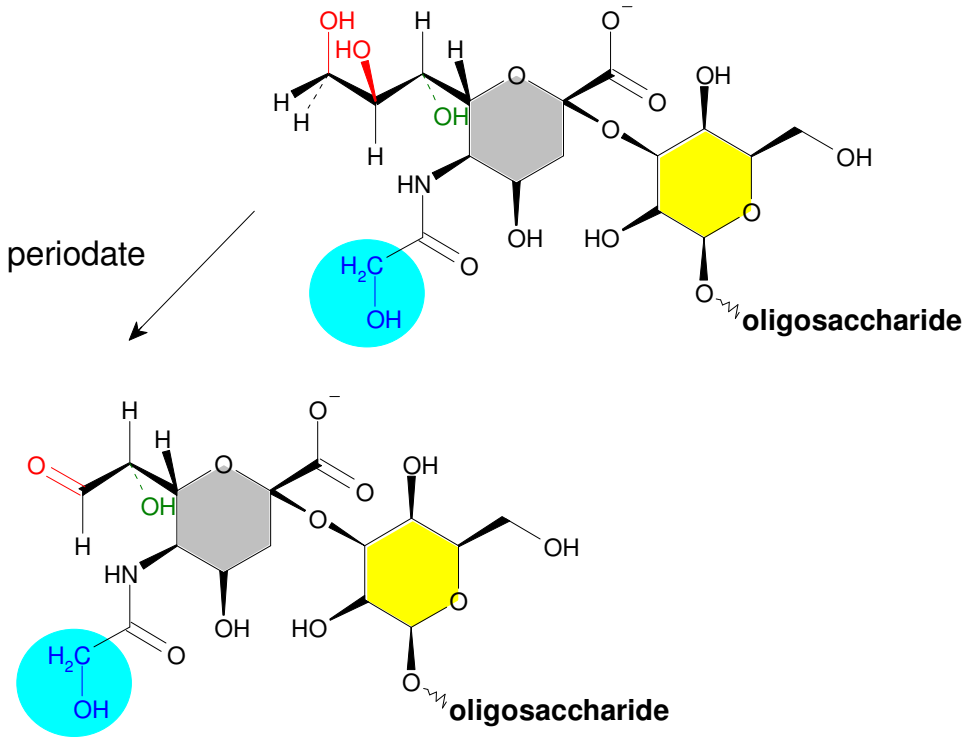

Figure 9: Periodate oxidation of a terminal *N*-glycolyl-neuraminic acid (NGNA) which is linked to a galactose residue.

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A characteristic feature of NANA and of NGNA is their flexible tail with three vicinal OH groups. The term "vicinal" means that the OH groups are attached to adjacent C-atoms. The two outermost OH groups are particularly reactive with sodium periodate because these two OH groups can easily adopt a synclincal conformation which can form a cis-complex with a periodate ion (IO_4^-). In such a situation, low concentrations of periodate and short reaction times are sufficient to achieve complete conversion of the vicinal OH groups into aldehyde functions.

Please, note:

- The protocol of periodate oxidation described in this manual is based on the assumption that the glycoprotein contains NANA or NGNA residues which are very easily oxidized by periodate.
- At least 10× higher periodate concentrations are required for oxidation of galactose or fucose residues, due to steric constraints in the rings (see **Figures 10 and 11**). For this reason, most authors prefer enzymatic oxidation of galactose residues by galactose oxidase (see literature on the next page).
- One terminal C-atom is lost from sialic acid (or from *N*-glycolyl-neuraminic acid, NGNA) by formation of formaldehyde. This formaldehyde must rigorously be removed by dialysis (or gel filtration) before the oxidized antibody can be coupled to the hydrazide on the AFM tip.

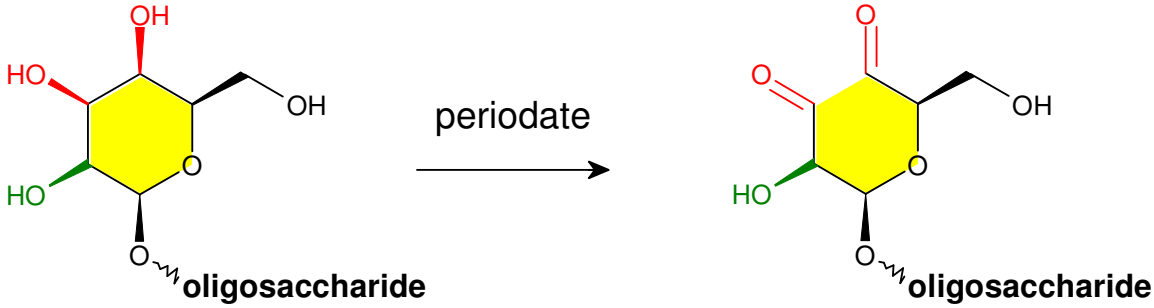

Figure 10: Periodate oxidation of a terminal galactose residue which is linked to the oligosaccharide chain of an IgG molecule.

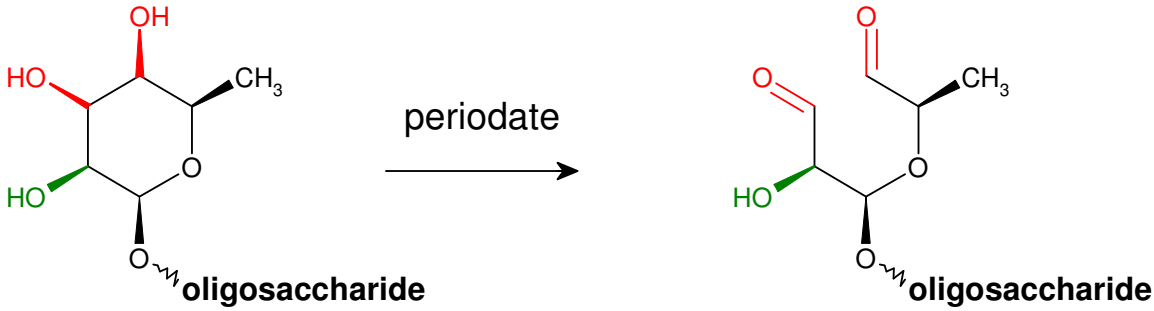

Figure 10: Periodate oxidation of a fucose residue which is linked to the oligosaccharide chain of an IgG molecule (see **Figures 4-7** for the location of fucose residues on antibody molecules).

Literature on derivatization of glycoproteins (mostly antibodies):

Agarwal, P., and Bertozzi, C. R. (2015) Site-specific antibody-drug conjugates: the nexus of bioorthogonal chemistry, protein engineering, and drug development. *Bioconjugate Chem. 26*, 176-192.

Andreana, P.R., Xie, W., Cheng, H.N., Qiao, L., Murphy, D.J., Gu, Q.-M., Wang, P.G. (2002) In situ preparation of beta-D-hydroxylamino carbohydrate polymers mediated by galactose oxidase. *Org. Lett. 4*, 1863-1866.

Endo, T., Wright, A., Morrison, S. L., Kobata, A. (1985) Glycosylation of the variable region of immunoglobulin G-site specific maturation of the sugar chains. Mol. Immunol. 32, 931-940.

Fu, Q., and Gowda, D. C. (2001) Carbohydrate-directed conjugation of cobra venom factor to antibody by selective derivatization of the terminal galactose residues. *Bioconjugate Chem. 12*, 271-279.

Lee, J. A., and Fortes, P. A. (1985) Labeling of the glycoprotein subunit of (Na,K)ATPase with fluorescent probes. *Biochemistry 24*, 322-330.

Lemieux, G. A., and Bertozzi, C. R. (1998) Chemoselective ligation reactions with proteins, oligosaccharides and cells. *TIBS 16*, 506-513.

Solomon, B., Koppel, R., Schwartz, F., Fleminger, G. (1990) Enzymic oxidation of monoclonal antibodies by soluble and immobilized bifunctional enzyme complexes. *J. Chromatogr. A 510*, 321-329.

Stadlmann, J., Pabst, M., and Altmann, F. (2010) Analytical and Functional Aspects of Antibody Sialylation. *J. Clin. Immunol. 30 (Suppl. 1)*, 15-19.

Stan, A. C., Radu, D. L., Casares, S., Bona, C. A., Brumeanu, T. D. (1999) Antineoplastic efficacy of doxorubicin enzymatically assembled on galactose residues of a monoclonal antibody specific for the carcinoembryonic antigen. *Cancer Res. 59*, 115-121.

Stan, A. C., Casares, S., Radu, D., Walter, G. F., and Brumeanu, T. D. (1999) Doxorubicin-induced cell death in highly invasive human gliomas. *Anticancer Res. 19*, 941-950.

Zeglis, B. M., Davis, C. B., Abdel-Atti, D., Carlin, S. D., Chen, A., Aggeler, R., Agnew, B. J., and Lewis, J. S. (2013) Chemoenzymatic strategy for the synthesis of site-specifically labeled immunoconjugates for multimodal PET and optical imaging. *Bioconjugate Chem. 25*, 2123-2128.

An excellent introduction to antibody glycosylation is found on the website http://www.imgt.org/IMGTeducation/IMGTlexique/G/Glycosylation.html

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