ATTO-TEC

ATTO 565 and ATTO 590

General Information

ATTO 565 and ATTO 590 are fluorescent labels belonging to the well known rhodamine dyes. A common feature of all rhodamine dyes is a carboxyphenyl substituent at the central carbon atom of the xanthen-chromophore.

The carboxy functionality (red) at the 2- or ortho-position has a significant impact on the physical and chemical properties of all rhodamines [1].

Due to the free ortho-carboxy functionality ATTO 565, ATTO 590, and all their derivatives exhibit special characteristics that need to be taken into account when dealing with these fluorophores. Both dyes carry an additional carboxy group in the 4- or 5-position within the phenyl ring as a site of coupling, making ATTO 565 and ATTO 590 suitable as fluorescent labels.

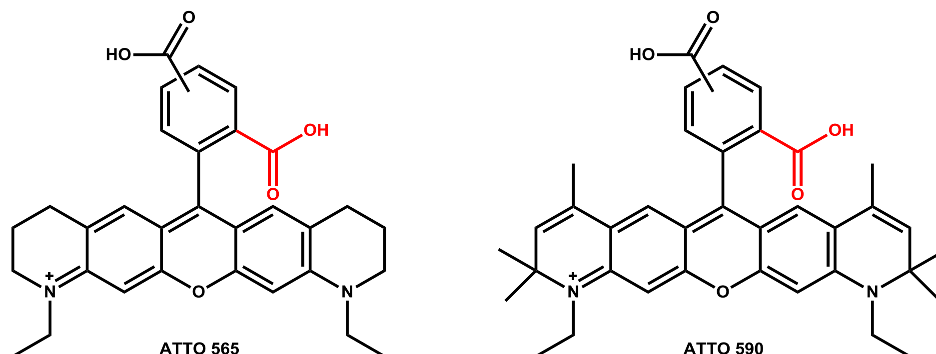

Figure 1: Chemical structures of ATTO 565 and ATTO 590.

Dependency of Absorption Wavelength on pH

The ortho-carboxy group is in close proximity to the chromophore and as a result the protonation-deprotonation equilibrium of this acid functionality strongly influences the optical properties of a rhodamine dye.

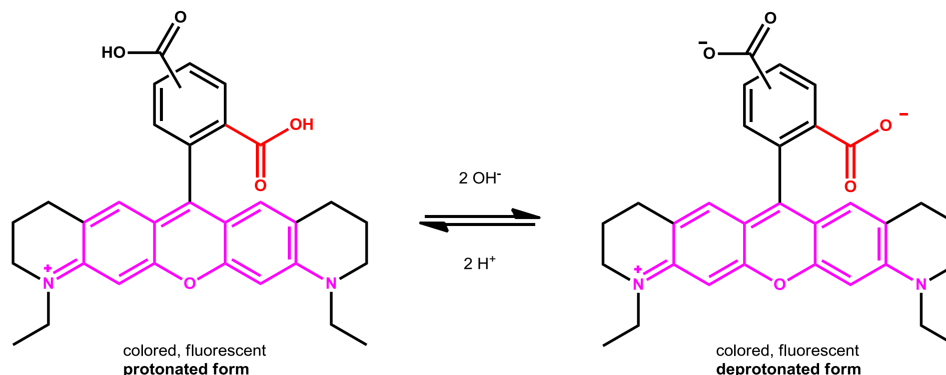

Figure 2: Protonation-deprotonation equilibrium of ATTO 565 (e.g. in ethanol).

[1] K. H. Drexhage, Structure and Properties of Laser Dyes, in: F. P. Schäfer, Dye Lasers, 1973, Springer Verlag, Berlin, Heidelberg.

Hence, the absorption maximum of ATTO 565 and ATTO 590 is different for the protonated and deprotonated form. For instance the absorption maximum of deprotonated ATTO 565 in Ethanol is shifted by 16 nm to shorter wavelength (hypsochromic) relative to the protonated dye.

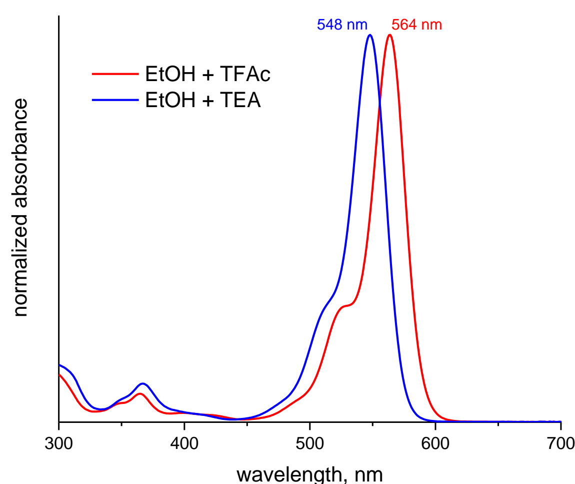

Figure 3: Comparison of the position of the absorption maximum of ATTO 565 in ethanol containing trifluoroacetic acid (TFAc) and in ethanol with triethylamine (TEA).

The Dye-Spirolacton Equilibrium

ATTO 565 and ATTO 590 in their deprotonated form can build a colorless spirolacton: After nucleophilic attack by the carboxylate anion at the centre carbon atom a five membered ring (lactone) is formed, interrupting the xanthene chromophore. As a result the compound turns colorless, i.e. no longer absorbs and fluoresces in the visible range of the spectrum (Figure 4).

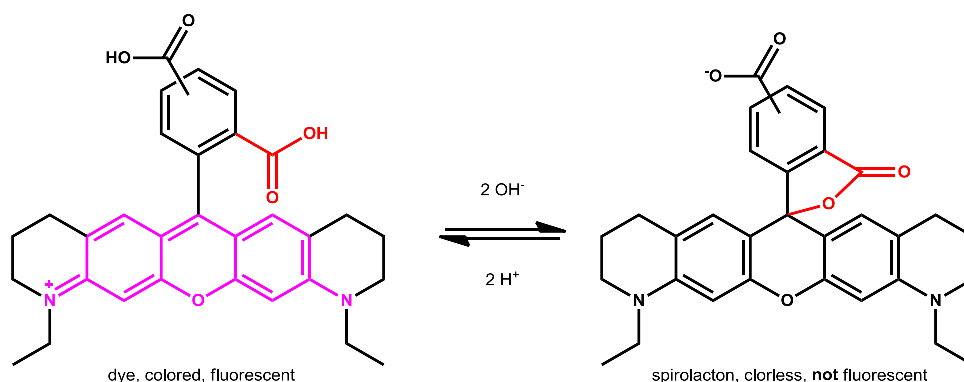

Figure 4: Dye-spirolacton equilibrium of ATTO 565 (e.g. in acetone).

The percentage of spirolacton in the equilibrium strongly depends on the solvent, pH, temperature and chemical structure of the dye. In polar aprotic solvents the equilibrium is almost completely in favor of the spirolacton. Consequently solutions of ATTO 565 and ATTO 590 in anhydrous acetone are virtually colorless.

Spirolacton formation is also possible in DMSO or DMF, particularly in the presence of bases or nucleophiles (Figure 5). One have to keep in mind, that commercial DMSO - independent of the purity assured by the supplier - contains variable amounts of basic or nucleophilic impurities which drastically alter the dye-spirolacton-equilibrium.

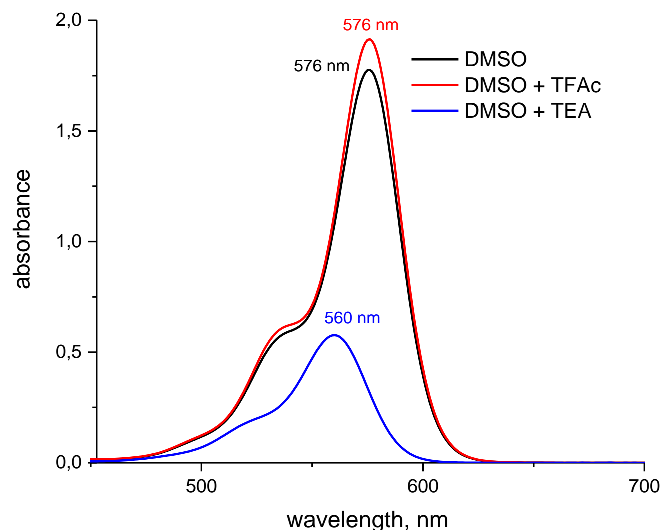

Figure 5: Absorbance spectra of ATTO 565 in DMSO, with trifluoroacetic acid (TFAc) and DMSO containing triethylamine (TEA).

However, in polar, protic solvents like water or ethanol, the equilibrium is almost completely in favor of the dye form.

Aggregation of Dyes / Intermolecular Interaction

In aqueous solution at high concentration the majority of organic dyes show a phenomenon called aggregation, a behavior strongly dependent on the structure of the dye and its hydrophilicity. In general: For hydrophobic dyes (e.g. ATTO 520) in aqueous solution dye aggregation is a lot more pronounced than for hydrophilic dyes (e.g. ATTO 532). As a result of the intermolecular interaction of the dye molecules the absorption of such a solution changes drastically. In the absorbance spectrum an additional absorption emerges at shorter wavelength predominantly due to the formation of dimeres (Figure 6).

When measuring the absorption of such solutions the spectrum obtained is a superposition of the absorption of dye monomer, dimer, and possibly higher aggregates. This phenomenon has to be taken into account in all measurements with the objective to determine the concentration of a dye in aqueous solution.

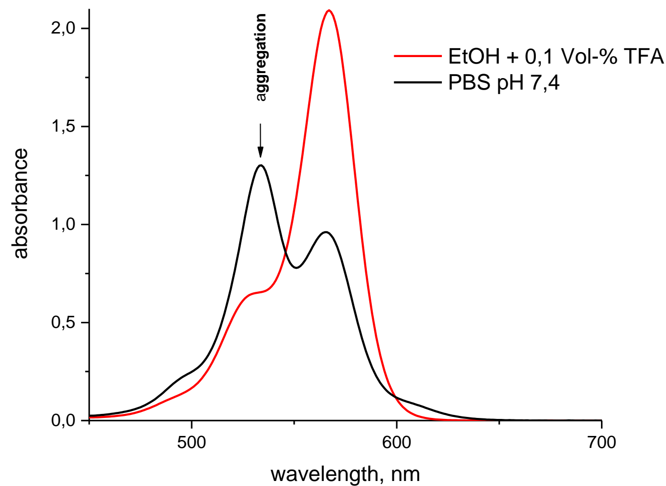

Figure 6: Absorbance spectra of ATTO 565 dissolved in PBS (pH 7,4) and in acidified (TFAc) ethanol. The concentration of dye in both solutions is identical.

The formation of a dimer out of two monomers is a chemical equilibrium and as such strongly dependent on dye concentration and temperature. Thus, dimers will be converted back to the monomers by simply diluting the dye solution. If the absorbance spectrum does not change with further dilution and increasing the pathlength accordingly, the spectrum obtained almost exclusively represents the absorption of the dye monomer. For most hydrophobic dyes as well as for ATTO 565 and ATTO 590 this is achieved at absorbance values of 0.04 at a pathlength of 1 cm (c = 10^{-7} – 10^{-6} mol/l).

In general, dimers and higher aggregates do not fluoresce, thus dimer formation will also alter the emission properties of such solutions.

Dye Interaction in Protein Conjugates (Intramolecular) / DOL-Determination

Dye NHS-esters easily react with amino groups of proteins forming dye-protein-conjugates. The covalently bound dye molecules at the protein can also interact with each other, leading to a similar observation, i.e. broadening of the absorption with a pronounced shoulder or additional maximum at shorter wavelength. This is illustrated in Figure 7, showing the absorbance spectrum of ATTO 565-streptavidin. As with an aqueous solution of sufficiently high concentration, an additional absorption at shorter wavelength is observed. In this case however, the dye interaction is intramolecular, hence dilution of the conjugate solution will not change the absorbance spectrum.

If an intramolecular interaction of dye molecules bound in the conjugate occurs the determination of the dye to protein ratio (degree of labeling, DOL) with the aid of the long wavelength absorption maximum of the dye will be defective. In such a case the determined absorbance at this position is too low and the error in DOL calculation can - depending on the distinct tendency for dye interaction - be off by 20 % and more.

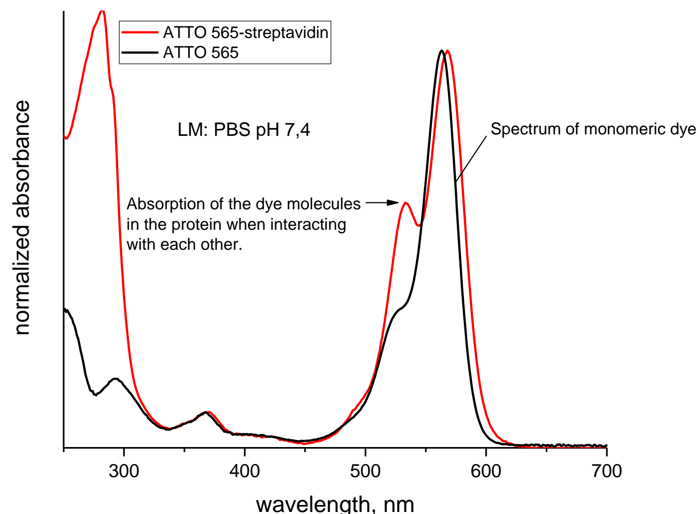

Figure 7: Comparison of the absorbance spectra of ATTO 565-streptavidin (DOL: 2,5) and ATTO 565 in PBS pH 7,4.

In such cases it is recommended to determine the DOL by measuring the amount of uncoupled dye. Therefore it is necessary to collect the second colored zone during gel filtration containing the unbound dye. The molar amount of dye can be calculated by measuring the absorbance of this solution and applying the Lambert-Beer law. Due to the tendency of ATTO 565 and ATTO 590 to form aggregates it needs to be assured that the absorbance of the dye solution does not exceed A = 0.04 (pathlength: 1 cm). Otherwise it is mandatory to dilute the solution accordingly. The difference of the initial molar amount of dye and the molar amount of unbound dye represents the molar amount of bound dye. The ratio of bound dye to the amount of deployed protein yields the DOL by eliminating the absorbance of the dye coupled to the biomolecule.

Determination of the Concentration of ATTO 565 and ATTO 590 Dye-Solutions

Owing to the characteristic features described above, when determining the concentration of a solution of ATTO 565 or ATTO 590, it is important to avoid lacton formation and to ensure that the dye is present in a defined protonated or deprotonated form.

To determine the concentration of a dye stock solution (e.g. in DMSO, DMF, acetonitrile) it is recommended to dilute an aliquot of the stock solution in acidified (0,1 vol.-% trifluoroacetic acid) ethanol for any absorbance measurements. For measurements in aqueous medium the dye solution must to be sufficiently diluted to ensure that the determined absorbance spectrum almost solely represents the absorbance of the dye monomer.