Spectroscopic aspects
of the cationic dye basic orange 21 (BO21)

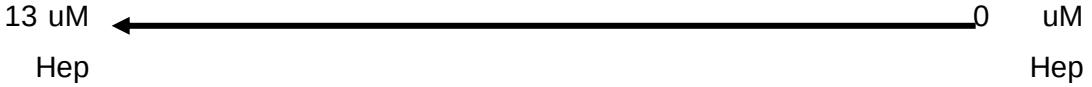
In this work, I investigated the spectroscopic aspects of the cationic dye, Basic Orange #21 (BO21) and the changes that could occur in those characteristics in the presence of biological molecules within the live cell in general, and various leukocytes (white blood cells) in particular. Leukocyte staining with BO21 can improve leukocyte differential counting, a routine process as first diagnostic for diseases and various infections.

This work is divided into two parts; the first is the main work for my thesis and the second is the appendix which contains some important results for future investigation.

Part 1: Study of the absorption characteristics of BO21.

This part contains results of my spectral investigation of the BO21 dye, including absorption spectrum of BO21 solution under several conditions (pH, viscosity) and various solvents (salts, proteins). The spectral investigation examined whether and how the various environments of the suspension BO21 will affect the absorption spectrum, i.e., will they cause an increase or decrease in the absorption spectrum or will they cause a blue or red shift in the spectrum. These changes are reflected in the hue observed by the eye after cell staining. In order to estimate the observed hue I converted the absorption spectrum to a color plane diagram composed of all colors perceivable to the human eye. Results of the measurement show negligible changes in absorption spectra as a function of different pH levels or the presence of salts. On the other hand, increased solution viscosity caused a shift of the absorption peak to red wavelengths.

In the main part of this work, spectral characteristics of BO21 are investigated in the presence of Heparin, an anion organic molecule common to a specific type of leukocyte. The shift of the absorption peak in short wavelengths was tested which is expressed by the red hue in the dye solution in the presence of Heparin (Figure 1).



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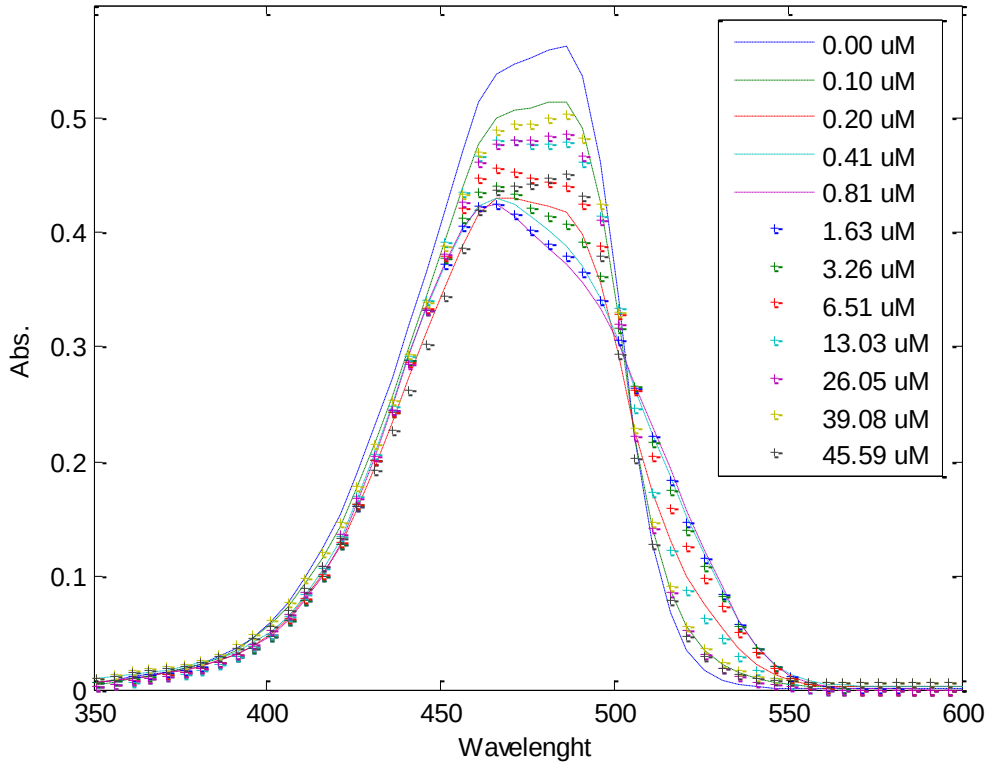

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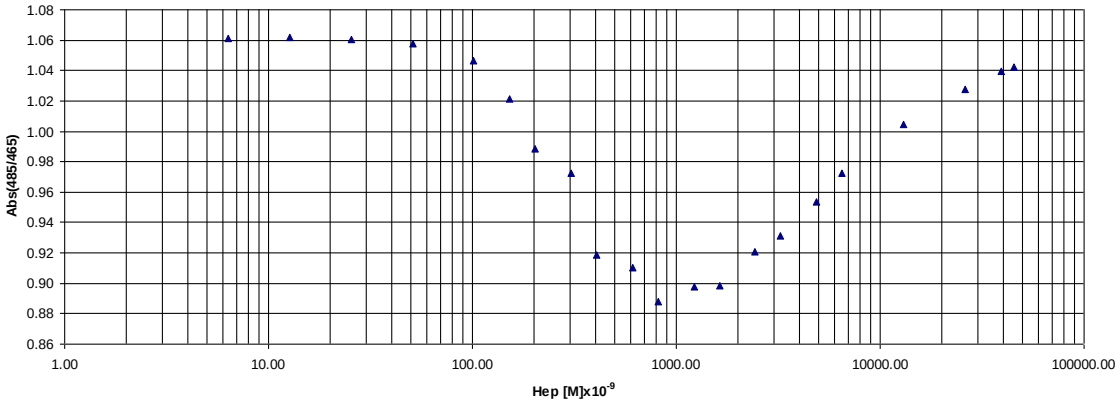

Figure 1. Absorption spectra of BO21 at various heparin concentrations. a) cuvettes sample picture. b) absorbance spectrum c) absorbance ratio Abs(485)/Abs(465) as function of log heparin concentration

The absorption spectrum may evolve from the formation of electrostatic bonds between the dye cations and polyanions and from effective aggregation of the dye cations due to their occupying adjacent sites on the polyanion. In order to distinguish between the two possible mechanisms, I performed two experiments. I isolated the electrostatic bonds by replacing the heparin with small anion molecules in the first experiment, while in the second, I isolated the dye aggregation by measurement of the absorption spectrum under high dye concentration. In order to measure absorption at high concentration I build a new narrow cuvette with width of 0.15mm (Figure 2). The conclusion reached from these experiments is that it is not the electrostatic bond which is responsible for the blue spectral shift of the dye but rather the aggregation of dye ions. In addition to these results we propose a mechanism which explains the shift phenomenon.

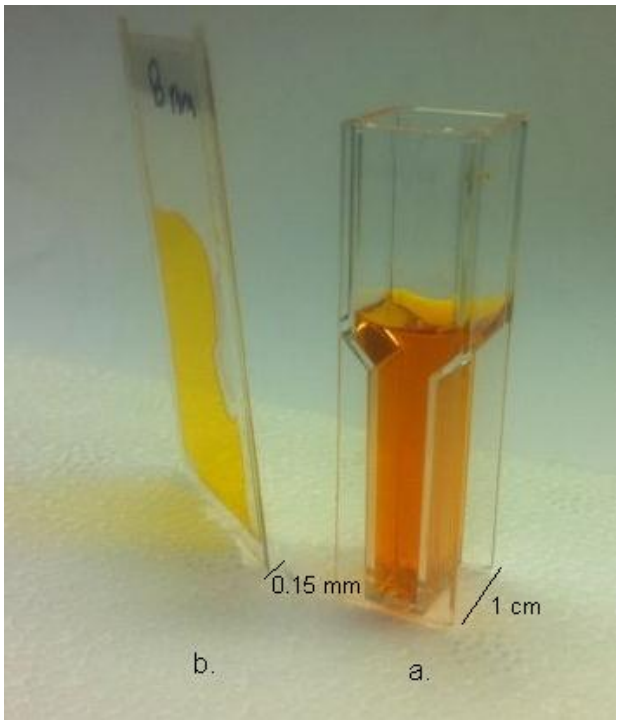

Figure 2. 8mM dye concentration in the two cuvettes, a) 10 mm width cuvette. b) 0.15mm width cuvette.

Further strengthening of the experimental results and proposed mechanism was obtained by the use of computational chemistry. The results and relevant discussions are given in the last chapter of the first part of this study. Briefly, the calculation indicates that parallel setting of the molecular electrical dipoles of BO21 of which the

dimers and trimers are composed, is indeed feasible. Surprisingly, the related binding energies are even lower than those calculated for anti-parallel arrangement.

From experiments in which I changed the ratio of concentrations between BO21 to heparin, by measuring concentration of fixed dye but with changes concentration of heparin and the complement experiment with the concentration of BO21 in it that has changed and the concentration of heparin remains constant, I observed changes in the influence of heparin on the spectrum which rely on the relative concentrations between free BO21 and bound BO21.

From these experiments I calculated the number of heparin binding sites available for BO21 and equilibrium bonding constant between heparin and BO21.

The practical aspect of this work is expressed in the attempt to apply obtained results for differentiation between main leukocyte sub populations: neutrophils, lymphocytes, monocytes, eosinophils and basophils, through the use of BO21 as a single differentiating dye.

Part 2: Appendix, Study of the emission and polarization properties of BO21

This part deals mainly in the fluorescence and polarization of BO21. I found the dye fluorescence to have lower quantum yield by two or three order of magnitude when compared to flourescein.

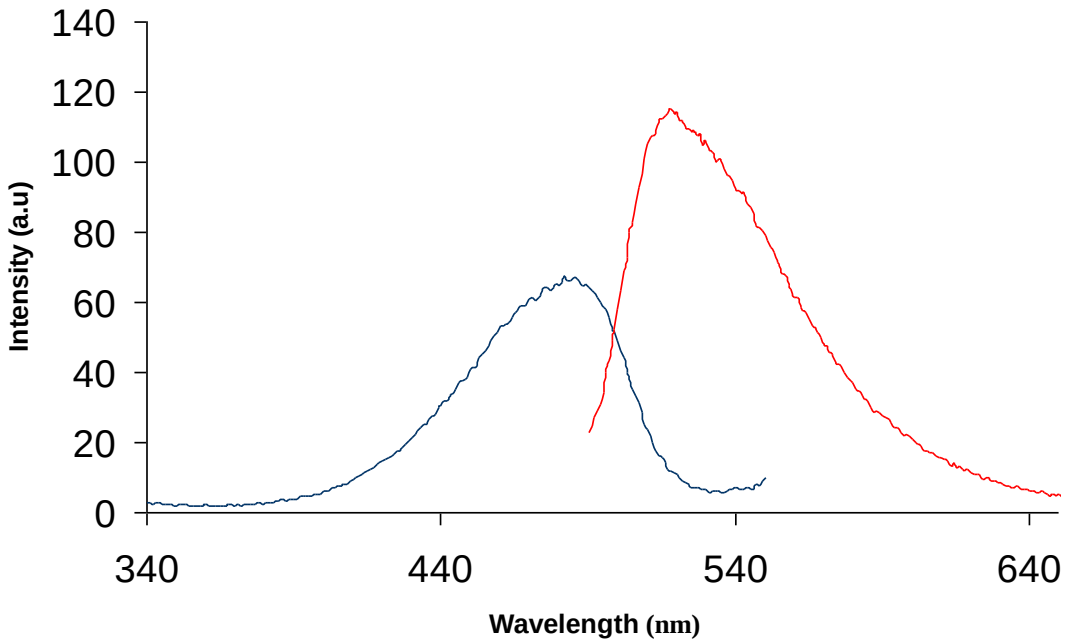

Figure 3. exitation (blue) and emission (red) spectrum of 10uM BO21 in water.

Fluorescence polarization measurements in water showed, contrary to all expectations, a polarization higher than 0.45. I proved that the source of this finding cannot be the short life time of BO21. However, the same measurement in the presence of heparin resulted in a polarization value lower by more than twice that obtained in water. In experiments I conducted to explain the mechanism behind this finding, I found that the presence of salts, alcohol, and most surprising, after heating the solution, the polarization level rises again to its high value. As a complement to the practical aspect measured in Part 1, the fluorescence spectra and emission polarization spectra of BO21 suspension cells were also examined. Preliminary findings of these measurements have shown that one can distinguish between types of leukocytes on the basis of fluorescence measurement of cells in suspension.

In this work some of the causes for the color change in leukocytes dyed with BO21 were studied. In my opinion, understanding this mechanism can contribute greatly to the use of differential staining of cells, both through the changes in visible color as well as fluorescence and polarization changes.