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SYNTHESIS AND CHARACTERIZATION OF BIARSENICAL DYES FOR SELECTIVE MODIFICATION OF PROTEINS

ABSTRACT

Protein fluorescent modification is one of the most popular techniques used in molecular biology studies. It can be performed using reactive derivatives of small fluorescent chemical dyes or by making a fusion with either fluorescent protein or enzyme that can attach fluorescent substrates. However, these methods have certain disadvantages. Addition of fusion protein usually changes the activity or cellular localization of the target protein. Selective modification with small, reactive fluorescent chemical dyes is not possible in live cells. Because of those problems researchers were looking for a method to specifically label protein in live cells with a small fluorescent chemical dye. One of the most popular method developed so far is based on high affinity of fluorescein modified with arsenic atoms at 4' and 5' positions (biarsenical probe) to a protein with tetracysteine tag, usually CCPGCC. This method was used in numerous studies in molecular biology, biochemistry, bioanalytics and many other fields. The aim of this PhD project was to develop new biarsenical probes, establish their physicochemical properties and apply them in molecular biology research. Specifically, the research was focused on searching of the most efficient biarsenical probe for inhibition of mutated protein tyrosine phosphatases (PTP), and preparation of zinc(II) or pH sensor that could be specifically localized in the selected cell compartment. In order to obtain the most efficient inhibitor of mutants of protein tyrosine phosphatases a library of biarsenical dyes was synthesized, based on fluorescein (FlAsH-EDT$_2$) derivatives and resorufin (ReAsH-EDT$_2$). Also several of the previously described mutants of TCPTP and HePTP protein tyrosine phosphatases with tetracysteine motifs were produced. Enzymatic activity assay, performed using *para*-nitrophenol (*p*NPP) and phosphorylated peptide as substrate have unequivocally proven that dyes with 2' and 7' substitutions are the best inhibitors of mutants of protein tyrosine phosphatases. Moreover complexes of all dyes with tetracysteine peptides

were characterized in terms of their physicochemical properties. Also for the first time the stability of the biarsenical dyes in aqueous solutions was established. Next objective was the development of pH sensor with the ability to localize in selected cellular compartments. The biarsenical modification of the most popular pH sensor – BCECF yielded a new compound BCECFlAsH-EDT_2. New sensor has similar physico-chemical characteristic as the parent sensor. The key parameter for measuring capabilities – the value of dissociation constant of hydroxyl group at position 3' is 6,68. Similarly to other biarsenical dyes, the fluorescence was quenched before binding to the tetracysteine motif. The sensor also retained the ability of ratiometric signal read-out. However the results obtained from analyzing ratiometric fluorescence read-out was not convergent with ones obtained from analyzing intensity at single wavelength. Detailed analysis of the results showed that it is caused by lack of linearity of the intensity ratio. Because of that, the final results were under- or overestimated. Taking this fact into consideration a new method for calculation of the dissociation constant was developed and successfully applied to calculate the dissociation constants of Zincon and FLIPE-1μ ratiometric sensors complexes. The last objective of the performed research was the development of zinc(II) ion sensor with the ability to localize in selected cellular compartments. At the beginning it was established that optimized tetracysteine sequence FLNCCPGCCMEP is able to bind zinc(II) ions in 1:1 stoichiometry with dissociation constant of ~10^{-11} M and that the first three cysteines residues are involved in the metal cooridination. It was also observed that binding of the zinc(II) to the tetracysteine motif is severely reducing the speed of the biarsenical binding. Next, using genetically encoded zinc(II) ion sensor eChZinc-2 it was proved that number of factors, such as keeping the cells in imaging buffer under the microscope or washing the cells with 1,2-dimercaptoethane solution after the biarsenical labelling does not changes the free zinc(II) in cytosol of HeLa cell line. In order to measure the concentration of free zinc(II) in HeLa cell nucleus a synthesis of ZnAF-F zinc(II) ion sensors was performed. The biarsenical modification of ZnAF-1F allowed for obtaining ZnAFlAsH-1F-EDT_2, whose physicochemical and coordination properties were established. What is important for further measurements the biarsenical zinc(II) sensor, even with attached zinc(II) ion was only slightly fluorescent. Only the sensor attached to tetracysteine motif and coordinating zinc(II) ion was strongly fluorescent. The value of dissociation constant of the complex is 1,4 nM, which allows its use in cellular conditions, where concentration of free zinc(II) is between 5 and 900 pM. In order to direct the sensor to the nucleus the HeLa cells were transfected with plasmid encoding histone 2B with C-terminal optimized tetracysteine sequence. Images obtained from confocal

microscopy not only confirmed the proper localization of the sensor but also allowed for calculation of free zinc(II) concentration in nucleus of two cells. The obtained values – 324 and 494 pM are well within expectation based on the current knowledge of zinc(II) homeostasis.