Name of the scholar: Ovais Manzoor

Name of the supervisor: Dr. Mohd Mohsin

Department: Biosciences

Title of thesis: "Development of Genetically Encoded FRET-based Nanosensor for in vivo Measurement of the Vitamins"

ABSTRACT:

In this study, FRET based genetically encoded nanosensors were developed that could successfully monitor vitamin B_{12} and vitamin B_1 levels in bacterial, yeast and mammalian cells. To develop these nanosensors, Group II periplasmic binding proteins (PBPs) for vitamin B_{12} and vitamin B_1, BtuF and thiB served as ligand sensing domains. Nanosensors were constructed by sandwiching the ligand sensing domains between cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP). Any change in metabolite concentration changes the resultant FRET ratio. The constructed nanosensors SenVitAL and FLIPT were specific to their ligand of interest and stable to pH within physiological range.

AIM OF THE STUDY:

Experiments were designed to carry out the measurement of vitamin B12 and vitamin B1 levels, ranging from nanomolar to millimolar concentrations. The influence of biologically relevant metal ions on the uptake of vitamins was also measured. Analysis of vitamins was done under in vitro and in vivo environment in both prokaryotic and eukaryotic cellular systems.

RESULTS:

To optimize these newly developed nanosensors for physiological conditions, fluorescence emission intensities were recorded in various buffer systems such as TBS, PBS and MOPS in the pH range of 4.5 to 8. From our data, the maximum stability of SenVitAL was found in MOPS buffer, in which sensor showed least FRET ratio variations at around physiological pH. Similarly, maximum stability of sensor protein FLIPT was found in PBS buffer. Therefore, after validation, pH 7.2 of MOPS and PBS buffer were selected for further *in vitro* and *in vivo* analysis of sensor proteins respectively.

SenVitAL sensor showed least sensitivity towards other vitamins such as vitamin B_1 and vitamin C. The sensor showed maximum FRET ratio change with vitamin B_{12}, indicating its specificity for it. Similarly, FLIPT sensor protein showed least or no significant change upon the addition of vitamins like vitamin B_{12}, vitamin C and maximum FRET ratio change was obtained only with the addition of vitamin B_1, clearly indicating that developed sensor was very selective and specific towards vitamin B_1.

In both sensors, it was found that upon the addition of several biologically relevant metal ions like Ca^{2+}, Mg^{2+}, K^+ and Na^+ of 1mM concentration each, no significant effect of FRET ratio

change was observed and therefore, the activity of SenVitAL and FLIPT sensor remained unhindered.

The physiological detection range of SenVitAL nanosensor increased due to generation of three affinity mutants. Among the three affinity mutant sensors, SenVitAL W44Q showed the highest affinity for vitamin B_{12} ligand. The calculated K_d values are (93 μM) WT, 5 μM (W44Q), 143 μM (P144G) and 126 μM (A225P). Furthermore, to detect vitamin B1 in physiological range, among different interactive amino acids of thiB binding pocket, only one residue was successfully mutated through site directed mutagenesis to generate a mutant nanosensor (W280S). The calculated K_d of wild type (WT) and mutant W280S were 529 nM and 421 nM respectively.

A significant increase in FRET ratio was observed by the addition of 1mM vitamin B_{12} to *E.coli* expressing SenVitAL that saturated at 35 minutes of incubation. Increase in FRET ratio proved that SenVitAL was able to monitor vitamin B_{12} change taken up by bacterial cells in the *E.coli* cytosol. Similarly, in case of FLIPT nanosensor, FRET ratio increased constantly for a time period of 90 minutes, thereafter no further increase was observed and nanosensor reached the saturation level.

Addition of the 5 mM vitamin B_{12} to the sensor (pYES-DEST-CFP-BtuF-YFP) getting expressed in yeast (*S. cerevisiae*/URA3 BY4742) cells showed clear change in the fluorescence emission intensity of CFP and YFP. FRET ratio was recorded and found to be increased from 0.765 at 0 sec. time to 1.70 at 1400 sec. Addition of vitamin B_1 to FLIPT expressing yeast cells lead to change in the emission intensities of CFP and YFP. YFP/CFP emission intensity ratio was recorded for 700 seconds and was found to increase with the addition of 10 mM vitamin B_1 from 0.830 at 0 second to 0.950 at 700 seconds.

Confocal images and data showed that the FRET ratio increased by the addition of vitamin B_{12} in time-dependent manner. The basal level of FRET ratio (535/485 nm) was 0.935 till 12 min that increased rapidly after addition of B_{12}, reaching to a saturation level of 1.342 at 18 min of incubation. Live cell imaging of HEK-293T transfected cells showed that FLIPT was predominantly distributed within the cytoplasm of transfected cells. With the addition of vitamin B_1, YFP emission increased and that of CFP decreased in a time dependent manner. Change in the YFP/CFP emission intensity ratio was recorded in a 0 to 5 min interval, which showed a constant increase with vitamin B_1 addition.

CONCLUSION:

It is concluded, that the two nanosensors developed in this study possess efficient ability to detect, monitor and measure the fluctuations of vitamin B12 and vitamin B1 levels within living cells. Creation of affinity mutants did not changed their potential of specificity, although a collection of sensors was created to detect the ligands of different concentrations. Monitoring the real time dynamics of vitamins in HEK-293T cells at real time is a big outcome of this study and in future, other human or animal cell lines can be used for experiments. Also, it needs to be recommended that definite signal peptides should be attached to these constructs to track the vitamin concentrations in sub-cellular compartments of a cell in future.