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Topic of Research: Construction of versatile FRET-based nanosensors to monitor the flux of Biotin and Folic acid

Findings

In the present work, we have developed genetically encoded FRET-based sensors for real-time monitoring and analysis of two clinically important vitamins biotin and folic acid in prokaryotic and eukaryotic cellular systems. The study was conducted at various stages including the designing and development of nanosensors, expression, and purification of nanosensor proteins, *in vitro* assays of nanosensors (Fluorescence emission scan, Buffer, and pH stability, specificity, effect of biologically relevant metal ions, affinity, and mutagenesis for affinity mutants) of the nanosensors and *in vivo* study of developed vitamin specific nanosensors in bacteria, yeast, and mammalian cells. To design and construct these sensors, firstly the ECFP, Venus, and Avd/ FolT gene were amplified and sequentially cloned into bacterial expression vector pRSET-B. Avd and FolT proteins were used as a ligand recognition element with two GFP variants, ECFP and Venus, connected to the protein's N- and C-termini, respectively, as donor and acceptor molecules and converted into FRET-based sensors. Affinity chromatography using nickel-NTA agarose beads was used to purify chimeric sensor proteins. The sensor protein's integrity, purity, and molecular weight were analyzed using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). OSenB and SenFol sensor proteins satisfy the required conditions for FRET-based nanosensors because the translated protein undergoes sufficient conformational changes upon binding to their respective ligands. OSenB and SenFol were profiled in various buffer systems to determine sensor protein's stability in the different pH ranges by monitoring the change in the FRET ratio. *In vitro,* assays showed that these sensor proteins are extremely selective and specific for biotin and folic acid because their binding to the respective proteins increases the FRET ratio. The nanosensor proteins were mixed with biologically relevant metal ions such as Na^{+}, K^{+}, Ca^{2+}, and Mg^{2+} and incubated to determine whether these ions influenced the sensors' fluorescence. Affinity mutants were created by using site-directed mutagenesis and compared to WT sensor proteins to change the sensor proteins' physiological detection range for biotin and folic acid. Due to

their lowest K_d values, the sensor mutants OSenB-44 and WT SenFol were chosen for *in vivo* testing in bacterial and eukaryotic systems, respectively. *In vivo,* analysis and real-time monitoring of biotin and folic acid dynamics in bacterial cells showed that the sensor proteins OSenB-44 and WT SenFol are effectively expressed. In addition, confocal imaging was performed to explore the nanosensors' cellular expression in bacteria. Eukaryotic expression vectors such as pYES-DEST52 and pcDNA3.1(+/-) were also used to generate constructs for studying the performance of these sensors in eukaryotes. Nanosensor constructs cloned into their respective plasmids were transferred and expressed in *S. cerevisiae* and HEK-293T cells. Confocal images of sensor-expressing yeast cells showed that chimeric proteins were expressed in the cytoplasm, but not in the vacuole. As a result, the sensors permit direct monitoring of vitamin uptake into the cytosol at a subcellular level. Similarly, the HEK293T cell line expressing the sensors constructs, and confocal microscopy imaging showed that the nanosensors were highly expressed within the cells. This shows that the sensors can non-invasively measure vitamin levels in numerous biological systems with high spatial and temporal resolution. These genetically encoded sensors monitor biotin and folic acid levels in given samples in real-time. The *in vivo* assays demonstrates that the sensors may be used non-invasively and with a great spatial and temporal resolution to detect biotin and folic acid levels in given experimental samples. These genetically encoded sensing tools employ fluorescent sensing assay technology to track and assess dynamic changes in cellular biotin and folic acid rates *in vitro* and *in vivo*, without degrading samples.