Rational Engineering of Protein Stability for Potential Application as Detectors in NASA Space Missions

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ABSTRACT – Protein-based diagnostics are a promising tool for detection of small molecules on space missions. One challenge hindering the use of proteins in this application is the instability of proteins in the harsh conditions of space. Site-specific PEGylation by click reaction with non-canonical amino acids, ncAA, provides a promising solution to this challenge by potentially enhancing protein stability without compromising activity. We present a process of site-directed PEGylation to stabilize proteins while maintaining their activity. The successful demonstration of this technology could improve both Lab-on-a-Chip technology and medical diagnostics, aligning with NASA’s strategic goals.

Proteins can be used in microarray chips to detect thousands of compounds and molecules instantly. This function could be useful in analyzing compounds and molecules on the surface of other planets. The proteins could be used to detect molecules which may indicate life in exploration of other planets, such as water, amino acids, DNA, carbon hydrochains, etc. Implementation of these protein chips into a rover, similar to the Life Marker Chip which NASA and the ESA are planning to incorporate into the ExoMars Mission, could reduce the electricity demand of the rover when compared to the impressive laser spectroscopy equipment included in the Mars Curiosity rover. Stabilizing the proteins on these chips could reduce the size of the chips by eliminating the need for excess proteins which would otherwise be necessary to compensate for the loss of activity over time. This reduction in size would make the chips much lighter and make it easier to include the chips in very small rovers and probes. Thus, enhanced protein stability has applications to several of NASA’s strategic goals, such as the following:

- NASA Strategic Goal 1.1: Expand human presence into the solar system and to the surface of Mars to advance exploration, science, innovation, benefits to humanity, and international collaboration.
- NASA Strategic Goal 1.5: Ascertain the content, origin, and evolution of the solar system and the potential for life elsewhere.

Similar technology using proteins as detectors is also being developed by NASA to monitor the transfer of biologic material from earth to space and to detect compounds or potential pathogens which may affect the health of a space flight crew. This will allow for treatment or prophylaxis before a serious problem results. As mission durations increase, monitoring and maintaining the health of the flight crew will be of increasing concern. As with the small molecule detection, increasing the stability of these proteins without compromising activity would improve the longevity and reliability of the equipment used in crew health monitoring and medical diagnostics. This could boost the potential for longer space missions.

PEGylation Background
Covalent attachment of polyethylene glycol chains to proteins, or PEGylation, has been demonstrated to improve long-term stability and activity of drugs inside the human body. PEGylation of proteins helps to increase the stability of proteins by shielding them from the environment and by locking in place the correct protein conformation. While PEGylation can be achieved with natural amino acids such as lysine, the limited number of natural amino acids means that a single amino acid is often included in several places on a folded protein’s exterior. This makes it difficult to use natural amino acids in rational site-specific attachment of PEG, as PEGylation near the active site may reduce the
protein’s activity. However, by using unnatural amino acids, the location of PEGylation on the protein can be controlled to find the optimal location for PEGylation that enables long-term stability and activity. The Bundy lab has demonstrated that cell-free protein synthesis can be employed to incorporate these unnatural amino acids at only the specific locations where they are desired.

**ncAA Incorporation using CFPS**

As a model protein for incorporation of a ncAA, we employed super-folder GFP with threonine at the 216 residue of replaced with an amber stop codon. The GFP template was then expressed in our CFPS system, which has been described previously. This system uses bioorthogonal machinery to incorporate a ncAA at the amber stop codon. Using an orthogonal tRNA to recognize the amber stop codon and an engineered synthetase to charge the orthogonal tRNA, p-proparglyoxyphenylalanine (pPa) was incorporated at the amber stop codon. This system is ideal because any proteins where the pPa is not incorporated truncate because of the stop codon. Following strep-tag purification of the CFPS product, we assessed the purified GFP using a SDS-PAGE protein gel and verify that the full length GFP that contains pPa was indeed purified. As shown in Figure 1, the results confirmed the effective purification and synthesis of full-length sfGFP with the pPa incorporated at residue 216 (MW of 27,105 Daltons). The concentrations used were at 5x and 1x the concentration predicted to be necessary for click conjugation of PEG as detailed in the following section.

**PEGylation via Click Reaction to ncAA**

The next step is to attach PEG to pPa on GFP via click chemistry. Work is continuing to attach PEG to our GFP construct and to assess the stability of the GFP-PEG conjugate. PEGylation will be accomplished using a copper-catalyzed click reaction. The specific reaction to be used is Azide-Alkyne Huisgen cycloaddition, one of the most frequently employed click reactions and used extensively for bioconjugation, immobilization, and purification of biomolecules. This “click” reaction, first reported by the Meldal and Sharpless labs in 2002, forms a 1,2,3-triazole through cycloaddition of an azide to an alkyne, and occurs at biologically friendly temperatures and pH through copper catalysis. These characteristics make click reactions extremely useful in PEGylation of proteins. Figure 2 illustrates a copper-catalyzed click reaction for GFP PEGylation, where the terminal alkyne shown is provided by the ncAA pPa. This reaction is useful for bioconjugation reactions because it can be used to covalently attach azide-PEG to pPa. However, the copper catalyst Cu(I) inactivates GFP. GFP activity is restored upon removal of the copper, so a second purification step will be included in order to remove the PEGylated GFP from the remainder of the click reaction solution. Following purification, the stability of the GFP will be assessed by measuring fluorescence after subjecting the protein to biologically harsh conditions.

**Conclusion**

PEGylation of proteins at rationally designated sites offers a method of stabilizing proteins for use as small molecule detectors. This technology would enable production of more stable protein sensors for use in NASA technologies such as Lab-on-a-Chip. The on-going work of PEGylating GFP will provide insight into the degree of stabilization afforded by PEGylation.
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References