REPORT DOCUMENTATION PAGE

Form Approved OMB NO. 0704-0188

1. REPORT DATE (DD-MM-YYYY)	2. REPORT TYPE	3. DATES COVERED (From - To)
13-03-2017	Final Report	1-Jul-2013 - 31-Mar-2014

4. TITLE AND SUBTITLE
Final Report: Improving Protein Stability and Activity Using Zwitterionic Peptides (research area 8)

5a. CONTRACT NUMBER
W911NF-13-1-0207

5b. GRANT NUMBER

5c. PROGRAM ELEMENT NUMBER
611102

6. AUTHORS
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAMES AND ADDRESSES
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS (ES)
U.S. Army Research Office
P.O. Box 12211
Research Triangle Park, NC 27709-2211

10. SPONSOR/MONITOR'S ACRONYM(S)
ARO

11. SPONSOR/MONITOR'S REPORT NUMBER(S)
63926-LS-II.3

12. DISTRIBUTION AVAILIBILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES
The views, opinions and/or findings contained in this report are those of the author(s) and should not contrued as an official Department of the Army position, policy or decision, unless so designated by other documentation.

14. ABSTRACT
This work established the use of polypeptides composed of repeating glutamic acid (E) and lysine (K) (poly(EK)) for the purpose of increasing protein stability. The addition of poly(EK) to the primary sequence at the C-terminus of a model beta-lactamase protein and a destabilized TEM-19 demonstrated significant ability in increasing protein stability while maintaining bioactivity. The use of fusion protein products allows for a one-step protocol resulting in a homogeneous product, a significant advantage over polymer-based approaches.

15. SUBJECT TERMS
Fusion proteins, genetic engineering, EK peptide and zwitterionic

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	15. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			Shaoyi Jiang
UU	UU	UU	UU		19b. TELEPHONE NUMBER 206-616-6509

Report Title

Final Report: Improving Protein Stability and Activity Using Zwitterionic Peptides (research area 8)

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Enter List of papers submitted or published that acknowledge ARO support from the start of the project to the date of this printing. List the papers, including journal references, in the following categories:

(a) Papers published in peer-reviewed journals (N/A for none)

Received	Paper
02/15/2017	2 Erik J. Liu, Andrew Sinclair, Andrew J. Keefe, Brent L. Nannenga, Brandon L. Coyle, François Baneyx, Shaoyi Jiang. EKylation: Addition of an Alternating-Charge Peptide Stabilizes Proteins, Biomacromolecules, ( ): 3357. doi:
TOTAL:	**1**

Number of Papers published in peer-reviewed journals:

(b) Papers published in non-peer-reviewed journals (N/A for none)

Received	Paper
TOTAL:	

Number of Papers published in non peer-reviewed journals:

(c) Presentations

Number of Presentations: 0.00

Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Received Paper

TOTAL:

Number of Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Peer-Reviewed Conference Proceeding publications (other than abstracts):

Received Paper

TOTAL:

Number of Peer-Reviewed Conference Proceeding publications (other than abstracts):

(d) Manuscripts

Received Paper

TOTAL:

Number of Manuscripts:

Books

Received Book

TOTAL:

Received	Book Chapter

TOTAL:

Patents Submitted

Patents Awarded

Awards

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Total Number:	

Names of Post Doctorates

NAME	PERCENT_SUPPORTED
Xuewei Xu	0.25
Jean-Rene Ella-Menye	0.35
FTE Equivalent:	**0.60**
Total Number:	**2**

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FTE Equivalent:	
Total Number:	

Names of Under Graduate students supported

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FTE Equivalent:	
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NAME

Total Number:

Names of personnel receiving PHDs

NAME

Total Number:

Names of other research staff

NAME	PERCENT_SUPPORTED

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Total Number:

Sub Contractors (DD882)

Inventions (DD882)

Scientific Progress

See the attached report

Technology Transfer

ARO Project Report (63926-LS- 249 II)
Revised on 2/15/2017
Principle Investigator (PI): Prof. Shaoyi Jiang, University of Washington, Seattle

Summary
This work established the use of polypeptides composed of repeating glutamic acid (E) and lysine (K) (poly(EK)) for the purpose of increasing protein stability. The addition of poly(EK) to the primary sequence at the C-terminus of a model β-lactamase protein and a destabilized TEM-19 demonstrated significant ability in increasing protein stability while maintaining bioactivity. The use of fusion protein products allows for a one-step protocol resulting in a homogeneous product, a significant advantage over polymer-based approaches.

Introduction
Among polymers conjugated to proteins, poly(ethylene glycol) (PEG) has been widely used to enhance protein stability, however, these stability enhancements come at the expense of bioactivity, biodegradability, or immunogenicity.[1-3] Particularly of note is increasing evidence suggesting antibodies against PEG, reducing the efficacy of protein-based therapeutics.[4] PEGylation has been used extensively over the past several decades, but several alternatives to PEG have been devised to address issues that have arisen through the use of PEG.[5-8]
Previous work in the PI's group has utilized zwitterionic poly(carboxybetaine) (pCB) and demonstrated its superhydrophilic and ultra low-fouling properties compared to PEG.[9,10] Chemical conjugation of pCB to chymotrypsin has prevlously demonstrated the ability to increase thermal and chemical stability of proteins in addition to increasing the protein's substrate affinity.[11] Further work in the PI's group has focused on poly(EK), as the positively-charged side group on lysine and the negatively-charged side group on glutamic acid present poly(EK) as a polypeptide analogue to pCB. These poly(EK) sequences have also been used to demonstrate zwitterionic nonfouling characteristic when applied to surfaces and particles.[12-14] Additionally bioinformatics studies have shown the predominance at which these charged E and K residues occur in similar amounts on protein surfaces, suggesting that these charged residues play an essential role in maintaining protein stability.[14] The naturally-derived poly(EK) material is ideally suited for medical applications due to its protein-mimicking properties, suggesting improved biocompatibility.[15] It also allows for poly(EK) to be synthesized utilizing several methods, including solid-phase peptide synthesis and genetic engineering techniques. In this work proteins are modified with poly(EK) utilizing both techniques. The use of solid-phase peptide synthesis allowed for chemical conjugation of poly(EK) to proteins whereas genetic engineering allowed for poly(EK) to be directly incorporated into the primary sequence of the resulting fusion protein at the protein terminus.

Results and Discussion

The initial approach utilized solid-phase peptide synthesis and chemical conjugation of the resulting poly(EK) to chymotrypsin. Peptide synthesis resulted in 5 kDa poly(EK) chains consisting of alternating E and K residues and an N-terminal cysteine residue. Chymotrypsin was modified with a bifunctional crosslinker targeting the surface amines on the protein, resulting in free maleimide groups that would target the free thiol on the cysteine residue of poly(EK) in a subsequent step. However, chemical conjugation of poly(EK) to chymotrypsin requires more complex chemistries than fusion proteins via genetic engineering. The use of solid-phase peptide synthesis makes it difficult to synthesize large polymers compared to using traditional polymerization techniques. Furthermore this approach still results in a heterogeneous product with batch-to-batch variation as all other chemical conjugation methods do. In parallel, additional work also supported by this ARO funding was performed to study the genetic engineering approach. ARO was acknowledged for financial support in the final publication[16]. The primary advantages to this approach over chemical conjugation is that it allows for a one-step expression of a homogeneous product, with the ability to generate poly(EK) tails of greater size. This method is also able to take advantage of well-established techniques in cloning and protein expression and purification. The model protein β-lactamase(Bla) and a destabilized mutant TEM-19 (TEM) had their C-termini modified with 10 kDa and 30 kDa poly(EK) polypeptides.[17] The resulting protein fusion products were conferred beneficial properties by the poly(EK) tails; notably the ability to increase stability while maintaining bioactivity. The addition of 10 kDa poly(EK) tails allowed for both enzymes to maintain quantitative bioactivity, while the addition of 30 kDa poly(EK) to Bla and TEM maintained 70 and 60 percent of native bioactivity, respectively. Furthermore, the addition of poly(EK) increased the substrate affinity of the enzymes. Appending 10 kDa and 30 kDa poly(EK) to Bla reduced the Michaelis-Menton constant K_m from 70 to 45 and 25 μM, respectively. This notable increase in substrate affinity coupled with the maintenance of bioactivity greatly increases the catalytic efficienty (k_{cat}/K_m) of the enzymes.

Additionally the addition of poly(EK) greatly enhanced thermal and salt stability, as shown in Figures **1** and **2**.The addition of 10 kDa poly(EK) increased the transition midpoint temperature by approximately 15°C without affecting the shape of the unfolding curve, whereas the addition of 30 kDa poly(EK) increased the transition midpoint temperature by approximately 20-30°C and exhibited a markedly different unfolding curve, particularly increasing stability at extreme temperatures. These results observed through using different sizes of poly(EK) indicate that there is a trade-off between enzymatic activity at moderate temperatures and increasing stability at more extreme conditions. This is further demonstrated through incubation at 95°C for an hour, as 30 kDa poly(EK)-modified protein retained over 30% of enzymatic activity, maintaining nearly three times of its inherent bioactivity when compared to the wild-type Bla enzyme. Increased stability was also demonstrated in high salt conditions, with 30 kDa poly(EK)-modified enzyme maintaining 80% activity after incubation in 2M NaCl at

90°C for an hour, compared to the complete elimination of enzymatic activity of the wild-type enzyme.

Conclusions and Future Work

This work demonstrated a new approach in increasing protein stability through the use of poly(EK) through the use of genetic engineering by generating fusion protein products. Poly(EK) was developed as a natural polypeptide analogue to pCB, and exhibited similar low-fouling properties. The use of genetic engineering was able to maintain enzymatic activity while increasing protein stability under stressful thermal and saline conditions. The poly(EK) polypeptide was encoded into the primary sequence of the enzymes at their C-terminus, and allowed for the recombinant expression of a well-defined protein fusion product. Various poly(EK) lengths were studied, and it was shown that shorter poly(EK) chains confer additional stability without impacting bioactivity whereas longer poly(EK) chains sacrifice a small amount of bioactivity in exchange for significant gains in thermostability. Furthermore the addition of poly(EK) was found to decrease K_m, demonstrating increased protein-substrate affinity. The improved stability properties coupled with bioactivity maintenance and enhanced kinetics are consistent with previous results observed through the conjugation of pCB to proteins. These marked improvements demonstrated through the use of a well-defined method shows the promise of poly(EK) as a material for improving protein properties in areas including drug delivery and protein therapeutics.

Further work will focus on further developing and characterizing the system. As Bla is a model system, a more advanced system will be used for further studies. These more advanced systems will also allow for *in vivo* characterization for poly(EK), including studying poly(EK) pharmacokinetics and biocompatibility. It is also desirable to develop poly(EK) for addition to the N-terminus in order to modify proteins with active sites or other sensitive structures near the C-terminus with poly(EK) with minimal detrimental effects. Currently the structural properties of poly(EK) are not well-understood, and further investigations to study the effects of poly(EK), including using circular dichroism to study secondary structure and molecular dynamics simulations to study interactions between poly(EK) and its surroundings, are needed.

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Figures

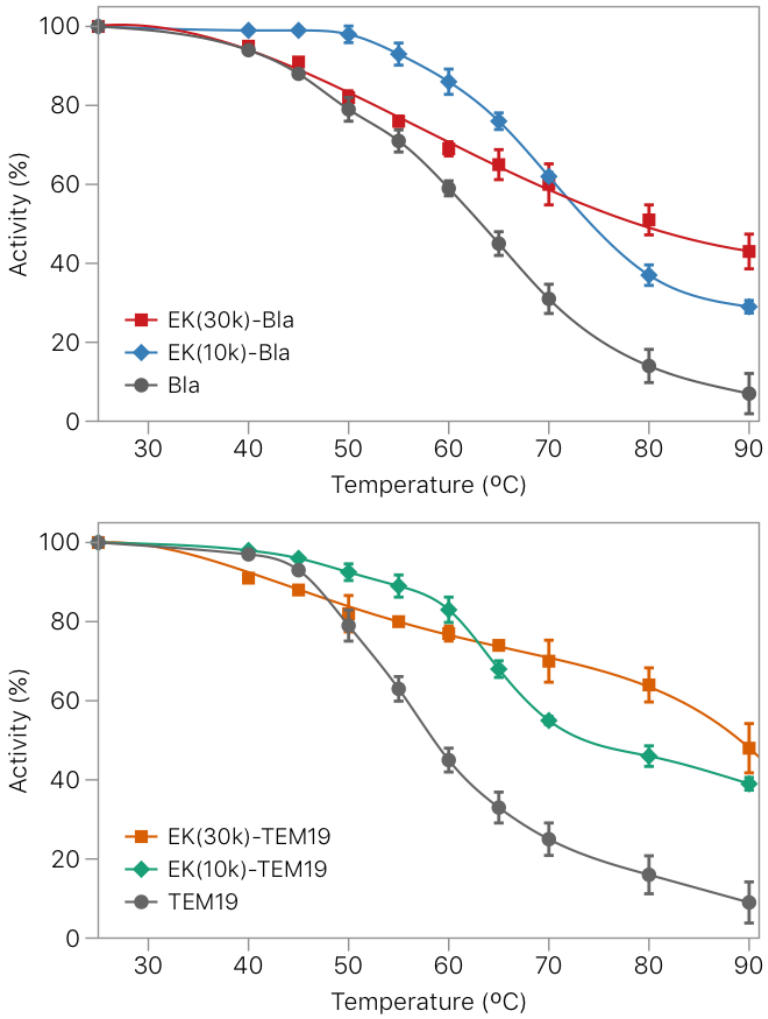

Figure 1. Relative thermal stability of Bla fusion products (top) and TEM fusion products (bottom). For both enzymes, the 10 kDa poly(EK) modification conferes a similar stability benefit throughout, while the EK(30k) modification increases stability under more extreme conditions above T_m. Curves are drawn as a visual guide.

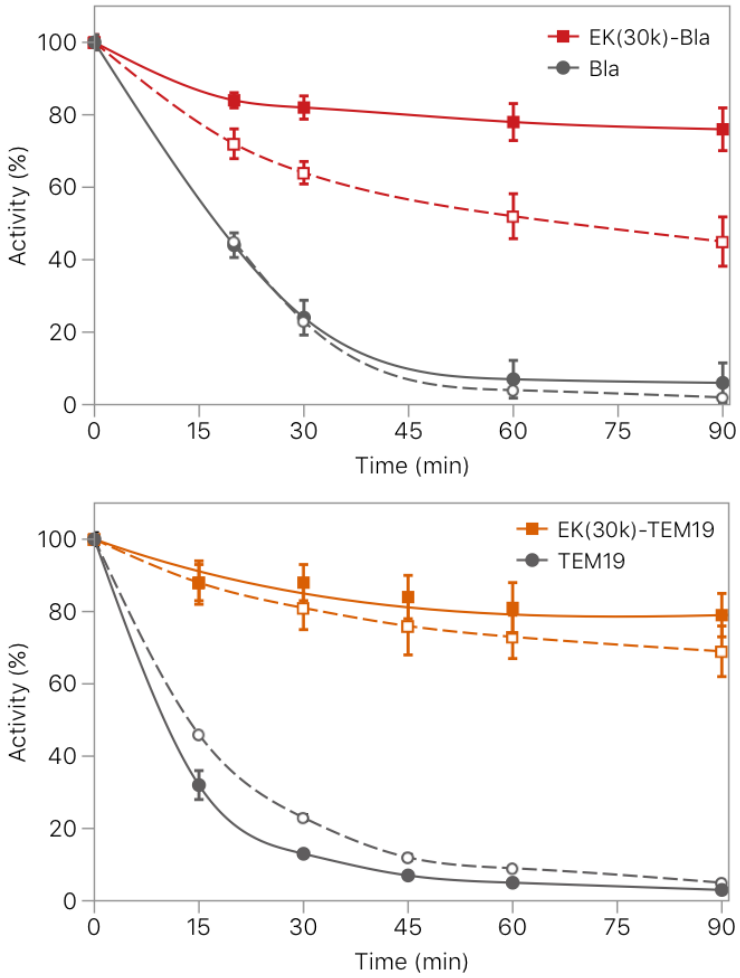

Figure 2. Relative temperature stability of Bla and 30 kDa-Bla (top) and TEM and 30 kDa-TEM (bottom), as activity retention after incubation under high salt (2M, filled data points) and normal salt (20mM, open data points) conditions at 80°C. Similar substantial activity loss is seen for unmodified proteins under both conditions, while proteins containing poly(EK) retain high activity, especially in high salt. Curves are drawn as a visual guide