Supporting Information

**Next generation salivary lubrication enhancer derived from recombinant supercharged polypeptides for xerostomia**

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Materials and Methods

*Cloning/Gene oligomerization*

The general protocol of molecular cloning can be found in a previous report[¹]. Information about the genes and the respective amino acid sequences of the monomeric K variant containing Cys are shown in **Figure S1**. In brief, the SUP monomer gene was excised from the pJET1.2 vector by digestion with *PflmI* and *BglII* and run on a 1% agarose gel in TAE buffer (per 1L, 108 g Tris base, 57.1 mL glacial acetic acid, 0.05 M EDTA, pH 8.0). The band containing the gene was excised from the gel and purified using the QIAGEN spin column purification kit. A host vector pJET1.2 containing the monomer gene fragment was digested with *PflmI* and dephosphorylated. The vector was purified by agarose gel extraction following gel electrophoresis. The linearized pJET1.2 vector and the SUP-encoding gene were ligated and transformed into chemically competent DH5α cells (Stratagene, Cedar Creek, TX) according to the manufacturer’s protocol. Cells were plated and colonies were picked and grown overnight in LB medium supplemented with 100 µg/mL ampicillin, and plasmids were isolated using the GeneJET Plasmid Miniprep kit. Positive clones containing the doubled gene fragment were verified by plasmid digestion with *PflmI* and *BglII* and subsequent gel electrophoresis. The DNA sequence of putative inserts was further verified by DNA sequencing (GATC, Konstanz, Germany). Further oligomerization was performed similarly with the procedure above, which is termed recursive directional ligation and was developed by Chilkoti and co-workers[²].

*Expression vector construction*
The pET25b(+) expression vector was digested with EcoRI and NdeI, dephosphorylated and purified using a micro-centrifuge spin column kit. The repetitive SUP gene was excised from the cloning vector with the same enzymes and purified by agarose gel extraction following gel electrophoresis. The linearized vector and SUP-encoding gene were ligated with T4 ligase (Thermo Scientific), transformed into DH5α competent cells and screened as described above. The constructs of pET25b-SUP were verified first by EcoRI and NdeI digestion and then sent for DNA sequencing.

Polypeptide expression and purification

E.coli BLR (DE3) cells (Novagen) were transformed with the pET25b expression vectors containing the respective ELP genes. For polypeptide production, Terrific Broth medium (for 1 L, 12 g tryptone and 24 g yeast extract) enriched with phosphate buffer (for 1 L, 2.31 g potassium phosphate monobasic and 12.54 g potassium phosphate dibasic) and glycerol (4 mL per1 L TB) and supplemented with 100 µg/mL ampicillin, was inoculated with an overnight starter culture to an initial optical density at 600 nm (OD600) of 0.1 and incubated at 37°C with orbital agitation at 250 rpm until OD600 reached 0.7. Polypeptide production was induced by a temperature shift to 30°C. Cultures were then continued for additional 16 h post-induction. Cells were subsequently harvested by centrifugation (7,000 x g, 30 min, 4°C), re-suspended in lysis buffer (50 mM sodium phosphate buffer, pH 8.0, 300 mM NaCl, 20 mM imidazole) to an OD600 of 100 and disrupted with a constant cell disrupter (Constant Systems Ltd., DaventryNorthants, UK). Cell debris was removed by centrifugation (25,000 x g, 30 min, 4°C). Polypeptides were purified from the supernatant under native conditions by Ni-sepharose chromatography. Product-containing fractions were pooled and dialyzed against ultrapure water and then purified by anion exchange chromatography using a Q HP column. Purified products were frozen in liquid nitrogen, lyophilized and stored at -20°C until further use.

Product Characterization
The concentrations of the purified polypeptides were determined by measuring absorbance at 280 nm using a spectrophotometer (Spectra Max M2, Molecular Devices, Sunnyvale, CA). Product purity was determined by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% polyacrylamide gel. Afterwards, gels were stained with Coomassie staining solution (40% methanol, 10% glacial acetic acid, 1 g/L Brilliant Blue R250). Photographs of the gels after staining were taken with a LAS-3000 Image Reader and the resulting images are shown in Figure S2 (Fuji Photo Film GmbH, Düsseldorf, Germany).

**Mass Spectrometry**

Mass spectrometric analysis was performed using a 4800 MALDI-TOF/TOF Analyzer in the linear positive mode. The polypeptide samples were mixed 1:1 v/v with α-cyano-4-hydroxycinnamic acid matrix (SIGMA) (100 mg/mL in 70% ACN and 0.1% TFA). Mass spectra were analyzed with the Data Explorer V4.9 (shown in Figure S3). Values determined by mass spectrometry are in good agreement with the masses that are calculated based on the amino acid sequence (shown in Table S1).

**Figure S1.** The gene and amino acid sequence of the monomer used in this study. Two cysteines flank both N- and C-terminus of the gene of interest.
Figure S2. Protein samples used in this study characterized by SDS-PAGE. M, Protein ladder. Lane 1 is K72, Lane 2 is K108, Lane 3 is K144, Lane 4 is K108cys and Lane 5 is K144cys. Lane 6 is K108cys with dithiothreitol (a reducing chemical agent), which prevents dimerization. Black arrows indicate monomeric and dimerized bands of K108cys. The amount of dimer was quantified to around 30% compared to the amount of monomer. Grey arrows show monomer and dimer bands of K144cys exhibiting a ratio of 50% to 50%. The electrophoresis behavior of supercharged proteins is different compared to conventional proteins under denaturing SDS-PAGE conditions due to their excessive amount of charges.
Figure S3. MALDI-TOF mass spectra of SUPs used in this study.

Table S1. Molecular mass analysis of supercharged polypeptides used in this study.

| Peptide | Sequence | M<sub>w</sub> calculated * (Da) | M<sub>w</sub> ms#(Da) | Average M<sub>w</sub> available during lubrication$ |
|---------|----------|-------------------------------|---------------------|---------------------------------|
| K72     | GAGP[(GVGVP)(GKGVP)]_{9}GWPH<sub>6</sub> | 36313                       | 36347 ± 50          | 36347 ± 50                      |
| K108    | GAGP[(GVGVP)(GKGVP)]_{12}GWPH<sub>6</sub> | 53870                       | 53782 ± 100         | 53782 ± 100                     |
| K108cys | CGAGP[(GVGVP)(GKGVP)]_{16}GWPH<sub>4</sub>C | 54167                       | 54118 ± 100         | 54118(70%)+ 108234(30%)         |
| K144    | GAGP[(GVGVP)(GKGVP)]_{16}GWPH<sub>6</sub> | 71294                       | 71321 ± 150         | 71321 ± 150                     |
| K144cys | CGAGP[(GVGVP)(GKGVP)]_{16}GWPH<sub>4</sub>C | 71860                       | 71826 ± 150         | 71826(50%)+ 143650(50%)         |

*average molecular weight calculated with ProtParam tool
#molecular weight determined by MALDI-TOF mass spectrometry
$ molecular weight calculated taking dimerization into consideration (MALDI TOF + SDS-PAGE)
Figure S4 XPS analysis elemental composition of S-SCF treated with buffer or different SUPs. O\textsubscript{1s} photo-electron peaks of S-SCF adsorbed to crystal surface with or without SUP treatment are decomposed in two compounds i.e. O=C-N involved in amide groups and glyco group (C-O-H) \cite{3} which could be calculated by integral of O\textsubscript{1s} at 532.7 ev.

Table S2 Elemental composition of S-SCF treated with buffer, K72, K108, K144, K108cys, and K144cys. ± indicates standard deviation over three measurements.

|     | S-SCF with buffer | S-SCF with K72 | S-SCF with K108 | S-SCF with K144 | S-SCF with K108cys | S-SCF with K144cys |
|-----|------------------|----------------|-----------------|-----------------|--------------------|-------------------|
| C   | 60.35±2.05       | 57.4±3.5       | 58.73±1.5       | 47.83±1.24      | 45.35±1.9          | 49.46±8.8         |
| N   | 11.22±0.37       | 9.2±2.2        | 9.68±0.8        | 9.21±1.85       | 8.49±0.6           | 7.8±2.7           |
| O   | O\textsubscript{total} 18.56±1.12 | 17.8±2.9       | 18.88±1.4       | 22.18±5.2       | 23.99±3.5          | 23.51±7.6         |
|     | %O\textsubscript{532.7}∗O\textsubscript{total} 4.8±0.3 | 6.4±1.0        | 7.74±2.4        | 9.99±1.9        | 11.49±0.6          | 10.88±2.3         |
| S   | 1.16±0.03        | 0.7±0.13       | 0.99±0.75       | 1.3±0.8         | 1.57±0.4           | 2.92±1.9          |
| Cl  | 3.46±0.16        | 7.3±5.5        | 3.53±0.09       | 5.98±2.9        | 8.21±0.9           | 3.99±2.4          |
| P   | 2.37±0.53        | 5.4±4.0        | 4.35±2.8        | 5.76±5.5        | 4.86±3.5           | 4.47±2.2          |
| Na  | 2.9±0.31         | 3.07±0.04      | 3.4±1.5         | 2.76±1.6        | 5.02±3.5           | 4.5±1.2           |
Figure S5. Surface topography of the surfaces as imaged by AFM under tapping mode. a) Bare Au-coated crystal. b) S-SCF treated with buffer. c) S-SCF treated with K72. d) S-SCF treated with K108. e) S-SCF treated with K144. f) S-SCF treated with K108cys. g) S-SCF treated with K144cys. h) Height as a function of width of the globular structures found in different S-SCF.

Figure S6 Zeta potentials of the SCFs in absence and presence of adsorbed SUPs. Silica spheres (diameter 1.7 μm) were coated with SCF by suspending in saliva for 2 h. Subsequently, the spheres were suspended in buffer or recombinant K72, K108, K144, K108cys, and K144cys solutions (0.05% w/v) for 2 min. After this coating step, the spheres were rinsed with buffer for 10 min. The zeta potential of the different spheres was measured in buffer (2 mM potassium phosphate, 1 mM CaCl$_2$, 50 mM KCl, pH 6.8) employing a Zetasizer nano series (Model Number ZEN3600, Malvern Ltd, UK). Error bars represent the standard deviation over three measurements with separately coated spheres. *Statistically significant (p<0.05) differences in zeta potential of SCF exposed to SUPs with respect to SCF exposed to buffer. # Significant differences (P<0.05) in zeta potential of SCF exposed to K108cys and K144cys with respect to K72, K108 and K144, respectively.

References

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