

1 Thermo-responsive, self-assembling biointerface for on demand release of 2 surface-immobilised proteins

3 Angela Saccardo,^a Mikhail Soloviev^b and Enrico Ferrari^{*a}

4 a. School of Life Sciences, University of Lincoln, Lincoln LN6 7TS, UK.

5 b. Department of Biological Sciences, Royal Holloway University of London, Egham TW20 0EX, UK.

6 * Address correspondence to eferrari@lincoln.ac.uk

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9 Abstract

10 Dedicated chemistries for on-demand capture and release of biomolecules at the solid-liquid interface
11 are required for applications in drug delivery, for the synthesis of switchable surfaces used in analytical
12 devices and for the assembly of next-generation biomaterials with complex architectures and
13 functions. Here we report the engineering of a binary self-assembling polypeptide system for
14 reversible protein capture, immobilisation and controlled thermo-responsive release from a solid
15 surface. The first element of the binary system is a universal protein substrate immobilised on a solid
16 surface. This protein is bio-inspired by the neuronal SNAP25, which is the protein involved in the
17 docking and fusion of synaptic vesicles to the synaptic membrane. The second element is an artificial
18 chimeric protein engineered to include distinct domains from three different proteins: Syntaxin, VAMP
19 and SNAP25. These native proteins constitute the machinery dedicated to vesicle trafficking in
20 eukaryotes. We removed approximately 70% of native protein sequence from these proteins and
21 constructed a protein chimera capable of high affinity interaction and self-assembly with immobilised
22 substrate. The interaction of the two parts of the engineered protein complex is strong but fully-
23 reversible and therefore the chimera can be recombinantly fused as a tag to a protein of interest, to
24 allow spontaneous assembly and stimuli-sensitive release from the surface upon heating at a
25 predetermined temperature. Two thermo-responsive tags are reported: the first presents remarkable
26 thermal stability with melting temperature of the order of 80°C; the second disassembles at a
27 substantially lower temperature of about 45°C. The latter is a promising candidate for remote-
28 controlled localised delivery of therapeutic proteins, as physiologically tolerable local increase of
29 temperatures in the 40-45°C range can be achieved using magnetic fields, infra-red light or focused
30 ultrasound. Importantly, these two novel polypeptides provide a broader blueprint for the engineering
31 of future functional proteins with predictable folding and response to external stimuli.

32 Introduction

33 Controlled release is increasingly studied in the context of protein therapeutics, for which
34 spatiotemporal- and dosage-controlled delivery would be desirable.¹ Controlled release of biologics
35 such as recombinant proteins would be particularly beneficial to enhance their therapeutic efficacy,
36 as they often present limited half-life and immunogenicity issues. Although improving the therapeutic
37 window can be achieved by a slow and prolonged release of drugs using passive delivery systems, a
38 triggerable drug delivery system that can respond to stimuli could control drug release more
39 effectively.² To address this, many remotely triggerable and stimuli-responsive materials have been
40 developed for the design of novel drug delivery systems.^{3,4} Stimuli-responsive interfaces have been
41 engineered to respond to either physiological changes (e.g., pH, redox potential, glucose
42 concentration, specific enzymatic activity) or external stimuli, such as electric or magnetic fields,
43 mechanical forces (e.g., ultrasound mediated), light and heat.

44 Thermo-responsive materials are particularly promising due to the range of technologies that are
45 already available to increase local temperatures on demand: i. focused ultrasound-mediated
46 cavitation of microbubbles can heat tissue above 56°C within seconds;² ii. tissue-penetrating near
47 infrared light can be used to deliver local heat via nanomaterial-mediated photothermal effect;⁵ iii.
48 magnetic fields can be used to increase temperatures in the presence of iron oxide nanoparticles, like
49 those used in magnetic resonance imaging.⁶ As a consequence of multiple ways to deliver controlled
50 heat locally, thermo-responsive materials such as hydrogels and polymers presenting sharp phase
51 transition temperatures have been synthesised and used to encapsulate or cross-link drugs, including
52 therapeutic proteins.¹ Although thermo-responsive interfaces have been described mostly for their
53 potential use in drug delivery, biomaterials that respond to thermal stimuli have also been applied in
54 other contexts. Examples include protein affinity purification,⁷ rapid diagnostic assays,⁸ remotely-
55 controlled protein pores,⁹ and remote control of enzymatic activity.¹⁰

56 To enable dynamic and reversible structural change or release of proteins from surfaces, several
57 protein or peptide conformational switches have been studied.¹¹ Bio-mimetic conformational
58 switches have been engineered from natural systems, such as barnacle adhesive proteins-inspired
59 self-assembling peptides,¹² or *de novo* designed from canonical and predictable structural motifs such
60 as α -helical coiled-coils and β -hairpins.¹³ Protein conformational switches can present on/off
61 biomolecular recognition ability that can be triggered to release or capture another protein on
62 demand, in response to external stimuli such as ion concentration¹⁴ or temperature.¹⁵⁻¹⁷

63 Protein conformational switches are particularly relevant for the design of switchable surfaces and
64 interfaces with programmable functionalities.¹⁶ Smart and functional materials that can be switched

65 remotely will be increasingly used in applications beyond drug delivery and they have been already
66 applied to control cell adhesion,¹⁸ assemble nanomaterials on surfaces,¹⁹ control biomolecular
67 interactions for analytical devices¹⁵ and set the on/off state of bio-valves between catalytic nanoscale
68 compartments.²⁰

69 We aimed to devise a binary protein complex capable of spontaneous self-assembly, on-demand
70 disassembly in response to physical stimulus and re-assembly (Figure 1). We draw inspiration from
71 neuronal SNARE complex, comprising three proteins: Syntaxin, SNAP25, and VAMP (Figure 2A).²¹ The
72 complex assembles via the formation of a parallel coiled-coil of four α -helices, two contributed by
73 SNAP25 (green in Figure 2A) and one each by Syntaxin and VAMP2 (red and blue respectively in Figure
74 2A). The two helices of SNAP25 are kept together by a \sim 70 amino acid long linker that spans from the
75 C-terminal of the first helix to the N-terminal of the second (unstructured, not represented in Figure
76 2A and shown as a sketch in Figure 3A). Syntaxin presents extra N-terminal domains with regulatory
77 function and a C-terminal transmembrane domain for anchoring to the cell membrane (both not
78 represented in Figure 2A and shown as a sketch in Figure 3A). VAMP2 presents a C-terminal
79 transmembrane domain which, in the living organism, is embedded in the membrane of synaptic
80 vesicles. The domains that assemble into the tight and stable coiled-coil represented in Figure 2A are
81 referred to as SNARE motifs: they are \sim 60 amino acids long and behave as unstructured proteins in
82 solution. The SNARE motifs adopt stable α -helical structures only upon assembly with the other SNARE
83 domains, driven by the alignment of several hydrophobic layers towards the core of the complex.²¹
84 The native SNARE proteins retain their ability to form supramolecular complexes *ex vivo* and *in vitro*,
85 but being a ternary system, the native SNAREs do not translate easily into biotechnology
86 applications.^{22–24} We attempted to re-engineer two of the three native SNAREs to yield a simple and
87 robust binary protein system. One of the two elements of the new system is capable of oriented and
88 permanent immobilisation at a solid-liquid interface through an added glutathione S-transferase (GST)
89 domain.²⁵ A new complementary protein was therefore created by combining Syntaxin SNARE domain
90 with that of VAMP using a designer linker made of a sequence of unstructured amino acids. We then
91 tested whether this newly engineered component retained the ability to spontaneously and
92 specifically self-assemble with the immobilised partner. We also tested whether it can be released in
93 response to a local increase of temperature. The disassembly temperature of the engineered complex
94 could be adjusted by as much as \sim 40°C by altering the polypeptide length of a single SNARE domain.²⁴
95 To illustrate the thermo-responsive release of immobilised recombinant proteins from solid surfaces
96 we tested two distinct polypeptide “tags” and showed that the immobilised tagged proteins are
97 released selectively in a temperature dependent manner. The low temperature tag unfolds and
98 disassembles at $T_m=42.5^\circ\text{C}$, whilst the high-temperature tag remains bound up to $T_m=79.6^\circ\text{C}$.

99 Importantly, the released tags do not aggregate, making possible the regeneration of the interface.
100 The low-temperature immobilisation system enables the decoration of dynamically switchable bio-
101 interfaces and it is suitable for the immobilisation of proteins or enzymes that can be released on
102 demand by a mild local increase of the temperature.

103 **Results and discussion**

104 **Reductionist re-engineering of ternary SNARE complexes yields temperature-** 105 **sensitive protein assembly**

106 First, we used minimised SNARE helices and their native ternary SNARE protein architecture to check
107 our hypothesis that thermal stability of the tetrahelical assembly can be regulated by adjusting just
108 one of the α -helices. Recombinant SNAP25, Syntaxin and VAMP2 (VAMP2-L) were expressed and
109 purified in *E. coli* and were used to characterise the thermal stability of the full-length SNARE complex
110 in solution (Figure 2). To adjust the thermal stability of the complex while preserving the structural
111 features that allow self-assembly, a shortened VAMP2 was synthesised (VAMP2-S). Whereas both
112 VAMP2-L and Syntaxin were designed to span the entire SNARE motif (54 amino acids), VAMP2-S was
113 only 25 amino acids long, with the C-terminal truncated just before the ionic layer. A rationale for the
114 truncated design is presented in Supplementary Figure 1.

115 The assembly of SNARE proteins mixtures was analysed using Synchrotron Radiation Circular
116 Dichroism (SRCD) (Figure 2B). This was enabled by the fact that individual SNARE proteins are
117 unstructured in solutions whereas the ternary complex formed by SNAP25, Syntaxin and VAMP2 is a
118 highly structured coiled-coil. The far-UV SRCD spectra of both complexes containing SNAP25, Syntaxin
119 and either VAMP2-L or VAMP2-S revealed α -helical structure. VAMP2-L complex yielded a more
120 intense CD signal at the same molar concentration of VAMP2-S complex, consistent with longer
121 structured α -helical domains. Unstructured individual SNARE proteins and binary mixtures from
122 control tests (Supplementary Figure 2) confirmed that the minimalist, engineered, ternary SNARE
123 complexes retain fundamental properties of the native SNARE complex, i.e, the ability of SNARE
124 domains to interact tightly only when four of them form a helical bundle.²⁶

125 Temperature stability of the assembled protein complex was strongly affected by the length of the
126 VAMP2 fragment used. Whilst both VAMP2-L and VAMP2-S complexes showed fully folded α -helical
127 structure at 25°C, their stability at elevated temperatures was clearly very different. Melting
128 temperature of the VAMP2-S complex was reduced compared to that of VAMP2-L complex by ~37°C
129 (Figure 2C).

130 Engineering of thermo-responsive, chimeric, binary protein complexes

131 The results of Figure 2 informed the design of SNARE-derived immobilisation “tags” that can respond
132 to different ranges of temperatures, with VAMP2-L and VAMP2-S suitable to be used for high- and
133 low-temperature release respectively. The full potential of an immobilised protein system would
134 benefit from being binary rather than ternary, so that one protein could be immobilised on a surface
135 of interest and the thermo-responsive tag for controlled release fused to a protein of interest.²² To
136 retain the thermal stability properties of the full-length and shortened SNARE complex, while reducing
137 it to a binary system, we extensively re-engineered the native SNAREs by generating chimeras
138 comprising sequences from all three proteins (Figure 3A). VAMP2-L (V-L) and VAMP2-S (V-S) were
139 fused to Syntaxin (S) in tandem and separated by the polypeptide linker natively found between the
140 helices of SNAP25 protein, to allow the assembly of these two helices in a parallel configuration (Figure
141 3B). The new VS recombinant proteins were engineered to have either 54 amino acid domain (VS-L)
142 or 25 amino acid long domain (VS-S), and they were successfully expressed in *E. coli* and purified.

143 HH was engineered to contain shortened SNARE domains from SNAP25 so that the α -helices would
144 be precisely 54 amino acids long and consistent with the longest domains of VS-L and VS-S. Since the
145 native domain architecture of HH remained unchanged, we also retained the native linker domain
146 (Figure 3A) connecting the two domains. The new HH was successfully expressed in bacteria.

147 To assess the assembly properties of the new binary SNARE mimics we used SRCD and we found that
148 HH assembles with either VS-L or VS-S to form α -helices similarly to their ternary counterpart (Figure
149 3C). The far-UV SRCD spectra typical of α -helices were observed only where the pairs were combined,
150 whereas no α -helices were detected with individual proteins, suggesting that the new fusions are also
151 unstructured in solution unless assembled in a complex with specific partners, which is a feature
152 inherited from native SNAREs (Supplementary Figure 4). The melting temperature of the full-length
153 and shortened binary complexes was studied using SRCD temperature scans and the values obtained
154 were 79.9°C and 43.3°C for HH/VS-L and HH/VS-S respectively (Figure 3D), which are very similar to
155 the values obtained for the corresponding ternary complexes. To prove that the stability of the
156 complex depends on the truncation of the V domain rather than the nature of the linker domain, we
157 tested an entirely different linker, made of glycine and serine residues only, to connect V with C-
158 terminal S domains. Far-UV SRCD spectra and temperature scans of the new full-length VS-L2
159 confirmed that there was no substantial difference between SV-L and SV-L2 (Supplementary Figure 5).
160 Together, these results confirm that the new binary protein complexes retain the ability to self-
161 assemble in solution and possess thermal stability properties similar to that of the ternary protein
162 complexes.

163 Thermo-responsive release of surface-immobilised proteins

164 HH was immobilised on porous Sepharose beads and the assembled VS-L and VS-S complexes' stability
165 was studied at different temperatures. Sepharose beads derivatised with glutathione (GSH) were used
166 to facilitate immobilisation of HH via a recombinant Glutathione S-Transferase (GST) tag fused at the
167 N-terminal of HH (Figure 4A). The high affinity and remarkable stability of the GST/GSH interaction,
168 combined with the high solubility of the GST tag, makes this system suitable for robust immobilisation
169 of recombinant proteins on GSH-modified resins or other surfaces, such as plates, biosensors,
170 magnetic beads. As GST also has high affinity to gold, this can also be used for direct decoration of
171 gold nanoparticles.²⁵

172 The ability of the GST-HH modified surface to specifically capture VS-L and VS-S was assessed using a
173 pull-down experiment. This was performed using Sepharose beads functionalised with either GST only
174 (negative control) or GST-HH. Upon incubation with VS followed by extensive washing, Sepharose-GST
175 was unable to bind VS-L or VS-S, whereas Sepharose-GST-HH could distinctively pull-down VS-L and
176 VS-S, suggesting specific binding via HH/VS interaction and no background binding contributed by the
177 GST tag or the surface (Figure 4B). As SDS-PAGE only provides a qualitative or semi-quantitative
178 estimation of the amount of protein captured by the beads, VS-L and VS-S were fluorescently labeled
179 to allow quantification of the captured protein. Labeling was achieved by chemical cross-linking Cy5-
180 maleimide to the thiol group of a cysteine residue deliberately introduced between the V domain and
181 the flexible linker of both VS-L and VS-S (Supplementary Figure 6). Sepharose-GST-HH was incubated
182 with an excess of fluorescent VS (VS-L-Cy5 and VS-S-Cy5), similarly to the pull-down described above.
183 This time, fractions of the Sepharose-GST-HH/VS complexes were incubated at a set temperature and
184 unbound protein removed using a washing buffer at the same incubation temperature. The residual
185 Cy5 fluorescence intensity measured on the Sepharose beads represents the proportion of VS still
186 bound to the surface at any tested temperature. The results of the pull-down performed at different
187 temperatures show that both VS-L-Cy5 and VS-S-Cy5 are released from the resin upon incubation at
188 80°C. However, a substantial proportion of VS-L-Cy5 remained bound to the surface at 65°C, whilst
189 the bound VS-S-Cy5 dropped steeply and virtually none was still bound to the beads at the same
190 temperature, with the majority released below 50°C (Figure 4C). The reduction of fluorescence
191 intensity observed between 20°C and 35°C is due to the well documented temperature dependence
192 of fluorescence quantum yield of carbocyanide dyes.²⁷

193 To test whether the system can work like an ideal thermo-responsive switch and capture another
194 payload after a release cycle, an alternative payload was synthesised by conjugation of Cy3 to VS-S
195 (VS-S-Cy3, Supplementary Figure 7). The Sepharose-GST-HH beads were first exposed to VS-S-Cy3,

196 then heated at 50°C for 20', washed and exposed to VS-S-Cy5 after cooling at 20°C. The beads were
197 imaged using confocal microscopy and successful exchange of payload was confirmed by the sharp
198 change of fluorescence emission (Figure 4D).

199 The ability of Sepharose-GST-HH/VS-S-Cy5 system to preserve capture and release properties upon
200 several cycles of heating at 50°C and cooling at 20°C was tested for up to 4 cycles (Figure 4E). The
201 results show no substantial changes in the behaviour of the interface, most likely due to the
202 unstructured nature of SNARE-derived peptides that do not aggregate even at higher temperatures.
203 We also tested the ability of Sepharose-GST-HH/VS-L-Cy5 system to withstand cycles of heating at
204 80°C and cooling at 20°C, and we observed that a significant amount of fluorescence intensity was lost
205 after one cycle, but the remaining constructs were stable over 5 cycles. We attributed this to partial
206 desorption of the incompletely folded recombinant GST portion of the recombinant protein at higher
207 temperatures (Supplementary Figure 8). The correctly folded GST remains on beads and no further
208 change in fluorescence is detectable. This is also consistent with the trend of the VS-L-Cy5 release
209 observed in Figure 4C, which increases at a higher rate above 50°C, although still exhibiting a marked
210 difference with the release trend of the shorter VS-S-Cy5.

211 Finally, we tested the release rate of Sepharose-GST-HH beads loaded with VS-S-Cy3 at three different
212 temperatures: 20, 37 and 45°C. At the lowest temperature, only background intensity of Cy3
213 fluorescence was measured in the buffer surrounding the beads after 25 minutes. A mild increase of
214 VS-S-Cy3 release was observed at 37°C (body temperature), suggesting that a delivery system based
215 on this technology would have only moderate off-target loss of the payload. The release at 45°C
216 (hyperthermia temperature) was instead substantial within the first 10 minutes and was complete
217 after 20 minutes, suggesting sustained payload release happens above the engineered threshold
218 temperature (Figure 5).

219

220 The pull-down results and release curves together confirmed that, upon immobilisation, the thermo-
221 responsive HH/VS interaction preserves the interesting thermal stability properties found in solution
222 and, therefore, can be used as an effective way to release proteins from a surface in response to a
223 local temperature increase. No irreversible protein aggregation was observed, unlike other common
224 protein-protein interactions for which extensive optimisation is required to limit aggregation
225 propensity.²⁸ The refolding ability of proteins at the interface and the regeneration of the surface
226 following subsequent cycles of capture and release was tested successfully, suggesting that the
227 interactions involved are fully reversible and therefore compatible with dynamic and programmable
228 surfaces involving biomolecules.¹⁶

229 Importantly, informed engineering of the coiled-coil structure of the neuronal SNARE complex allowed
230 modulation of the disassembly temperature down to physiologically attainable conditions for *in vivo*
231 applications. Research into stimuli-responsive nanomaterials suggested many approaches to
232 engineering environmentally controlled molecular systems. Temperature and pH are the two stimuli
233 most physiologically relevant to drug delivery and release applications. Such systems are typically
234 based on temperature-sensitive spontaneously aggregated or polymerised molecules which undergo
235 depolymerisation or disassembly stimulated by the change in the external conditions. One group of
236 applications rely on spontaneously formed hydrogels which entrap typically small molecule drugs for
237 delayed, sustained or environmentally dependent release.^{29,30} Another category relies on micellar
238 delivery systems which are typically used for the delivery of poorly soluble low molecular weight (LMW)
239 therapeutics and for sustained drug delivery in diverse scenarios, including controlled delivery of
240 multiple drugs for synergistic effect, as elegantly exemplified by Emamzade *et al.*³¹ Another common
241 approach relies on self-assembled nanoparticles, optionally with added stimuli sensitivity, such as
242 Redox or enzyme sensitive nanoparticles.³²⁻³⁵ Polymeric carriers are especially suitable for payloads
243 such as conventional organic drugs which are physically entrapped in the polymer networks. Such
244 entrapment is strongly affected by the size and physical properties of the LMW drugs, and typically no
245 defined molecular ratio of polymer to drug could be specified. In contrast, the described system, which
246 relies on engineered interacting proteins, includes defined molar ratio of the captured protein to the
247 molecular anchor and is likely unaffected by the size or nature of the protein payload. The high
248 specificity and affinity of the interaction between the two protein components exceeds that of typical
249 polymers by far and the stimuli-sensitivity of their interaction can be engineered in a rational manner
250 as described in this paper. Another key distinction is that our system allows multiple rounds of re-
251 assembly, whilst hydrogels, micelles and nanoparticle-based systems assume single use; whilst that
252 may not have implications in therapeutic applications, the reversibility of the protein interface
253 provides additional advantages for biotechnology applications. Importantly, the proteinaceous nature
254 of the SNAREs mimic-based system reported here makes it particularly suitable for the thermo-
255 responsive release of proteins, thus expanding the use of thermo-responsive drug delivery towards
256 the vast arsenal of bio-therapeutics. We envisage that combining the thermo-responsive capabilities
257 described here with designer nanocarriers will be especially useful for the development of
258 hyperthermia-directed protein therapeutics.³⁶ Elsewhere, peptide-lipid hybrid nanoscale thermo-
259 responsive vesicles were tested *in vivo* and successful release of doxorubicin following hyperthermia
260 treatment was observed.³⁷ Magnetic hyperthermia, which makes use of iron oxide nanoparticles to
261 induce local increase of temperature, is clinically approved in Europe for the treatment of

262 glioblastoma³⁸ and it represents a promising approach for magneto-responsive local release of
263 therapeutics, towards which the thermo-responsive protein interface described here may contribute.

264 The use of thermo-responsive, nanoparticle-based systems for local delivery is not limited to magnetic
265 materials activated by alternate magnetic fields but, for example, nanomaterials with the ability to
266 extensively absorb energy in the near-infrared (NIR) spectrum can be used to trigger a localised and
267 controlled increase of temperature *in vivo*, as radiation in this window has minimal absorbance by
268 tissues and can penetrate to a depth of micrometers to centimeters, making photo-thermal drug
269 release possible.² Dedicated nanomaterials such as gold nanorods with a strong absorbance in the NIR
270 range have been developed and their ability to trigger conformational changes on gold-conjugated
271 biomolecules in response to NIR irradiation has been previously reported *in vitro* and *in vivo*.^{39,40} We
272 believe that the thermo-responsive protein release tag properties reported here make it a suitable
273 interface for magneto-responsive and photo-thermal therapy.

274 **Experimental**

275 Cloning, protein expression, purification and analysis

276 DNA inserts were purchased as synthetic genes (Dundee Cell Products) and introduced into pGEX-KG
277 GST gene fusion system (Addgene). The resulting sequences are reported in Supplementary Table 1
278 and secondary structure data are presented in Supplementary Figures 2 and 3. All plasmids were
279 expressed in BL21(DE3)pLysS *E.coli* strain (Thermo Fisher Scientific); the expressed recombinant
280 proteins were purified by affinity chromatography using Glutathione Sepharose 4B resins (GE
281 Healthcare). The GST tag was removed by thrombin cleavage, with the exception of GST and GST-HH
282 for which the tag was preserved. Fluorescently labeled proteins were incubated with an excess of Cy5-
283 or Cy3-maleimide at this stage. All proteins were further purified by size exclusion chromatography
284 using ÄKTA Pure chromatography system (GE Healthcare) equipped with a Superdex 75 10/300 GL
285 column. All proteins were stored and analysed in 100 mM NaCl, 20 mM HEPES pH 7.3. Protein
286 concentration was determined using BCA assay (Thermo Fisher Scientific). SDS-PAGE was performed
287 using 12% RunBlue SDS protein gels (Expedeon), stained using InstantBlue (Expedeon) and imaged
288 using a ChemiDoc imaging system (Biorad). Alternatively, an Odyssey Imaging System (LI-COR
289 Biosciences) was used for visualisation of fluorescently labeled protein. Quantification of fluorescent
290 protein in solution or immobilised on beads was done using an Infinite 200 Pro plate reader (TECAN).

291 Synchrotron radiation circular dichroism

292 Far UV SRCD spectra were recorded at the B23 beamline for SRCD of the Diamond Light Source (UK)⁴¹
293 using protein solutions at the concentrations indicated in the figure legends diluted in 25 mM NaCl, 5
294 mM HEPES pH 7.3 in a rotating cylindrical cuvette with 0.2mm optical path (Starna) at 20°C. SRCD
295 spectra of thermal unfolding were recorded from the same solutions in the 25-95°C temperature
296 range with an interval of 2.5°C. Temperature was raised at the rate of 1°C per minute and the solutions
297 were allowed to equilibrate for 2 min before collecting the spectra. The thermal unfolding curves were
298 plotted by selecting the data points at 222 nm over the range of temperatures scanned.⁴² The melting
299 temperature (T_m) of the complexes was obtained by interpolation of the temperature at which half of
300 the signal was lost. All SRCD spectra were processed using the software CDApps.⁴³

301 Protein pull-down and controlled release

302 5 μ M GST-HH and 7.5 μ M VS were incubated at room temperature for 2 hours with Glutathione
303 Sepharose 4B beads (GE Healthcare) under constant agitation. Excess protein was removed by
304 repeated centrifugation and washing using 100 mM NaCl, 20 mM HEPES pH 7.3. Total protein load
305 was assessed following complete denaturation in hot RunBlue LDS sample buffer (Expedeon) followed
306 by SDS-PAGE using 12% RunBlue SDS protein gels (Expedeon), stained using InstantBlue (Expedeon)
307 and imaged using a ChemiDoc imaging system (Biorad). Controlled release was obtained by incubation
308 of the GST-HH/VS beads at a set temperature for 20 minutes, followed by a wash at the same
309 incubation temperature. The beads for confocal imaging were prepared in the same way, but exposed
310 to VS-S-Cy3 first and to VS-S-Cy5 after release at 50°C and cooling at 20°C. Repeated heating and
311 cooling cycles were performed on 5 different sets of solutions, each subjected to 0 to 4 cycles as
312 described for the pull-down above before analysis of retained protein was performed. To obtain
313 release curves, Sepharose-GST-HH beads were loaded with VS-S-Cy3 using the same approach above.
314 Three fractions were diluted 5 times and incubated at 20, 37 and 45°C. The beads were quickly
315 sedimented by centrifugation every 5 minutes and the concentration of VS-S-Cy3 released into the
316 buffer was assessed. Quantification of retained fluorescent protein or protein fluorescence released
317 in solution was conducted using an Infinite 200 Pro plate reader (TECAN).

318 Confocal microscopy

319 Images were obtained using a Leica TCS SP8 confocal microscope (20X objective) from VS-S-loaded
320 Sepharose-GST-HH beads dispersions on a glass coverslip. False colour scanning images were obtained
321 using 552 nm laser excitation and 554-630 nm emission window (Cy3 channel) and 638 nm laser and
322 650-750 nm emission (Cy5 channel).

323 **Conclusions**

324 The results reported here show that modulation of the release temperature of engineered SNARE
325 complexes can be achieved by changing the length of just one SNARE motif which could also be fused
326 to Syntaxin SNARE motif to form a binary interaction complex. The engineered protein interface can
327 be expressed by recombinant means and its thermo-responsive properties adjusted within a broad
328 range of temperatures in the 40-80°C range. To fully exploit the potential of this new protein interface,
329 the immobilisation on surfaces other than GSH-crosslinked Sepharose beads should be explored
330 further. For example, GST could be replaced by available peptides with affinity to specific materials or
331 the SNAREs directly linked to the surface using available chemical bioconjugation methods.^{44,45} Once
332 fused to thermo-responsive VS proteins, other recombinant proteins could be potentially immobilised
333 and released from HH-modified materials, irrespective of HH's immobilisation method. On the other
334 hand, HH-activated materials could be used for the immobilisation and release of any VS-tagged
335 recombinant protein, highlighting the modularity and flexibility of the system described here. It is
336 likely that thermo-responsive SNARE-derived tags will have a role in future design of interfaces with
337 programmable, protein-mediated functions

338 **Conflicts of interest**

339 There are no conflicts to declare.

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419 **Figure legends**

420 **Figure 1.** Schematic of the reversible, thermo-responsive protein release system. (A) Reversible
421 temperature sensitive release of a payload (e.g., therapeutic protein) in response to heat activation
422 of a molecular thermal switch (T) which opens at temperatures above $\sim 45^{\circ}\text{C}$; the system can be loaded
423 again by dropping the temperature below the threshold. (B) Engineering of the molecular thermal
424 switch: the self-assembling SNARE proteins ternary complex has been re-engineered into a new binary
425 protein complex capable of sequence specific self-assembly. Tuning the temperature stability of the
426 complex is achieved by modifying the interface region (T). The complex re-assembles if cooled to
427 below the temperature threshold.

428 **Figure 2.** Length of SNARE motif affects thermal stability of the SNARE complex. (A) Structural
429 representation of the SNARE complex (based on PDB ID: 1SFC). The structures highlighted in colour
430 indicate the portions of SNARE motifs that have been used in this work: blue indicates 54 or 25 amino
431 acid long VAMP2 motifs, Syntaxin protein is highlighted in red and SNAP25 motifs in green. The parts
432 of the ribbons highlighted in yellow correspond to the ionic layer. (B) Far-UV SRCD spectra of VAMP2-
433 L and VAMP2-S complexes, each molecule at $2\ \mu\text{M}$ concentration, $\text{pH}=7.3$, 20°C . The peak at 222 nm
434 is highlighted and indicates the wavelength that was used to quantify unfolding and disassembly
435 shown in panel C. (C) CD temperature scan of VAMP2-L and VAMP2-S SNARE complex. The data
436 represent unfolding expressed as % of increase of CD signal at 222 nm over a value of 100 extrapolated
437 to infinite temperature. The melting temperature T_m is derived by fitting the data to a sigmoid and
438 represents the temperature value at which 50% unfolding is achieved.

439 **Figure 3.** Engineering of a binary SNARE complex with altered thermal stability. (A) Schematic of the
440 native SNARE proteins and their domains, used as building blocks for the engineering of binary artificial
441 complexes. Colour code: blue = domain V from VAMP2, red = domain S from Syntaxin, green = linker
442 and the two SNARE domains from SNAP25. Cylinders represent α -helices, the yellow rectangle
443 represents the membrane into which the SNARE proteins are embedded via an α -helical
444 transmembrane domain (VAMP2 and Syntaxin) or palmitoylated cysteine residues (4 zig-zag segments
445 on the SNAP25). The amino acid numbers of the domains effectively used for the engineering of the
446 binary complexes are displayed near the coloured domains, which also correspond to the colour coded
447 highlights of Figure 2A. (B) Schematic of the recombinant SNARE mimics. Cylinders represent SNARE
448 domains highlighted in panel A, whereas the green lines correspond to the naturally occurring linker
449 between the two α -helices of SNAP25 with four cysteine residues mutated into alanines (orange dots,
450 also highlighted by arrows in the HH sketch) and one introduced cysteine residue exposing a free thiol
451 group (SH) for site directed labelling (yellow dot in VS-L and VS-S). (C) Far-UV SRCD spectra of binary

452 complexes (8 μ M of each molecule, pH=7.3, 20°C) and (D) temperature scans have been obtained as
453 in Figure 2 and resulted in very similar assembly and disassembly properties.

454 **Figure 4.** Assembly of binary SNARE complexes on GSH activated Sepharose beads and thermo-
455 responsive release of VS. (A) Schematic of HH immobilisation strategy via GST/GSH interaction. The
456 structure of GST is based on PDB ID 1UA5. (B) SDS-PAGE of the pull-down of VS-L and VS-S by
457 functionalised Sepharose beads. Lanes 1 and 2 show eluates from Sepharose-GST incubated with VS-
458 L and VS-S respectively: for both lanes, only a band corresponding to GST was visible (28.6 kDa,
459 indicated by the arrow on the right), suggesting that there is no specific interaction of VS with GST or
460 the surface. Lanes 3 and 4 show eluates from Sepharose-GST-HH incubated with VS-L and VS-S
461 respectively: besides the bands corresponding to GST-HH protein (45.3 kDa, indicated by the arrow),
462 one intense extra band per lane was detected, corresponding to the mass of VS-L protein (21.3 kDa,
463 lane 3) and VS-S protein (17.2 kDa, lane 4), suggesting specific binding via HH/VS interaction. The lane
464 with the protein marker (PM) allows comparison of the relative mass of the proteins loaded on the
465 SDS-PAGE (relative masses of the standards are listed on the left). (C) Residual fluorescence intensity
466 from Sepharose-GST-HH bound VS-L-Cy5 and VS-S-Cy5 after incubation at different temperatures.
467 Data points and error bars represent the average of three measurements and standard error
468 respectively. (D) Confocal microscopy of representative Sepharose-GST-HH beads exposed to VS-S-
469 Cy3 first (upper panels), regenerated by heating and exposed to VS-S-Cy5 (lower panels). Left and right
470 panels show the fluorescence at the Cy3 and Cy5 emission wavelengths respectively. Scale bars are 50
471 μ m. (E) Fluorescence intensity of captured VS-S-Cy5 after Sepharose-GST-HH was heated and cooled
472 for 0 to 4 cycles. Average of three measurements, error bars represent the standard error.

473 **Figure 5.** Release of VS-S-Cy3 into the buffer upon incubation at three representative temperatures of
474 loaded Sepharose-GST-HH beads.

Figure 1

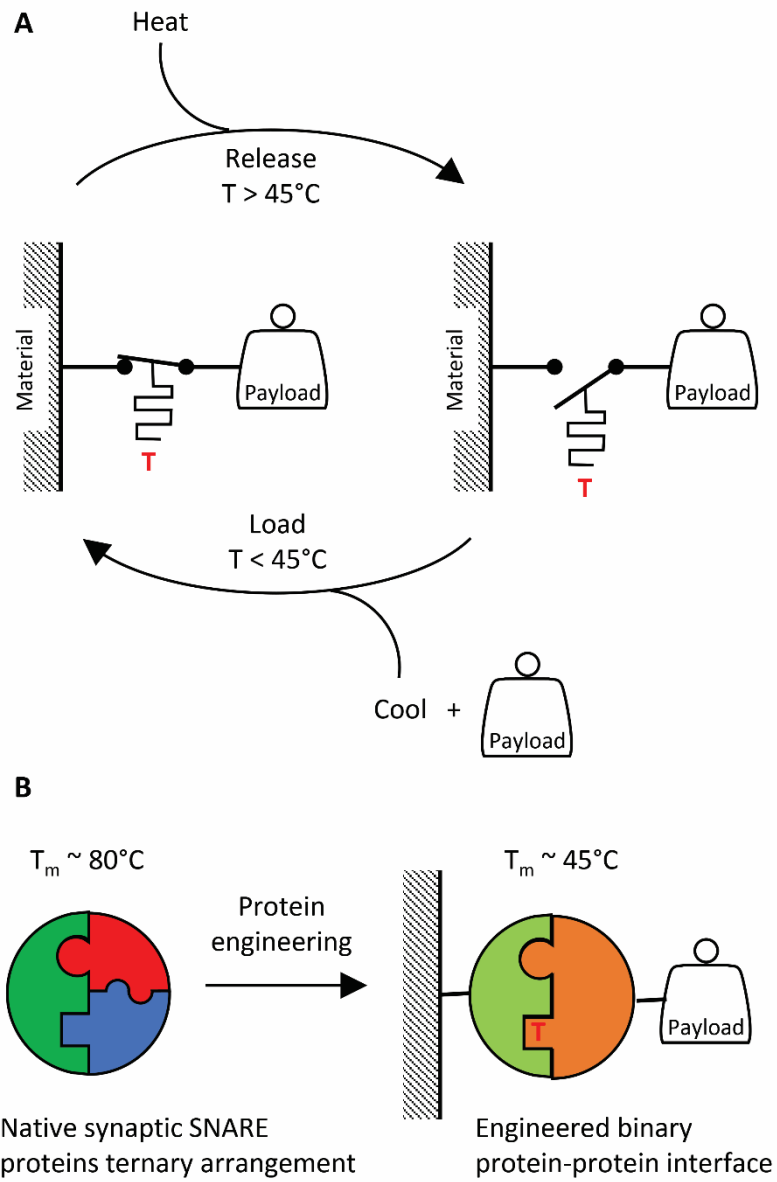


Figure 2

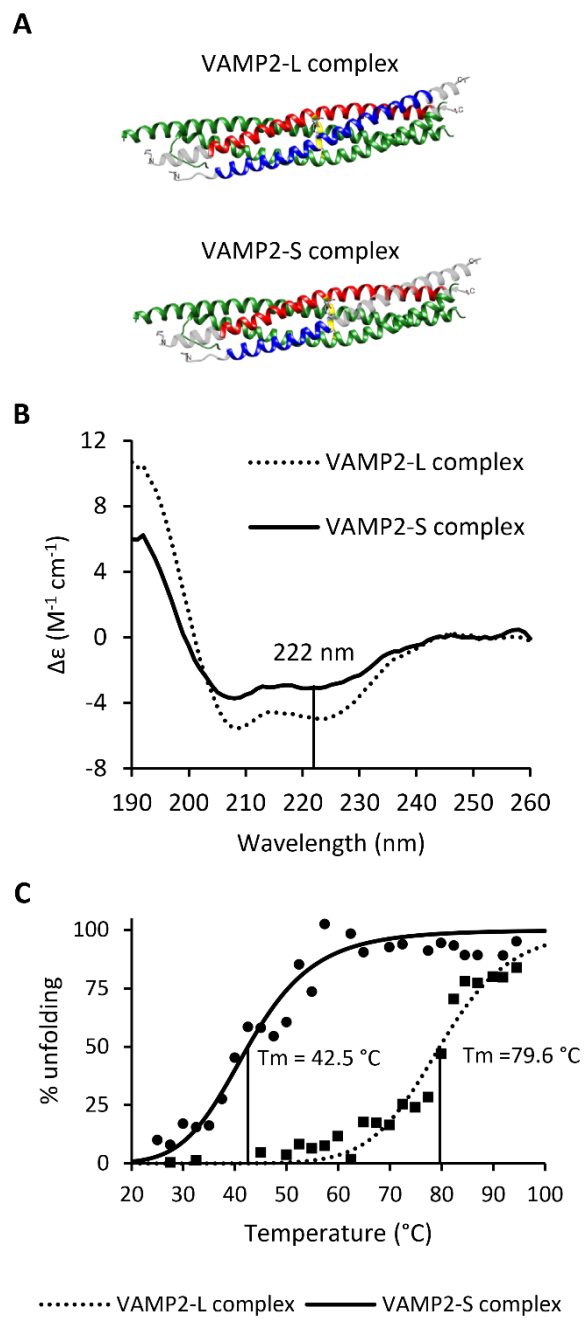


Figure 3

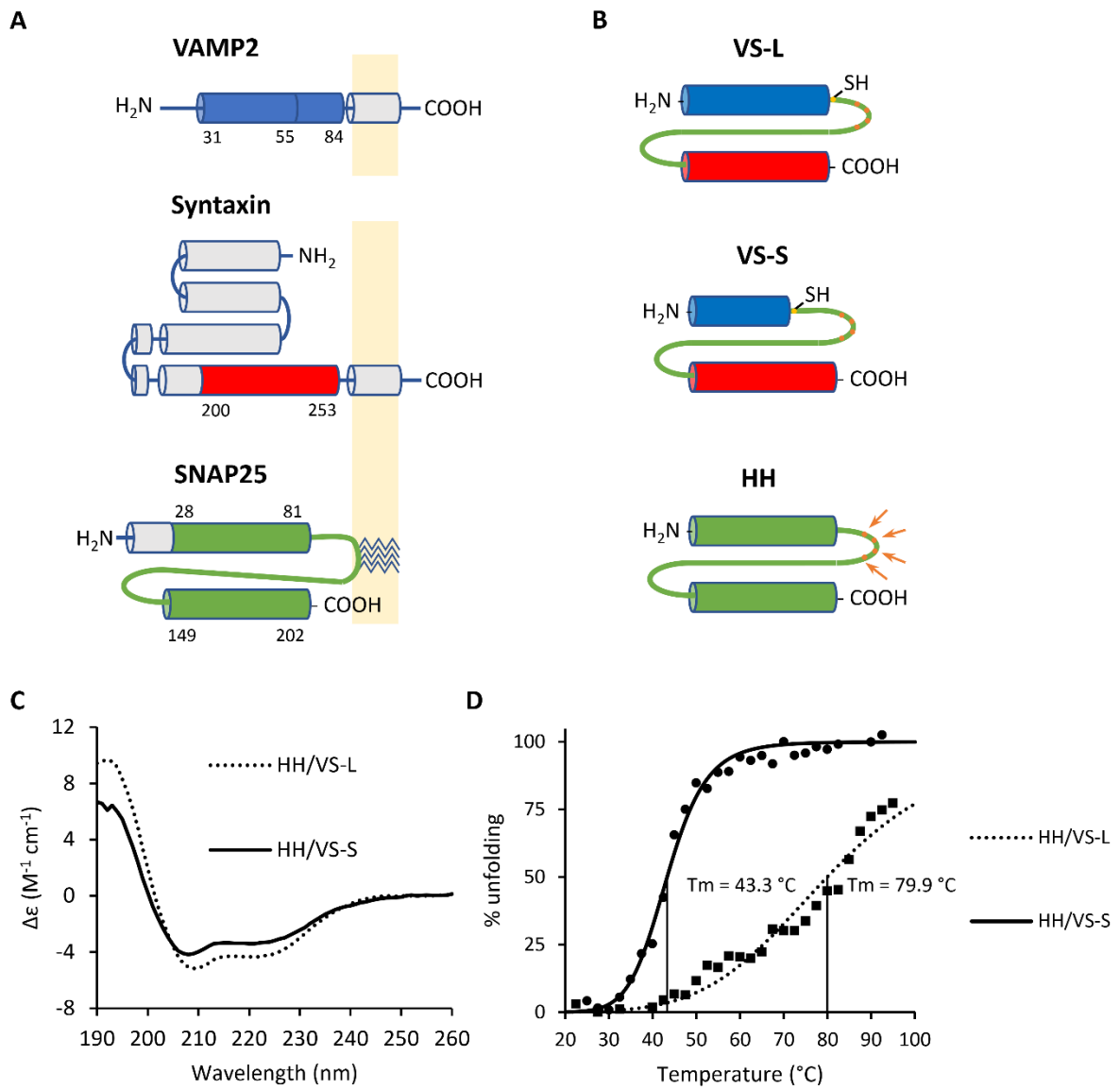


Figure 4

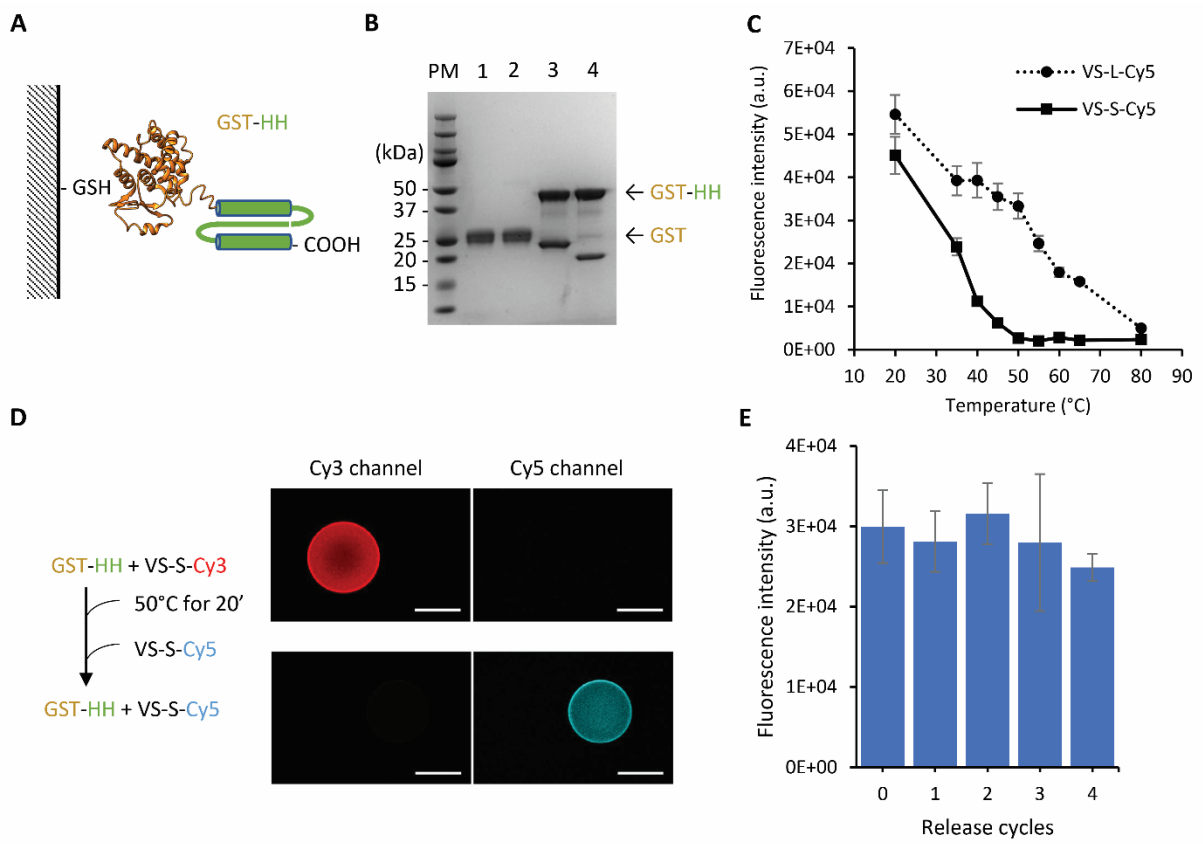


Figure 5

