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## **Coiled-coil biomaterials for biological applications**

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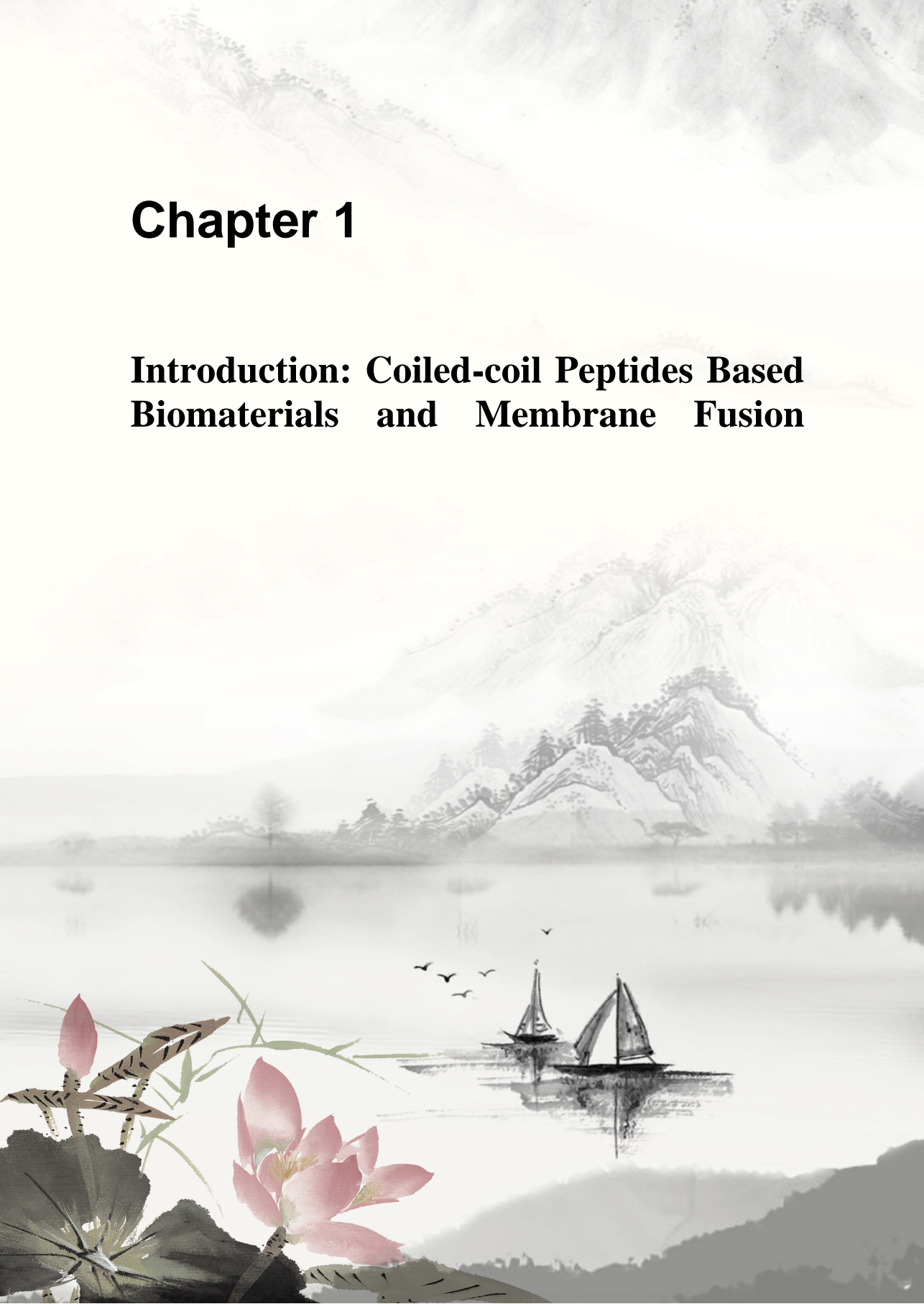
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# Chapter 1

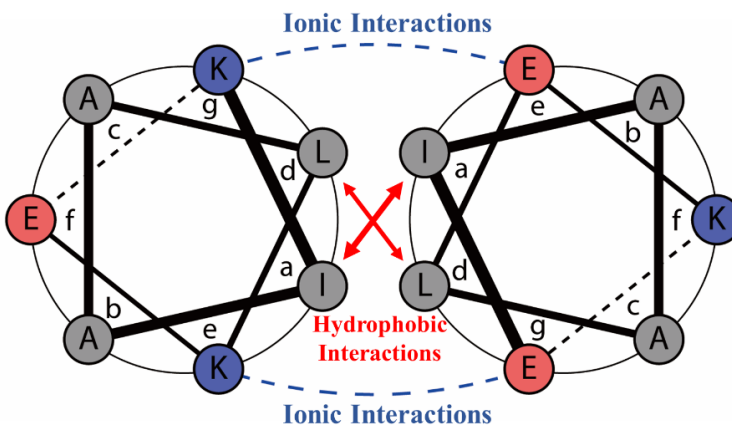
## Introduction: Coiled-coil Peptides Based Biomaterials and Membrane Fusion



## 1.1 Coiled-coil peptides

The coiled-coil motif was first named and described by Crick,<sup>1,2</sup> and Linus Pauling,<sup>3</sup> independently in 1953. After more than 60 years of research, the coiled-coil still arouses the interest of many researchers and has become more and more popular, for both fundamental and applied research.<sup>4-5</sup> The coiled-coil is a common structural motif in proteins,<sup>6</sup> with about 3-5% of amino acids in proteins forming such structures.<sup>7</sup> This motif is especially common in a large class of fibrous proteins like tropomyosin,<sup>8-9</sup> myosin,<sup>10-11</sup> intermediate filaments,<sup>12-13</sup> and prokaryotic surface proteins,<sup>14-15</sup> where the coiled-coil has been described as the main structural element.

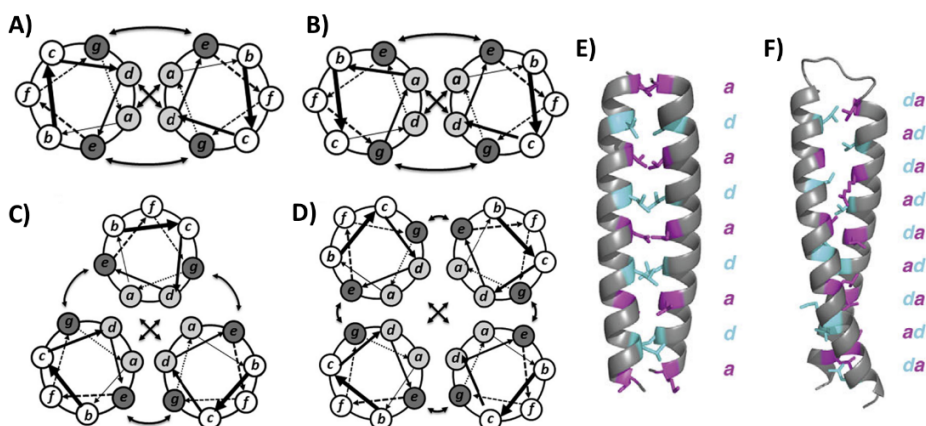
A coiled-coil contains two or more  $\alpha$ -helices which wrap around each other and form a left-handed supercoil. Crick described the structure as having “knobs-into-holes” packing since the  $\alpha$ -helices twist, at an angle of around  $20^\circ$ , with respect to each other, which interlocks the side chains of the amino acids on neighbouring peptide chains.<sup>6-7</sup> The reason for this twist is that, in  $\alpha$ -helices, each complete turn of a helix usually contains 3.6 residues, while in a coiled-coil there is a lower number of residues in each turn - 3.5. A repeat of seven residues, which is known as a heptad repeat, is common in many coiled coils. In that case, each heptad in a coiled-coil contains two helical turns. This heptad repeat is usually denoted as  $(abcdefg)_n$  where each letter represents each position of the coiled-coil and ‘n’ is the number of repeats. Heptad repeats can be shown using a helical wheel diagram (Figure 1).



**Figure 1.** Helical wheel diagram of a parallel heterodimeric coiled-coil.

Figure 1 shows an example of a parallel heterodimeric coiled-coil. In such a structure, positions *a* and *d* usually contain hydrophobic amino acids, thus forming the non-polar core of the  $\alpha$ -helix. Therefore, hydrophobic interactions and van der Waals interactions could induce coiled-coil formation in an aqueous solution. Positions *e* and *g* normally have amino acids containing complementary charged side chains and then the coiled-coil structure can be stabilized by interhelical electrostatic interactions. Since positions *b*, *c* and *f* are exposed to the solvent, hydrophilic amino acids are preferred to increase the solubility of the peptide. Previously, these positions were thought to be less important for defining the properties of the coiled-coil, however, some recent studies have shown that *b*, *c* and *f* residues can affect coiled-coil specificity and stability.<sup>16-17</sup>

Most coiled coils, both from nature or developed via artificial design, contain two to four helices and these structures have cyclic or dihedral symmetry.<sup>18</sup> However, coiled coils containing five or more helices have also been reported in numerous studies.<sup>18-21</sup> Figures 2A-2D show helical wheel diagrams of different structures of coiled coils; those with different oligomeric states or orientations.<sup>5</sup> All of these structures present ‘knobs-in-holes’ interactions (figure 2E & 2F).



**Figure 2.** Differently structured coiled-coils: (A) parallel dimeric coiled-coil; (B) antiparallel dimeric coiled-coil; (C) parallel trimeric coiled-coil; (D) parallel tetrameric coiled-coil. The ‘knobs-in-holes’ arrangement of a parallel coiled-coil (E) and an antiparallel coiled-coil (F). The parallel coiled-coil contains *a* and *d* layers while the antiparallel coiled-coil has mixed *ad* layers in its ‘knobs-in-holes’ arrangement. Figures are adapted from references.<sup>4-5</sup>

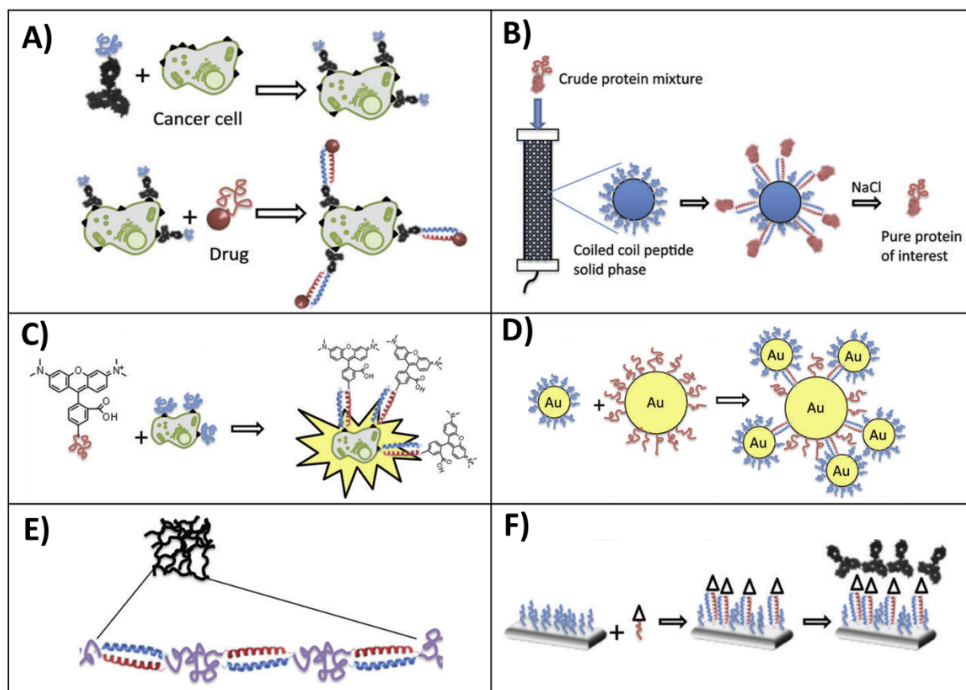
Many structural features can influence the stability of coiled coils.<sup>7, 22</sup> For example, by introducing more hydrophobic residues in the *a* and *d* positions, coiled-coil



stability can be increased. Additionally, the salt bridges between residues in *g/e'* positions can also influence the coiled-coil stability. Most importantly, increasing the length of the coiled-coil forming peptides can not only increase the area of the coiled-coil hydrophobic core but will also increase the number of salt bridges. Many studies have shown that increasing the length of the coiled-coil forming peptides is a facile way to increase the stability of coiled coil.<sup>7, 22-25</sup> Therefore, coiled-coil stability can be tuned by changing the length of the coiled-coil peptides.

## **1.2 Coiled coils for use in biomaterials**

Peptide- and protein-based biomaterials have seen rapid development over the last two decades because such biomaterials not only have potential applications in biotechnology but they can also help researchers to learn how natural biomaterials work in biology.<sup>26</sup> Coiled coils have become one of the most attractive building blocks for peptide- and protein-based biomaterials due to their excellent self-assembly properties, in addition to their specific recognition and responsiveness. The coiled-coil building block is widely used in drug delivery, protein purification, protein labeling, nanoparticle self-assembly, biosensing, and hydrogel formation, amongst others (Figure 3).<sup>5</sup>



**Figure 3.** Examples of coiled-coil peptide-based biomaterials: A) drug delivery; B) peptide purification; C) protein labelling; D) nanoparticle self-assembly; E) hydrogel formation, and; F) biosensing. The figure is adapted from reference.<sup>5</sup>

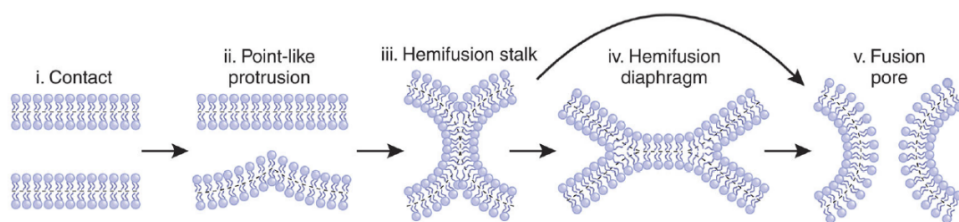
The E/K coiled coil, is a *de novo* designed parallel heterodimeric coiled-coil, developed by Hodges and coworkers.<sup>22, 27-29</sup> The coiled-coil forming peptides that make up this assembly were named peptide E and peptide K, because they are rich in glutamic acid and lysine respectively. This heterodimer, and others which are similar, have become very popular in a multitude of applications because of its stability and specificity. For example, a two-stage drug-free macromolecular therapeutic system has been developed by Wu *et al.*<sup>30</sup> In this work, the coiled-coil forming peptides E and K, were separately modified with the 1F5 anti-CD20 antibody and an HEMA copolymer, respectively. The therapeutic process contains two stages. In the first stage, peptide E modified antibodies selectively bind to CD20 on the surface of Raji B cells. Next, coiled coil formation between peptides K and E crosslinks CD20 receptors due to the action of the HEMA copolymer, resulting in apoptosis. In another study, coiled-coil motifs were used to develop a drug delivery system using the pH-sensitive nature of the K<sub>3</sub>/E<sub>3</sub> coiled coil.<sup>31</sup> Here, peptides E<sub>3</sub> and K<sub>3</sub> were attached to either a synthetic polymer or a drug/dye. By forming a coiled-coil, a polymer drug conjugate was formed which entered cells

via endocytosis. Next, these drugs could be released from the polymer complex due to the lower pH in endosomes/lysosomes compared to the rest of the cell. The pH change results in the dissociation of the  $K_3/E_3$  coiled coil and concomitant drug release.

The coiled-coil motif has also been used in various hydrogels.<sup>32-37</sup> Hill and coworkers designed a thermo-responsive hydrogel using coiled-coil domains as physical crosslinks.<sup>32</sup> Due to the reversible self-assembly properties of coiled-coil peptides, the formation and dissociation of the hydrogel can be easily controlled by adjusting the temperature. These hydrogel biomaterials can be used to control small molecule release, which also has potential applications for drug delivery.

### 1.3 Membrane fusion

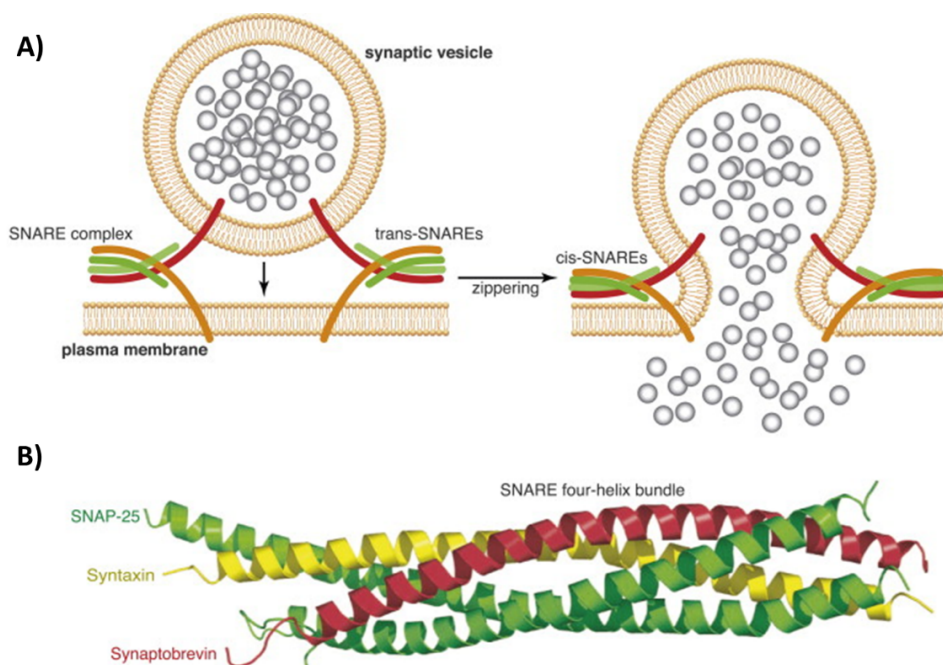
Membrane fusion is a fundamental and essential process in life.<sup>38-42</sup> It occurs between cellular plasma membranes, intracellular compartments, cellular membranes and intracellular compartments, and lipid-covered vesicles and cellular membranes.<sup>43</sup> Neurotransmitter release,<sup>44-45</sup> fertilization,<sup>46-47</sup> endocrine hormone secretion,<sup>48-49</sup> and enveloped virus infection<sup>50-51</sup> are examples of biological processes that require membrane fusion. The process of membrane fusion can be described using the fusion-through-hemifusion pathway model, which is shown in Figure 4.<sup>52</sup> This process starts from a pre-fusion contact, (i), in which two lipid membranes are forced close to each other. Because of the energy barrier caused by the hydration repulsion between the proximal leaflets of the membranes, a point-like membrane protrusion state, (ii), is formed which can lower the energy barrier for the transition into the hemifusion stalk state, (iii). The hemifusion stalk state represents the two outer leaflets fused while the inner leaflets are still unfused. The fusion pore can then either directly form from the hemifusion stalk or go through a hemifusion diaphragm state (iv) obtained by hemifusion stalk expansion. After the fusion pore, (v), is formed, complete membrane fusion is achieved. During hemifusion-stalk to fusion pore formation and expansion, high energy barriers exist because of the necessary membrane curvature deformations. Studies have shown that lipid composition influences the curvature of the lipid membrane and assists membrane deformation, thus reducing the energy barrier and facilitating membrane fusion.<sup>53-57</sup> Lipids with short and unsaturated fatty acids can increase the fluidity of the lipid membrane and a higher membrane fluidity has a positive effect on membrane fusion.<sup>58-60</sup> Therefore, the lipid composition is a very important parameter in membrane fusion.



**Figure 4.** Lipid membrane fusion via the fusion-through-hemifusion pathway. The figure is adapted from reference.<sup>52</sup>

#### 1.4 SNARE assisted membrane fusion

SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptors) proteins play a key role in mediating membrane fusion in eukaryotic cells.<sup>61-62</sup> SNARE proteins were first characterized in the late 1980s, and these SNAREs were found to mediate a variety of intracellular trafficking processes.<sup>63-64</sup> For instance, efficient membrane fusion between synaptic vesicles and presynaptic plasma membranes, a process induced by SNARE proteins, allows fast neurotransmitter release and thus efficient synaptic transmission.<sup>44-45, 65</sup> The SNARE complex contains two parts: the transport vesicle SNARE (v-SNARE) and target membrane SNARE (t-SNARE). Usually, the SNARE complex contains one v-SNARE with two or three t-SNAREs. The first identified structure of a SNARE complex in the presynaptic neuron was composed of vesicle-associated membrane protein (VAMP), syntaxin 1 and SNAP-25.<sup>66</sup> VAMP is located on the synaptic vesicle and therefore acts as the v-SNARE while syntaxin and SNAP-25 are located on the presynaptic plasma membrane and are known as t-SNAREs. VAMP and syntaxin contain transmembrane domains (TMD), which anchor them into the lipid membrane, while SNAP-25 is connected to membranes via covalent bonds between palmitoyl side chains of the membrane and multiple cysteine side chains of the protein. Some studies have shown that the TMD of the SNARE proteins within the complex could reduce the energy barrier and facilitate fusion pore formation.<sup>67</sup> By forming a four-helix coiled-coil structure (Figure 5), the v-SNARE and t-SNAREs could interact with each other and bridge two membranes thus facilitating fusion. Other SNARE proteins have similar structures and mediate membrane fusion in quite similar manners.



**Figure 5.** A) Membrane fusion induced by a SNARE complex which contains a tetrameric coiled-coil motif formed by syntaxin, and SNAP-25 on target membrane and synaptobrevin (also known as VAMP) on the synaptic vesicle. The fusion pore is formed by the coiled-coil complex ‘zipping up’ from the N- to the C-terminus and dragging the two membranes into close proximity. B) The tetramer coiled-coil complex of the SNARE protein. The figure is adapted from reference.<sup>68</sup>

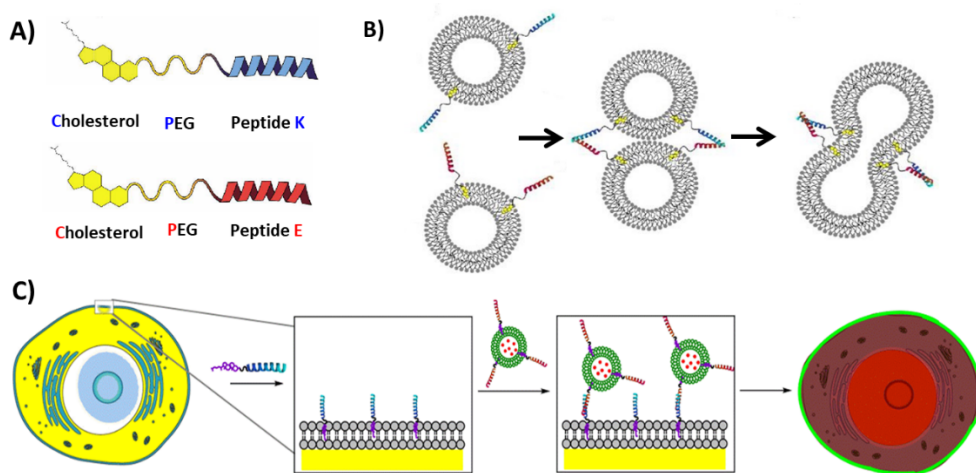
### 1.5 Membrane fusion triggered by lipid-anchored coiled coils

In our group, we have designed a coiled-coil peptide mediated membrane fusion system inspired by SNARE proteins.<sup>69-70</sup> A lipid anchor was conjugated to the coiled-coil forming peptides K and E by a short poly(ethylene glycol) (PEG) spacer (Figure 6). In one study examining the effect of the lipid anchor, cholesterol was found to be the best anchor as it triggers liposome membrane fusion with the highest efficiency.<sup>71</sup> The spacer length has also been investigated and the results show that short coiled-coil peptides prefer a longer spacer in order to reach each other and form a coiled-coil interaction, while longer coiled-coil peptides do not need such a long spacer.<sup>72</sup> The length of coiled-coil peptides was also found to play an important role in fusogenicity: four heptad coiled coils can trigger both liposome-liposome and cell-liposome membrane fusion while three heptad coiled-coils can only induce liposome-liposome membrane fusion.<sup>71, 73-74</sup> The K/E coiled-coil triggered membrane fusion system was also found to have potential



applications in drug delivery.<sup>74-76</sup>

Mechanistic studies show that the stability of the coiled-coil and the membrane affinity of peptide K are two crucial factors that affect fusogenicity.<sup>77-79</sup> As previously mentioned, several energy barriers exist during the membrane fusion process. A more stable coiled-coil can bind the two separate membranes more tightly and overcome these energy barriers more easily. Furthermore, peptide K interacts with membranes and induces a positive curvature, thereby destabilizing the membrane, lowering the energy barrier for fusion pore formation.<sup>77</sup>



**Figure 6.** Cartoon pictures illustrating A) the structure of CPK and CPE, B) liposome membrane fusion triggered by CPK and CPE, C) cell-liposome membrane fusion triggered by CPK<sub>4</sub> and CPE<sub>4</sub>.

## 1.6 Aim and outline of this thesis

In this thesis, we used the K/E coiled-coil motifs to facilitate various novel applications.

In **chapter 2**, a magnetic-activated cell sorting (MACS) system based on the K<sub>3</sub>/E<sub>3</sub> coiled-coil is described. For this project, iron oxide particles (IOPs) were functionalized with coiled-coil forming peptides and coiled-coil formation on the IOPs surface was demonstrated. Using these coiled-coil forming peptide-functionalized IOPs and an external magnetic field, cell sorting assays were performed with three different cell lines, showing that coiled-coil decorated cells can be separated from non coiled-coil decorated cells. Finally, we will show that cells that express peptide K<sub>3</sub> on the cell membrane can be enriched using this

approach.

In **chapter 3**, we compare the fusogenicity of different coiled-coil pairs formed by different lengths of K and E peptides. Using circular dichroism (CD) spectroscopy and dynamic light scattering (DLS), the secondary structure and stability of the individual K and E peptides, together with their coiled-coil complexes has been studied in order to probe the relationship between coiled-coil length and stability. We subsequently investigated the fusogenicity of these coiled coils by performing lipid-mixing and content-mixing assays using liposomes. After demonstrating their fusogenicity in liposome systems, we prove that all K-lipopeptides can be used to decorate cell membranes, and we subsequently performed liposome-cell docking and evaluated the docking efficiency by flow cytometry. Finally, we selected the coiled-coil pairs which exhibited good liposome-cell docking and used them for cell-liposome membrane fusion studies. These studies showed that liposome-loaded propidium iodide (PI) can be released into cells, and the cell-liposome fusion efficiency can be obtained by comparing the PI fluorescence intensity inside the cells.

The membrane affinity of peptide K is crucial for coiled-coil triggered membrane fusion because it can induce positive curvature of the lipid membrane, which lowers the energetic barrier and thus facilitates fusion pore formation. In **chapter 4**, we describe a membrane fusion system comprising different dimeric  $K_4$  peptides, which interact with  $E_4$  peptides resulting in membrane fusion. The secondary structure of three different  $K_4$  dimers, and their self-assembly with  $E_4$ , was investigated. These  $K_4$  dimers form either temperature-dependent aggregates or concentration-dependent homodimers in buffered solutions. Furthermore, all  $K_4$  dimers are capable of forming coiled coils with  $E_4$ . The membrane affinity of these three  $K_4$  dimers was studied by performing a tryptophan fluorescence titration assay, before the fusogenicity of different  $K_4$  dimer- $E_4$  coiled coils was examined by liposome fusion assays. Finally, we studied cell-liposome membrane fusion triggered by these coiled coils.

Since a  $K_4$  dimer- $E_4$  coiled-coil shows enhanced fusogenicity for both liposome-liposome and cell-liposome membrane fusion (**chapter 4**), in **chapter 5** we used this coiled-coil complex to trigger l-form cell-cell membrane fusion. L-forms are cell wall-deficient bacteria that can survive without their original cell wall.<sup>80-81</sup> Therefore, the study of l-form fusion may be a novel tool to study the origin of life questions.<sup>82</sup> Two phenotypes of l-forms containing different fluorescence markers

and different antibiotic resistance markers were produced. We studied the possibility of decorating l-form membranes with E<sub>4</sub> via a membrane labelling assay. Then, l-form cell-cell fusion was performed using the K<sub>4</sub> dimer-E<sub>4</sub> coiled-coil pair and the fusion efficiency was determined. In order to obtain stable l-forms expressing double markers, a fused l-form enrichment protocol was developed. After enrichment, more than 97% of double marker expressing l-forms were obtained in the total l-form population. Finally, we investigated the fused l-form cell division in medium and chromosome segregation in the solid-state.

In **chapter 6**, the main results and conclusions of the studies described in this thesis are summarized and future perspectives of coiled-coil based biomaterials are discussed.

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