Coiled-coil biomaterials for biological applications

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Chapter 6

Summary and Perspectives

Coiled coils are an important structural motif in proteins, playing an essential role in a multitude of biological processes. Moreover, the programmable assembly properties of coiled coils makes it an ideal building block for designing responsive bioactive materials. In this thesis, the heterodimeric coiled-coil pair "E/K" was used for the development of a novel cell sorting protocol and controlling membrane fusion between liposomes and complex biological membranes.

In **chapter 2**, we designed a magnetic-activated cell sorting (MACS) method based on the short coiled-coil-forming peptide pair K_3 and E_3. Divinyl sulfone modified dextran (Dextran-DVS) was synthesized and used as a coating for magnetic iron oxide particles (IOPs). This coating strategy not only makes IOP functionalization trivial but also permits the number of functional groups on the IOPs to be controlled by changing the degree of substitution (DS) of dextran-DVS. Introduction of a cysteine into peptides E_3 and K_3 enabled the facile synthesis of coiled-coil peptide-modified magnetic particles IOPs-E_3 and IOPs-K_3. Successful functionalization of the magnetic particles was demonstrated by tryptophan fluorescence measurements and an IOP surface fluorescence-labelling assay. A MACS assay was performed using both IOPs-E_3 and IOPs-K_3 and cells functionalized with the complementary lipopeptide (denoted CPE_3 and CPK_3). Using IOPs-E_3, a mixture of CPK_3-decorated cells and non-decorated cells were efficiently separated after applying an external magnetic field. The novel MACS protocol was tested on three different cell lines (Hela, CHO and NIH3T3) and high levels of selection were obtained. Another advantage of this system is that the IOPs can be easily dissociated from the cells via trypsinization resulting in separated cells with high viability. When MACS was performed using IOPs-K_3, poor cell separation was observed, likely due to electrostatic interactions between the positively-charged K_3 and the negatively-charged cell membrane. It is believed that these electrostatic interactions interfere with the cell separation process. Finally, for future experiments peptide K_3 was expressed at the cell membrane of three cell lines and IOPs-E_3 was used for separation and enrichment. Using MACS, the population of K_3 expressing cells was enriched. This study shows the advantages of using coiled coils as a novel noncovalent conjugation material for cell sorting. We believe this MACS system has potential applications in a wide range of biomedical areas, such as cell selection after genetic modification.

In **chapter 3**, we studied the fusogenicity of coiled-coil pairs as a function of peptide length. For this study, three-, four- and five-heptad variants of K and E peptides were synthesized. The secondary structure of these peptides shows that the

four and five heptad peptides tend to form homodimers, with peptide K being more prone to homodimerization than peptide E. K_n peptides were also found to have higher thermal stabilities when compared to E_n peptides of the same length, and longer peptides had higher thermal stabilities than shorter peptides. For the thermostability of the E_n/K_n coiled coils, the same trend was observed. Chemical denaturation experiments using GdnHCl showed that higher-order self-assemblies may exist for peptides K_5 and E_5. Lipopeptide variants (*i.e.* CPK_n and CPE_n) were synthesized with different peptide lengths and fusogenicity was investigated by performing lipid and content-mixing assays. The K_5-E_4 coiled-coil pair had the best fusogenicity in these liposome fusion assays. Subsequently, cell membrane labelling assays were performed, showing that all CPK_n are able to decorate cell membranes and form coiled coils with the complementary peptide E_n. Liposome-cell membrane docking assays using CPK_n-modified cells and CPE_n-modified liposomes revealed that the coiled-coil pairs with four- and five-heptads induced the highest amount of cell-liposome docking. Selected coiled-coil pairs were further investigated for their capacity to trigger cell-liposome fusion. The CPK_4/CPE_4 coiled-coil pair was optimal for efficient cell-liposome membrane fusion, while CPK_5/CPE_4 induced liposome-liposome fusion most effectively. The results show that coiled-coil pairs containing peptide K_5 induce high levels of cell-liposome docking, but this does not translate into efficient drug delivery into cells. This is probably because peptide K_5 forms homodimers which weakens its interaction with lipid membranes, possibly resulting in less efficient fusion pore formation. In this study, we obtained new insights and information regarding the structure, self-assembly, thermostability, and fusogenicity of different lengths of coiled-coil peptides. These results illustrate that fusogenicity is not only related to coiled-coil stability but is also influenced by competing peptide-peptide and peptide-membrane interactions. This study will aid the design of better fusogens with potential applications in membrane fusion mediated drug delivery over complex biological membranes.

In the E/K coiled-coil peptide-mediated membrane fusion system, peptide K not only forms coiled coils with peptide E in order to bring two lipid membranes together but also interacts with the lipid membrane, facilitating efficient membrane fusion. In **chapter 4**, three K_4 dimers were designed, synthesized and characterized to enhance fusion as coiled-coil formation and membrane interaction can occur simultaneously. The parallel K_4 dimer (denoted PK_4) formed peptide aggregates in

a buffered solution, which dissolved upon the addition of the complementary peptide E_4GW, resulting in heterodimeric coiled-coil formation. The linear K_4 dimers (denoted NLK_4 and CLK_4) assembled into 'tetramer-like' homodimers. These highly stable structures did not form heterodimeric coiled coils with peptide E_4, which is likely to be detrimental for efficient membrane fusion. The highest membrane affinity was obtained for PK_4 in comparison to the K_4 monomer and linear K_4 dimers. The fusogenicity of all K_4-dimer based coiled-coils was evaluated by performing lipid and content-mixing assays. As expected, the PK_4/CPE_4 coiled-coil pair showed the best capacity for triggering both lipid mixing and content mixing, while the linear K_4-dimer based coiled-coils induced only a low level of fusion. This is consistent with the hypothesis that the membrane affinity of peptide K is related to the fusogenicity of the resulting heterodimeric coiled-coil. Fusion of liposomes with cells was achieved with PK_4/CPE_4 resulting in highly efficient propidium iodide (PI) delivery. This study will therefore help us understand the relationship between peptide self-assembly, membrane affinity, and fusogenicity of coiled-coil peptides, which may aid in the development of membrane fusion based drug delivery systems.

Since PK_4/CPE_4 proved to be more fusogenic than CPK_4/CPE_4, PK_4/CPE_4 was used to induce fusion between cell wall deficient bacteria (*i.e.* L-forms) in **chapter 5**. Two L-form strains containing double identifying markers were designed. For this study, one strain expresses eGFP and is resistant to hygromycin, while the other strain expresses mCherry and is resistant to apramycin. A L-form membrane fluorescence labelling assay showed that CPE_4 can be used to modify L-form membranes with coiled coils. A fusion assay between the two L-form strains was performed using the PK_4/CPE_4 pair. Fused L-forms were obtained and enriched in a liquid medium containing both antibiotics. After several generations of selection, the purity of the doubly fluorescent cells was > 97%. The process of cell division was tracked by time-lapse confocal microscopy showing the viability of the fusant. We also found that the fused L-forms stably express genes from the two sets of chromosomes and chromosome segregation was found to be slow. The work in this chapter presents a new method for making genomic combined hybrid species by cell-cell membrane fusion, which is important for understanding the evolution of protocells and designing synthetic cells.

The studies described in this thesis also leave some questions open. We discovered a new fusogenic peptide, PK_4 and showed that the PK_4/CPE_4 coiled-coil pair shows

the highest fusogenicity of all tested coiled coils to date. However, the introduction of this peptide makes the membrane fusion system more complex, as it now contains three components. In future studies, we could therefore conjugate a cholesterol anchor to the PK_4 peptide, which may simplify the current system and more easily facilitate drug delivery applications. Another issue in the L-form membrane fusion study was that chromosome segregation was difficult in fused L-forms after double antibiotic enrichment. Future studies on this issue can focus on investigating the genomic sequences of fused and enriched L-forms.

In this thesis, we built coiled-coil peptide-based biomaterials for a range of applications. By knowledge-guided design and modification, coiled coil induced membrane fusion systems are expected to achieve drug delivery *in vivo*. Finally, L-forms obtained by coiled-coil induced fusion of different strains are expected to be an ideal model for studying questions related to the origin of life and the discovery of novel antibiotics.