CHAPTER 6

Summary and Perspectives

Gold nanoparticles (GNPs) are metallic nanoparticles that are facile to synthesize, have controllable shapes and sizes, are easy to functionalize, and possess extraordinary optical properties. Due to these characteristics, GNPs are extremely attractive tools for use in a multitude of applications spanning nanophotonics to sensing, to the treatment of autoimmune diseases. One major drawback is that their inherent instability under physiological conditions prevents their widespread use. It was previously shown that coating GNPs with biocompatible molecules can significantly reduce both their aggregation and cytotoxicity. However, commonly used silica or polymer coatings are not optimal due to the fact that surface functionalization, with therapeutic moieties for example, is not straightforward. Additionally, the thickness of these coatings is typically high, which is a disadvantage when GNPs are intended for use in sensing, for instance. A solution to this is to stabilize GNPs with peptides. A set of cysteine-containing oligopeptides has been developed to tackle this task. The cysteine side chain binds to the gold surface to form an Au-S bond, and the rest of the sequence self-assembles on the gold surface yielding β-sheet-like secondary structures. These oligopeptides possess a net negative charge, which induces electrostatic repulsion of individual GNPs, ensuring colloidal stability. Unfortunately, these oligopeptides have only been used to stabilize small (≤30 nm) spherical GNPs and reports of stabilization of larger GNPs, or other shapes, are not available. We have demonstrated this approach can be revised to create a peptide-based stabilizer suitable for a wide range of GNP shapes and size by using peptide amphiphiles. These molecules possess the features necessary for effective stabilization of gold surfaces: β-structured self-assembly behavior and a high net charge. Moreover, it was suggested that hydrophobic interactions between alkyl chains can contribute to the stabilizing effect of these peptide amphiphiles. This thesis has designed a series of peptide amphiphiles and characterized

their self-assembly properties, before exploring their suitability as coatings for GNPs of different shapes and sizes. Finally, the functionalization of the peptide amphiphiles was demonstrated to evaluate cellular immune responses to different GNPs.

To make peptide amphiphiles suitable for binding to a gold surface, the molecules had to be functionalized with a thiol group (HS-). A detailed study focusing on the effects that compositional changes (in both the alkyl chain and the peptide segment) impose on self-assembly is presented in **Chapter 2** of this thesis. To assess the effects of thiolation, a set of five peptide sequences (**V_3A_3**, **A_3V_3**, **A_6**, **$(VA)_3$**, and **$(AV)_3$**) and alkyl chains of two lengths (C16 and C11) were studied. Furthermore, dimeric species formed *via* thiol oxidation were compared to their monomeric counterparts. The self-assembly properties were compared to conventional palmitoylated peptide amphiphiles. As a general trend, thiolation lowered the amount of β-structure and the level of self-assembly was decreased, with the exception of those formed by one peptide segment, **$(AV)_3$**. Additionally, the fibers that formed were shorter in length. Dimerization of the C16 alkyl chain exhibited a pronounced effect on fiber appearance, in comparison to their monomeric counterparts, but not on the β-structure content. A change towards a shorter thiolated alkyl chain (C11) revealed a more complex nature of self-assembly: no common trends could be drawn for the five peptide segments, except for the fact that the content of α-structure increased in all cases. In general, dimerization of the C11 alkyl chain had a positive effect on both the fiber appearance and β-structure content. Interestingly, the **A_6** peptide amphiphiles demonstrated the highest α-content, which was reflected in the appearance of the fibers formed. The combined analysis of data obtained using circular dichroism (CD) spectroscopy, Fourier-transform infrared (FT-IR) spectroscopy, and transmission electron microscopy (TEM) allowed for the five peptide sequences to be divided into two groups: those with a

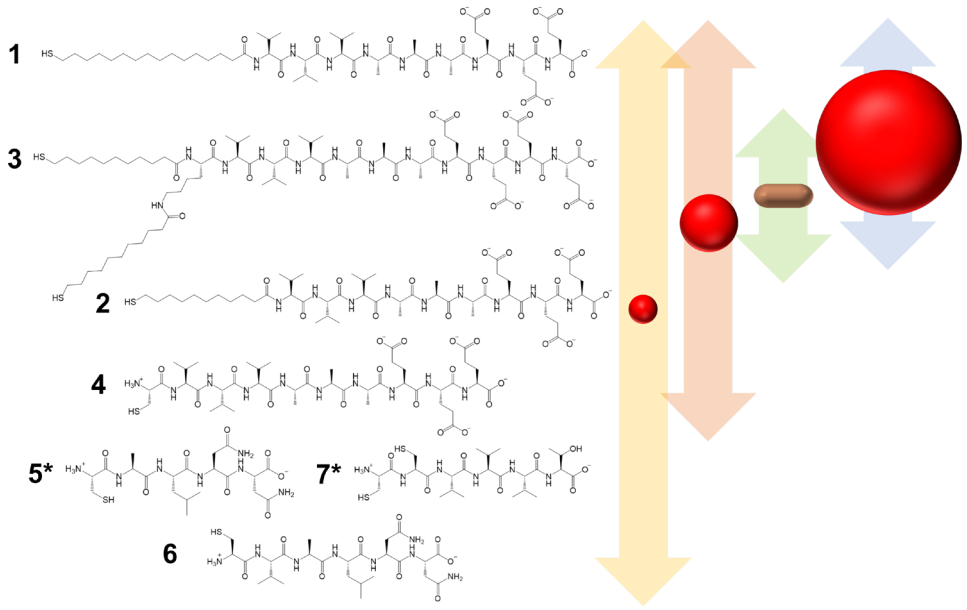
peptide-driven mode of self-assembly (**V_3A_3** and **A_3V_3**) or those where self-assembly is dependent on the alkyl chain composition (**A_6**, **$(VA)_3$**, and **$(AV)_3$**). The **V_3A_3** peptide domain exhibited minimal changes in terms of the self-assembly behavior in response to changes to the alkyl chain and so was presumed to be the most suitable candidate for stabilizing gold surfaces. This peptide amphiphile was subsequently employed in coating experiments, which are described in **Chapter 3**.

Chapter 3 of this thesis showcased the use of negatively charged, thiolated peptide amphiphiles as a stabilizer for spherical GNPs of 20, 40 and 100 nm diameter. These peptide amphiphiles were examined alongside well-known peptide stabilizers. The surface-curvature effect governed the stabilizing capacity of the peptidic stabilizers: higher net charge and more hydrophobic bulk were required for lower surface curvatures. Peptides with a lower net charge could only stabilize smaller GNPs (20 nm in diameter). An increased surface charge provided by the $CV_3A_3E_3$ peptide, was sufficient for effective stabilization of 40 nm GNPs, but failed to stabilize larger particles (100 nm in diameter). Thiolated peptide amphiphiles were capable of stabilizing all three GNP sizes, and maintained high colloidal stability due to formation of self-assembled monolayers (SAMs) on the gold surface. The surface-curvature effect was reflected in the SAM coverage density: it decreased from 3.72 to 2.17 peptide/nm^2 while the GNP size increased from 20 to 100 nm in diameter. The same trend was observed for the β-content: it decreased from 73.8% to 41.8%. It was proven that these SAMs are impermeable to competing thiols dissolved in water, this is because water is depleted from the gold surface due to the high density of the alkyl chains. Only the double chain variant (mercaptoundecanoyl)$_2$-$KV_3A_3E_4$ was capable of preserving the colloidal stability of all sizes of coated GNPs at high NaCl concentrations. The obtained results demonstrate that peptide amphiphile-coated GNPs are

stable under physiologically relevant conditions and suitable for biological and biomedical applications.

The same coating approach was used to explore the potential of thiolated peptide amphiphiles as stabilizers for gold nanorods (GNRs): **Chapter 4** of this thesis covers this study. GNRs are typically synthesized using a different chemical method called seed-mediated overgrowth, and the resulting surface chemistry is drastically different to that of spherical GNPs. GNRs are typically protected by a surfactant bilayer composed of cetyltrimethylammonium bromide (CTAB). In this chapter, this bilayer was shown to have limited permeability for peptides, whereas the three thiolated peptide amphiphiles that were tested effectively displaced CTAB, leading to a SAM formation at the GNR surface. The coating procedure was accompanied by more intense fiber formation in comparison with coated GNPs. This behavior was attributed to the charge of the original stabilizer: negatively charged citrate *versus* positively charged CTAB. The charge defined whether repulsive or attractive electrostatic interactions occurred upon addition of negatively charged thiolated peptide amphiphiles. Therefore, the coating procedure for GNRs was optimized to yield fiber-free samples. As in the case of peptide amphiphile-coated GNPs, SAM coverage densities and the β-content for GNRs were high (2.62-3.87 peptide/nm^2 and 51-58%, respectively depending on the peptide amphiphile composition). Interestingly, all three coatings were resistant to displacement by competing thiols, but only the double-chain (mercaptoundecanoyl)$_2$-K$V_3A_3E_4$ amphiphile was capable of preserving the GNR surface charge and colloidal stability at elevated NaCl concentrations. These observations help in determining how different thiolated peptide amphiphiles are displayed on a gold surface: due to the bidentate nature of (mercaptoundecanoyl)$_2$-K$V_3A_3E_4$, the coverage density is lower in comparison to the single chain counterparts, although this does not prevent effective hydrogen bonding as reflected in the high β-

content. On the other hand, the higher coverage density observed for the mercaptoundecanoyl-$V_3A_3E_3$ amphiphile resulted in packing defects as is evident from the lower β-content. However, extending the length of the alkyl chain, to obtain mercaptohexadecanoyl-$V_3A_3E_3$, allowed for an even higher coverage density and fewer packing defects, although the charge display on the gold surface was still obstructed, as it was rapidly screened upon addition of NaCl. A high negative charge and the presence of a hydrophobic segment make thiolated peptide amphiphiles superior GNR stabilizers in comparison with commonly used PEGs. SAMs formed by peptide amphiphiles exhibit similar stabilizing capacities to PEG, however peptide amphiphiles are up to 8 times shorter, meaning coating thicknesses are decreased. This feature is especially attractive for applications of GNRs in bio-sensing and nanophotonics where the coating thickness affects measurement accuracy.



Scheme 6.1. Summary of peptides and peptide amphiphiles tested in this thesis as stabilizers for differently sized GNPs and GNRs. Arrows indicate the stabilizing capacity of different molecules with respect to GNP size and shape. GNPs and GNRs are shown to scale (from the left to right: 20 and 40 nm in diameter, 45 by 15 nm GNRs, 100 nm in diameter).

Since peptide chemistry offers a facile route to modification of a peptide sequence with various ligands, peptide amphiphile-coated GNPs and GNRs can be decorated with one or more functionalities. To demonstrate this, peptide amphiphile-coated GNPs of different sizes, and GNRs, were equipped with model epitopes derived from chicken ovalbumin ($OVA_{257-264}$ and $OVA_{323-339}$) and the evoked immune responses were studied *in vitro* and *ex vivo* using OT-I and OT-II mouse lines as described in **Chapter 5** of this thesis. The epitopes used stimulate either cytotoxic or helper T-epitopes and so it was possible to split the immune response into two components and draw conclusions regarding the impact of the shape and size of the epitope carrier particle. It was shown that peptide amphiphile-coated GNPs and GNRs do not cause cytotoxicity or induce inflammation through the IL-1β pathway. Furthermore, GNPs of 15 nm in diameter did not activate bone-marrow derived dendritic cells (BMDCs) and as a consequence did not evoke significant cytotoxic or helper T-cell responses. Although, at elevated concentrations 15 nm GNPs did show an increase in helper T-cell response but their effect was minimal in comparison to the performance of larger particles: GNPs of 40 nm in diameter and GNRs of 45 by 15 nm in size. Surprisingly, for GNPs of 40 nm in diameter, strong BMDCs activation through IL-12 secretion did not translate into 40 nm GNPs inducing superior cytotoxic and helper T-cell responses. Furthermore, GNRs delivered more antigen to the BMDC lysosomes than 40 nm GNPs. This is of importance since MHC-II is loaded with antigens inside lysosomes, thus GNRs elicited a stronger helper T-cell response. However, a different trend was observed when GNPs and GNRs were used to induce a cytotoxic T-cell response. 40 nm GNPs evoked a considerably stronger cytotoxic T-cell response than GNRs, indicating that the mass (or volume) of a delivery vehicle defines MHC-I antigen presentation. This can be explained by the fact that MHC-I and MHC-II utilize different pathways for presentation of the processed

antigens: MHC-I is loaded with epitopes present in the cytosol. Our findings indicate that shape and size of an antigen delivery vehicle do influence immune response and there are two key conclusions: (i) there is a size threshold below which delivery efficacy drops (~15 nm in diameter); (ii) there is a mass (or volume) threshold below which no effective MHC-I antigen presentation is possible (~40 nm in diameter). We believe these findings will have implications for vaccine development, and will help to form a better understanding of cellular uptake, as well as antigen egress from lysosomes into the cytosol.

In future, expanding the pool of displayed functionalities will accelerate the use of peptide amphiphile-coated GNPs and GNRs. For example, the thin, yet highly stable coatings are amenable to fulfilling GNPs potential in biosensing at the single molecule level and for target-specific imaging or biomarker detection assays. In addition, this system has demonstrated great potential as a model system to unravel the fundamental principles behind the behavior and function of biomacromolecules and cells.