CHAPTER 1

Introduction

Since the first synthesis of colloidal gold was reported in 1951,[1] researchers worldwide have been exploring the potential of gold nanoparticles (GNPs) in multiple fields of materials science. The appeal of GNPs arises from their distinct features, such as their facile synthesis, programmable shape and size, unique optical properties, and ease of surface functionalization, in combination with their large surface area. In more recent years, synthesis protocols for different GNPs have been developed, including for: spherical GNPs from 1 to 200 nm diameter,[2] gold nanorods (GNRs) with programmable dimensions,[3] and unconventional shapes including nanourchins, nanostars, nanodogbones, nanodumbbells, nanocubes, and nanotriangles.[4] With this shape and size diversity comes a wide range of optical properties. GNPs are plasmonic: the electrons on the surface form an electron cloud that oscillates in a manner that is dependent on particle shape and size.[5] Thus, every GNP shape has a characteristic positioning of the absorbance band that defines their localized surface plasmon resonance (LSPR). Spherical GNPs exhibit LSPRs located between 500-600 nm, while LSPRs of more complex shapes are shifted to the near infra-red region (NIR).

GNPs have found applications in a multitude of different fields. They are attractive for applications including drug delivery and vaccine development due to their high relative surface area that can be functionalized with targeting ligands or therapeutic molecules.[6-9] [10] In materials science, GNPs are commonly used in detection assays where the readout is based on optical property changes triggered by GNPs agglomeration.[11, 12] Non-spherical gold nanoparticles have been applied in biomacromolecule sensing,[13, 14] nanophotonics,[15] and in the enhancement of Raman scattering.[16] Differently shaped GNPs are also used in plasmonic photothermal therapy (PPTT).[17-19] GNPs are also widely employed as contrast agents in imaging,[20-22] labeling,[23] and tracking[24] of cellular processes *in vitro* and *in vivo*. GNPs of a size (or shape) range covering the nanometer-

to-submicrometer scale can be used to study size (or shape) effects on cellular uptake,[25-27] clearance from the bloodstream,[28] evoked immune response,[8, 9] or nanoparticle-associated impairment of native cellular function.[29-32] In addition, several groups have investigated the formation of a protein corona around GNPs and GNRs as a function of the applied surface coating and tried to unravel the effects it imposes on the particles' biological fate.[33-36]

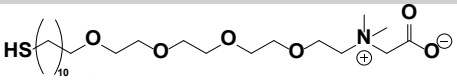
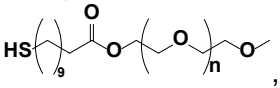
One drawback of using GNPs for *in vitro* and *in vivo* applications is that 'as-synthesized' GNPs are not stable in saline, leading to GNP aggregation.[37, 38] Moreover, this aggregation behavior is believed to be the reason these particles are cytotoxic.[31, 35] In order to minimize cytotoxicity and to make GNPs biocompatible, the GNP surface has to be stabilized with alternative ligands. Silica or polymer coatings are commonly used to efficiently prevent GNP aggregation and abolish cytotoxicity,[31, 35, 39, 40] but they have disadvantages. The control over the silica coating thickness is poor, and the coating thickness commonly exceeds 10 nm.[41, 42] Moreover, silica coatings are porous which can lead to accumulation of undesired molecules within the shell. Polyethyleneglycols (PEGs) have been shown to induce immune responses upon persistent exposure, thus the use of thiolated PEGs as GNP stabilizers can have adverse effects.[43] Additionally, it is often desirable for these coatings to be further functionalized, with targeting ligands for example, and functionalization of long PEG molecules is not a trivial task.

In 2004, Levy *et al.* proposed the use of cysteine-containing oligopeptides to stabilize spherical GNPs: these peptides displace citrate from the gold surface and form a protective coating.[44] The study evaluated 58 different peptides and devised rules for the design of GNP-stabilizing peptides. Firstly, the presence of cysteine at the N-terminus is essential for establishing Au-S bonds. Only free thiols (HS-groups) of cysteine side chains participate in

binding, while disulfide-bound cysteines do not form Au-S bonds. The presence of a free amine at the N-terminus was found to aid coordination of the thiols to the gold surface. Secondly, charged residues (lysine or aspartate, for example) should not be placed in the middle of the peptide, as they induce repulsion within the peptide shell leading to GNP aggregation. Moreover, it is crucial to have a C-terminal carboxyl moiety but not a C-terminal amide. This facilitates electrostatic repulsion between individual GNPs and helps to maintain their colloidal stability. Thirdly, the rest of the peptide sequence should consist of non-polar residues. It is beneficial if they are capable of self-assembling into β-structures both in solution and on the gold surface. These β-structures, or β-sheets as they are often referred to in literature, are a distinct peptide secondary structure that exhibit intermolecular hydrogen bonding between neighboring peptide backbones. Due to these tight interactions, water molecules are depleted from the gold surface, thus a high colloidal stability is maintained. The peptide sequences that have been reported to efficiently stabilize GNPs are presented in **Table 1.1**. Such peptide include CALNN, CCVVVTT,[44] and the amyloid-inspired CFGAILSS.[45] These provide 'protein-like' surface chemistry implying that GNPs coated with these peptides exhibit long term-stability under biologically relevant conditions and are resistant towards freeze-drying, repetitive centrifugation, and can be purified *via* size-exclusion chromatography. Interestingly, α-helical peptides were also used coat GNPs, but they exhibited a much lower coverage density:[46] 0.72 peptide/nm^2 versus 2.55 peptide/nm^2 reported for CFGAILSS.[45] This difference indicates that peptides capable of self-assembly into β-structures provide higher coverage densities and potentially better colloidal stabilities. Although these peptides were widely used for stabilizing GNPs and imparting acquired functionalities (**Table 1.2**), use of these peptides for stabilization of GNPs larger than 30 nm, or for non-spherical GNPs has not been reported.

Another widely used approach utilizes short PEG molecules (≥ tetraethyleneglycol) conjugated to a thiolated alkyl chain (≥ C8).[47-50] These molecules possess a terminal thiol enabling binding to a gold surface (**Table 1.1**). GNPs stabilized with these molecules and equipped with an active moiety, such as an enzyme substrate, have been reported.[47] These molecules were shown to stabilize small GNPs (up to 25 nm in diameter) with comparable efficiency to peptides, but they also failed to efficiently stabilize larger GNPs (35 nm in diameter).[48] Interestingly, Schulz *et al.* reported the use of PEG_{2000} conjugated to 11-mercaptoundecanoic acid as a surface stabilizer for GNRs.[51] This amphiphilic stabilizer provided a ~3x higher coverage density, and hence higher colloidal stability, than non-functionalized thiolated PEG_{2000}. It was suggested that the amphiphilic PEG penetrates the hydrophobic CTAB bilayer at the GNR surface more readily than regular, hydrophilic PEG.

Table 1.1. Commonly used peptide sequences and PEG-based compounds for stabilization of GNPs and GNRs.

Peptide sequence	**GNP size, nm**	**Reference**
CALNN NNLACCALNN CCVVVT	12.3	44
CFGAILSS	5; 8; 10; 25	45, 47
CLPFFD-amine*	25	52
Chemical structure		
	15; 25; 35	48
 , n = ~45	12; 30 ~85 by 25 (GNRs)	50 51

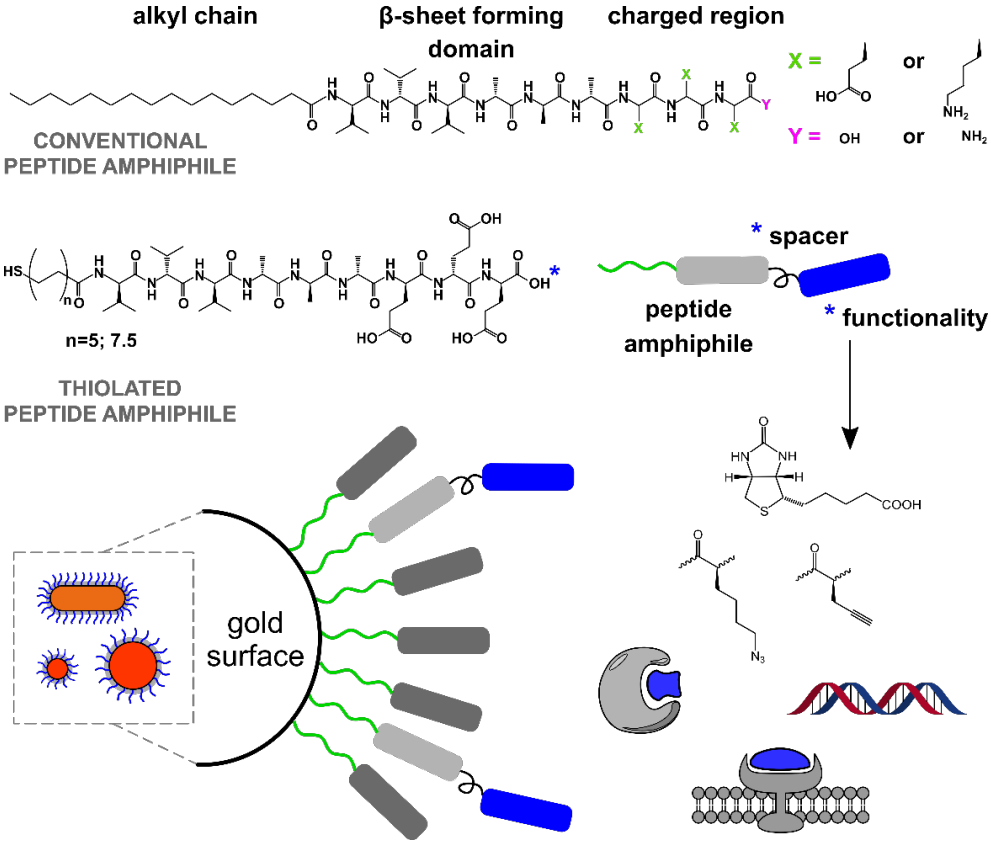
*- this peptide had an amide group at the C-terminus.[52]

Table 1.2. Additional functionalities appended to CALNN-stabilized GNPs.

Functionality	Comments	Reference
Biotin	Streptavidin docking proved availability of biotin displayed on the GNP surface	44, 54
Oligonucleotides	Interactions between GNPs decorated with complementary strands led to controllable GNP assembly proving that the proposed derivatization route was successful	53
TAT peptide*	These GNPs permeated the cellular membrane and can be used as transporters	54
Epitopes**	Upon contact with specific antibodies, GNPs agglomerate and their optical properties change, which provided the readout in the immunoassay	10, 55
Enzyme substrates***	Caspase-3 cascade was triggered by these GNPs though the cleavage of the substrate	56

*- The human immunodeficiency virus (HIV)-derived trans-activator of transcription peptide (TAT) is a cell-penetrating peptide with the following peptide sequence: GRKKRRQRRRPPQ.[54] **- epitopes were derived from *haemophilus influenzae* hemeagglutinin, *herpes simplex* virus glycoprotein D, and c-Myc protein,[10] or abscisic acid glucose ester was used.[55] ***- the following substrates were used: PFFDVED and KYDDVED.[56]

Although several stabilizers have been developed for GNPs and GNRs, a new approach is needed for larger GNPs (> 30 nm in diameter). We propose to expand the peptidic stabilizer approach by using peptide amphiphiles (**Scheme 1.1**). Peptide amphiphiles are composed of a β-sheet forming peptide sequence coupled to an alkyl chain.[57] They are capable of self-assembling to form a range of structures including 1D cylindrical fibers and well-defined 2D antiparallel β-sheet ribbons.[58] Moreover, peptide amphiphiles can form hydrogels upon the addition of salts;[59, 60] and serve as templates for the mineralization of various inorganic compounds, *e.g.* hydroxyapatite and $CaCO_3$.[61, 62] These applications are possible due to the robustness of the peptide amphiphile self-assembly, which is beneficial for



Scheme 1.1. The structure of a typical conventional peptide amphiphile comprises an alkyl chain, β-sheet forming peptide domain, and charged region. Lysine residues provide positive net charge, while glutamate residues provide a negative charge. The design of thiolated peptide amphiphiles is based on a similar structure: a thiol group (HS-) is introduce at the alkyl chain terminus, and a functionality is coupled to the N-terminus of the peptide segment. The thiol groups enable binding to a gold surface in such a way that alkyl chains are located close to the gold surface and the peptide segments are exposed to the aqueous environment. Examples of functionalities include biotin, “click”-handles, oligonucleotides, enzyme substrates, and receptor-specific ligands including B- or T-cell epitopes, *etc*.

their use as a GNP stabilizer. In addition to their other benefits, peptide chemistry offers easy chemical routes to extend the peptide sequences with various ligands thus functionalization of peptide amphiphile-coated GNPs and GNRs with active moieties is possible. We have designed peptide amphiphiles to contain a terminal thiol group that binds to the gold surface.

The alkyl chains subsequently undergo hydrophobic collapse and are sequestered from the aqueous environment. The middle segment of the peptide amphiphile, formed by a β-sheet forming motif, enables intermolecular hydrogen bonding resulting in dense peptide packing. Additionally, we designed the peptide amphiphiles to exhibit a high net charge. These charged residues, which can be either positive or negative, are located at the C-terminus and are therefore exposed to the aqueous environment and promote electrostatic repulsion, thereby aiding colloidal stability.

The experimental work presented in this thesis focuses on the design of peptide-amphiphile ligands capable of stabilizing GNPs of differing sizes and shapes. A large part of this thesis is devoted to determining the optimal conditions for coating GNPs with peptide amphiphiles and evaluating the stabilizing effects that these thiolated peptide amphiphiles afford. Peptide amphiphile-coated GNPs are also demonstrated to be suitable for use in *in vitro* experiments, which facilitates the study of how the physico-chemical properties of GNPs influences T-cell immune responses.

In **Chapter 2** the design and synthesis of thiolated peptide amphiphiles is described. The changes of peptide amphiphile self-assembly behavior in response to variations in the alkyl chain length and composition of the β-sheet forming domain is studied. A comprehensive analysis of data obtained using circular dichroism (CD) spectroscopy, transmission electron microscopy (TEM), and Fourier-transform infrared (FT-IR) spectroscopy is presented.

Chapter 3 describes the coating procedure used to prepare peptide amphiphile-coated spherical GNPs with a size range of 20 to 100 nm. Using dynamic light scattering (DLS), ultraviolet–visible spectroscopy (UV-Vis), and

TEM it was shown that thiolated peptide amphiphiles stabilize all GNP sizes tested. CD and FT-IR were used to assess the self-assembly of these amphiphiles on the gold surface into a self-assembled monolayer (SAM). Two assays: an NaCl-induced aggregation assay and a thiol displacement-induced aggregation assay, were employed to obtain insight into the colloidal stability of the peptide amphiphile-coated GNPs.

In **Chapter 4** thiolated peptide amphiphiles were used for GNR stabilization. UV-Vis spectroscopy, TEM analysis, and zeta-potential measurements were performed to demonstrate the successful displacement of CTAB from the GNR surface and to evaluate the colloidal stability of the resulting particles. Determination of coverage densities of the peptide amphiphiles on the GNR surface, combined with FT-IR data revealed that densely-packed SAMs similar to those formed on the surface of spherical GNPs were also formed on GNRs.

In **Chapter 5** peptide amphiphile-coated GNPs and GNRs were equipped with chicken ovalbumin-derived epitopes in order to study the effect of GNP size and shape on immune response. DLS, UV-Vis spectroscopy, TEM, and inductively coupled plasma mass spectrometry (ICP-MS) were used to characterize epitope-decorated GNPs and GNRs. The cytotoxicity of the particles in bone marrow-derived dendritic cells (BMDCs) was probed with a lactate dehydrogenase (LDH) release assay. Cellular uptake was visualized with TEM and quantified with ICP-MS. Activation of BMDCs through upregulation in cellular receptors, clusters of differentiation CD80 and CD86 was monitored using flow cytometry, while activation through IL-12 and IL-1β cytokine secretion was evaluated with an enzyme-linked immunosorbent assay (ELISA). Efficacy of antigen presentation mediated by the corresponding major histocompatibility complex class I (MHC-I) was assessed using flow cytometry. GNP- and GNR-triggered T-cell immune

responses were studied *in vitro* using cells derived from ovalbumin transgenic mice. The immune responses elicited by different GNPs and GNRs were compared on the basis of T-cell proliferation rates, which were derived from a $CD25^{+}CFSE^{low}$ T-cell subset using flow cytometry.

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