INTERACTIVE RAFT POLYMER-DECORATED GOLD NANOPARTICLES

FOR BIOMEDICAL USE

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Pharmacist

Thesis submitted to obtain the degree of doctor in Pharmaceutical Sciences

2016

Promoter:

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Ghent, May 19th, 2016

The Promoter
Prof. Dr. Ir. Bruno G. De Geest

The Author
Zhiyue Zhang
Four years ago, I was busy with my master defense in China. Now, it is the doctoral defense in Belgium. My life have changed so much! Birthday is always an entertaining reminder, but here I would like to choose this 4 years period of time to look back and reflect on where I am and where I have been.

Return to the 26th of September in 2011, I sent my application letter to Dr. Ir. Bruno G. De Geest and later he replied and agreed with my doctoral applications after interview via Skype. Since then, we have worked on the grant applications, discussion on my doctoral research, manuscript submission to the journals and PhD defense preparation. I would like to express my sincerest gratitude to you, Bruno, my supervisor, for supplying me an opportunity to be a doctoral student in the laboratory of pharmaceutical technology. Combined with your experience, support, guidance and encouragement, I improved my knowledge and skills in drug delivery field and also my personality. I also would like to thank you for what you did in my private life, including picking me up in the first day I arrived at Gent, looking out housing for me, testing my second-handed car and giving advices for my daily life. THANK YOU!

11th of October in 2012 is my first day in the current group, and later I was introduced to Prof. Jean Paul Remon and Prof. Chris Vervaet from Department of Pharmaceutics, and Prof. Thomas De Beer from Department of Pharmaceutical analysis. I’d like to express my sincere gratitude to all of you for creating such friendly work atmosphere and effective cooperation in both research and life.

In the coming days, I was introduced to my group members. Marijke, thank you for booking my first laboratory notebook in Ghent University; Lien, thank you for installing the PC connection to our university internet; Benoit, thank you for showing me the complicated road to the offices; Nane, thank you for showing me the storage place for chemicals; Andre, thank you for your encouragement for practicing my oral English. All of you helped me a lot in the research, especially in the beginning of my study in the lab.

Later, I met our wonderful secretaries, Ilse and Katharine, and our technician, Christine. I am grateful to all of you for helping a lot not only on documents, but also on private issues.

When I was really into the research, I cannot forget to mention the great collaborators I had the privilege to work with. In particularly, I would like to thank Prof. Richard Hoogenboom from the Department of Organic and Macromolecular Chemistry at Ghent University. You are gratefully acknowledged for helpful discussions, which decisively contributed to the success of my research project.

I appreciated Dr. Samarendra Maji, Dr. Qilu Zhang, Dr. Victor De la Rosa from Department of Organic and Macromolecular Chemistry at Ghent University, Riet De Rycke from VIB-
Inflammation Research Centre, Belgium, for their scientific cooperation and helpful discussions.

As time went on, our group became bigger and more international. Here I would like to thank my group members, Ruben, Hui, Sabah, Simon, Lutz, Garima and Alexandra. It was a pleasure to have you in the group. Thanks for the nice atmosphere, the interesting discussions, the sharing of frustrations, the support... I wish you the best of luck with your future research carrier and life.

I also have to appreciate all my colleagues in the laboratory of Pharmaceutical Technology. Thank you for sharing your life and occasionally lab equipment and helping me.

I gratefully acknowledge the Chinese Scholarship Council for a PhD scholarship and Ghent University for BOF co-funding.

I greatly appreciate all my Chinese and Belgian friends. Without you, my life isn’t so funny and dramatic in Ghent.

Looking back over the past four years, I am proud of meeting so many intelligent, kind people who had an incredible patience for me. you have helped me to feel like a part of a family that I could always count on.

My parents, sister and parents-in-law are the persons who deserve all of my acknowledgements. Without your continuous love, support and encouragement this thesis would not exist. I’m very grateful to you all.

I am very grateful to my wife Hui Li, thank you for loving me, thank you for your support, thank you for being who you are, and thank you for sharing these last nine years with me.

I would like to thank my new-born son, Leo Zhang, who motivated me to finish the PhD thesis as soon as possible to take care of him and his mother.

Here, I would like to express my sincere gratitude to the members of the reading and examination committee for providing valuable suggestions to improve my thesis.

ZHIYUE ZHANG

GHENT, BELGIUM

2016
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<tr>
<td>ACN</td>
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<td>AFM</td>
<td>atomic force microscopy</td>
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<td>AIBN</td>
<td>2,2’-azobisisobutyronitrile</td>
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<td>ALS</td>
<td>automatic liquid sampler</td>
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<tr>
<td>APMA</td>
<td>N-(3-aminopropyl)methacrylamide</td>
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<td>Au</td>
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<tr>
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<tr>
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<td>chain transfer agent</td>
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<td>Δf</td>
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<td>DAD</td>
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<tr>
<td>DLS</td>
<td>dynamic light scattering</td>
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<td>dimethylsulfoxide</td>
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<tr>
<td>DP</td>
<td>degree of polymerization</td>
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<tr>
<td>HA</td>
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<td>HAuCl₄</td>
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<td>HEA</td>
<td>2-hydroxyethylacrylate</td>
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<td>--------------</td>
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<tr>
<td>1H-NMR</td>
<td>proton nuclear magnetic resonance</td>
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<td>HPMA</td>
<td>N-(2-hydroxypropyl)methacrylamide</td>
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<td>ICP-MS</td>
<td>inductively coupled plasma mass spectrometry</td>
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<tr>
<td>LC-MS/MS</td>
<td>liquid chromatography–mass spectrometry / mass spectrometry</td>
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<td>LCST</td>
<td>lower critical solution temperature</td>
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<td>LiCl</td>
<td>lithium chloride</td>
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<td>[M]</td>
<td>monomer</td>
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<td>MAA</td>
<td><em>Maackia amurensis</em> agglutinin</td>
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<td>MBTTC</td>
<td>methyl 2-(butylthiocarbonothioylthio)propanoate</td>
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<tr>
<td>MEA</td>
<td>methoxyethylacrylate</td>
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<td>Mn</td>
<td>number average molecular weight</td>
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<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
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<tr>
<td>Mw</td>
<td>weight average molecular weight</td>
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<td>NaBH₄</td>
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<td>NaCl</td>
<td>sodium chloride</td>
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<td>PABTC</td>
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<td>phosphate buffered saline</td>
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<td>PDT</td>
<td>photodynamic therapy</td>
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<tr>
<td>PEG</td>
<td>poly(ethylene glycol)</td>
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</tr>
<tr>
<td>PEI</td>
<td>poly(ethylene imine)</td>
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<tr>
<td>PEtOx</td>
<td>poly(2-ethyl-2-oxazoline)</td>
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<tr>
<td>PI</td>
<td>propidium iodide</td>
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<tr>
<td>PDM</td>
<td>poly(2-(2-methoxyethoxy)ethyl methacrylate)</td>
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<tr>
<td>PMeOx</td>
<td>poly(2-methyl-2-oxazoline)</td>
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<tr>
<td>PMMA</td>
<td>polymethylmethacrylate</td>
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<td>PMT</td>
<td>photomultiplier tube</td>
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<tr>
<td>polyDEGA</td>
<td>poly(diethyleneglycol acrylate)</td>
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<tr>
<td>polyNIPAm</td>
<td>poly((N)-isopropylacrylamide)</td>
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<td>polyNVP</td>
<td>poly((N)-vinylpyrrolidone)</td>
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<td>poly²PropOx</td>
<td>poly(2-n-propyl-2-oxazoline)</td>
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<td>PPE</td>
<td>poly((p)-phenyleneethynylene)</td>
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<td>PPEGMA</td>
<td>poly(poly(ethylene glycol) methacrylate)</td>
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<td>PVA</td>
<td>poly(vinyl alcohol)</td>
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<tr>
<td>P4VP</td>
<td>poly(4-vinylpyridine)</td>
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<tr>
<td>PVP</td>
<td>poly((N)-vinylpyrrolidone)</td>
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<tr>
<td>QCM</td>
<td>quartz crystal microbalance</td>
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<tr>
<td>RAFT</td>
<td>reversible addition-fragmentation chain transfer</td>
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<tr>
<td>RID</td>
<td>refractive index detector</td>
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<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate- polyacrylamide gel electrophoresis</td>
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<tr>
<td>SEC</td>
<td>size exclusion chromatography</td>
<td></td>
</tr>
<tr>
<td>SEM</td>
<td>scanning electron microscope</td>
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<tr>
<td>SNA</td>
<td><em>Sambucus nigra</em> agglutinin</td>
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<tr>
<td>SPR</td>
<td>surface plasmon resonance</td>
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<td>Abbreviation</td>
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<tr>
<td>SSC</td>
<td>side scatter</td>
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<tr>
<td>TA</td>
<td>tannic acid</td>
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</tr>
<tr>
<td>TCC</td>
<td>thermostatted column compartment</td>
<td></td>
</tr>
<tr>
<td>Tcp</td>
<td>cloud point temperature</td>
<td></td>
</tr>
<tr>
<td>TEM</td>
<td>transmission electron microscope</td>
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<tr>
<td>TGA</td>
<td>thermogravimetric analysis</td>
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<tr>
<td>THF</td>
<td>tetrahydrofuran</td>
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<tr>
<td>TOAB</td>
<td>tetraoctylammonium bromide</td>
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<tr>
<td>UV-Vis</td>
<td>ultraviolet-visible</td>
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<td>ζ</td>
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CHAPTER 1

GENERAL INTRODUCTION AND AIM OF THE THESIS
INTRODUCTION

Gold is the quintessential noble element, and has been regarded as one of the most precious materials in the world since its first extraction in the 5th millennium B.C. in Bulgaria.\textsuperscript{1} By nature, it is highly unreactive and as such, historical artifacts made of gold can retain their brilliant luster for thousands of years without tarnishing or deterioration.\textsuperscript{2} While gold has many favorable properties as a bulk metal, including high electrical conductivity, reflectivity, malleability and chemical inertia, new properties and potential applications emerge when it is finely divided into fragments of size reaching down to 100 nm, in which the large surface area-to-volume ratio introduces a large number of surface sites with high curvatures.\textsuperscript{1}

Although bulk gold is known to be chemically inert, its properties on the 1-100 nm scale become dependent on its size and shape.\textsuperscript{3-8} The nanometer scale incorporates collections of atoms or molecules, however, their properties are neither those of the individual constituents nor those of the bulk,\textsuperscript{9} but result from the interface of atoms on the surface of nanoparticles.

The first scientific article on gold nanoparticles was reported by Michael Faraday in 1857,\textsuperscript{10} in which the synthesis of gold colloids was demonstrated in an aqueous medium, attributing the red color to the colloidal nature of gold nanoparticles. The next major advances came in 1908, when Mie rationalized their visible absorption using Maxwell’s electromagnetic equations for spherical particles,\textsuperscript{11} making it possible to predict the optical properties of gold nanospheres. According to the Mie theory, if a spherical nanoparticle is much smaller than the wavelength of light, an electromagnetic frequency induces a resonant coherent oscillation of the free electrons at the surface of particles, which is called the surface plasmon resonance (SPR) (Figure 1). When gold nanoparticles are illuminated at a proper optical frequencies, the conduction band electrons in the gold are excited, resulting in a resonant, coherent oscillation of these electrons. This resonance condition leads to light extinction.\textsuperscript{12} For small (~30nm) gold nanoparticles, the SPR causes an absorption of light in the blue-green portion of the spectrum (~450nm) while red light (~700nm) is reflected, yielding a red color. These effects have already been used by the Romans dating back to the 4\textsuperscript{th} century to produce artifacts (Figure 2) with colors that depend on the direction of the incident light.
Figure 1. (a) Schematic representation of surface plasmon resonance phenomenon. Gold nanoparticles strongly absorb and scatter visible light. Upon light absorption, the light energy excites the free electrons in the gold nanoparticles to a collective oscillation, the so-called surface plasmon. (b) Size-dependent surface plasmon resonance of gold nanoparticles, with a red shift in absorption maximum occurring for larger particles.

With an increase in particle size, the SPR peak shifts to longer wavelengths, in which case red light is absorbed and blue light is reflected, yielding solutions with a blue or purple color. Basically, the agglomeration of gold nanoparticles shortens the distance between the gold cores, also leading to a red shift of SPR peak. From the Mie theory, the frequency of the plasmon band varies between spherical and non-spherical nanoparticles of various shapes, such as gold nanorods. In the latter case, two plasmon bands are observed corresponding to the length and width of gold nanorods, respectively. The SPR can be tuned by varying the size and shape of gold nanoparticles, leading to particles with tailored optical properties.

Figure 2. Photograph of the he Lycurgus Cup, a 4th century Roma chalice made of glass, impregnated with gold and silver nanoparticles, that changes color depending on the direction of the incident light: On the left, the cup is illuminated from the outside, and on the right it is illuminated from the inside.
SYNTHESIS OF GOLD NANOPARTICLES

Numerous synthesis methods for gold nanoparticles have been reported, including both ‘top-down’ (physical manipulation) and ‘bottom-up’ (chemical transformation) strategies. The Top-down approach uses larger (macroscopic) initial structures, which can be externally-controlled in the process of nanostructures. The Bottom-up approach includes the miniaturization of materials components (up to atomic level) with further self-assembly process leading to the formation of nanostructures.\(^1\),\(^{19}\)

**Physical methods**

Physical methods enable further manipulation of the structure and hence the properties of gold nanoparticles.\(^1\),\(^{20}\) In the photoreductive process, UV irradiation can significantly improve the quality of gold nanoparticles,\(^{21\text{-}23}\) and the size and morphology of gold nanoparticles can be controlled by the very slow irradiation.\(^{24\text{-}25}\) The presence of an ultrasonic field allowed the control of the rate of AuCl\(^-\) reduction in an aqueous solution and the size of the formed nanoparticles by varying the temperature of the solution, the intensity of the ultrasound and the positioning of the reactor.\(^{26\text{-}28}\) Thermolysis has shown capable to significantly reduce average particle size and polydispersity by regulating the growth of the gold nuclei.\(^{29\text{-}30}\) Control of the particle size can also be provided by radiolysis.\(^{31\text{-}32}\)

**Citrate reduction**

Since Faraday prepared gold colloids by reduction of an aqueous solution of chloroaurate with phosphorus dissolved in carbon disulfide,\(^10\) similar approaches have been reported for the preparation of colloid gold based on the reduction of gold salts by reducing agents.\(^9\),\(^{33\text{-}35}\)

Among the conventional methods of synthesis of goldNP by reduction of gold(III) derivatives, the most commonly used synthesis route for colloidal gold in aqueous solution is the reduction of tetrachloroauric acid (HAuCl\(_4\)) by trisodium citrate (Na\(_3\)Ct), which has been referred to as the ‘Turkevich method’, named after John Turkevich who described the reaction in 1951.\(^36\) After dissolving HAuCl\(_4\), the solution is rapidly stirred while a reducing agent is added, causing Au\(^{3+}\) ions to be reduced to Au\(^+\) ions. Meanwhile a disproportionation reaction occurs whereby 3Au\(^+\) ions give rise to Au\(^{3+}\) and 2Au\(^0\) atoms, the latter can act as
center of nucleation around which further Au\(^+\) ions get reduced. In this method, citric acid acts both as reducing and stabilizing agent and provides gold nanoparticles with diameters of 20 nm. Further studies by G. Frens enabled control over particle size by varying the feed ratio of gold salt to sodium citrate in 1973.\(^{37}\) In the case of the Turkevich method, a large number of contributions deal with the particle growth process or the molecular reduction mechanism, emphasizing the general importance of this synthetic procedure. Until today, the original synthesis protocol was modified numerous times allowing for the fabrication of gold nanoparticles in a wide range of sizes and being elaborated on in numerous applications.\(^{38-40}\)

**Functionalization of gold nanoparticles**

*Self-assembled monolayer formation*

Gold surfaces are easy to prepare by vacuum evaporation and also easy to clean because of the inertness of gold toward corrosion or oxidation, and are convenient substrates for self-assembled monolayers (SAM) formation.\(^{41}\) Thiolated-SAMs on gold surfaces, were first reported by Nuzzo and Allara in 1983,\(^{42}\) and can be formed using organosulfur compounds. They combine a high structural order and ease of preparation and analysis (schematically represented in Figure 3A). The strong specific interaction between the sulfur atom and the gold surface allows to form a densely packed monolayer in the presence of many thiol-functional groups. The ability to modify the functional group enables extensive variation of the monolayer properties.\(^{43}\) Indeed, changing the chain length of a homologous series of \(n\)-alkanethiols has reported to result in different ellipsometric thicknesses of monolayers on a gold surface (Figure 3B).\(^{44}\)
The Brust-Schiffrin method

The stabilization of gold nanoparticles with alkanethiols was first reported by Mulvaney and Giersig in 1993, and they provided the initial proof of using thiols of different chain lengths as stabilizer of colloidal gold. A significant breakthrough in the field of gold nanoparticles synthesis was achieved in 1994 by Brust and Schiffrin, who reported a two-phase synthetic strategy utilizing strong thiol-gold interactions to decorate gold nanoparticles with thiol-containing ligands, named as ‘the Brust-Schiffrin method’. In this method, the gold salt of AuCl is transferred from aqueous phase to organic phase (i.e. toluene) with the help of the surfactant tetraoctylammonium bromide (TOAB) and then reduced by sodium borohydride (NaBH₄) in presence of dodecanethiol. A rapid change in color from orange to deep brown takes place in organic phase upon addition of NaBH₄, yielding gold nanoparticles with controlled diameters in the range of 1.5-5 nm. The particle size can be tuned by various conditions, such as gold/thiol ratio, temperature, and reduction rate. The obtained nanoparticles can be thoroughly dried and then re-dispersed in organic solvents without any aggregation or decomposition. Brust et al. also developed a related one-phase method that is carried out in polar solvent and shares the positive attributes of the two-phase approach.
POLYMER COATED GOLD NANOPARTICLES

Interfacing gold nanoparticles and biological systems has gathered great interest and intensive investigation.\textsuperscript{39, 40, 48} One key requirement is the implementation of an effective surface-modification strategy that renders these materials hydrophilic and compatible with a biological environment. We should stress that aggregation is often observed for the widely used citrate-stabilized gold nanoparticles in even slightly acidic buffers or in the presence of low concentration of added electrolytes.\textsuperscript{49} In addition, thioalkyl ligand stabilized gold nanoparticles synthesized using a two-phase (toluene-water) procedure are hydrophobic and not compatible with biological media. A judicious surface functionalization strategy is therefore critically important to promote water-solubility and bio-compatibility (Figure 4).\textsuperscript{50, 51}

![Figure 4](image)

**Figure 4.** Schematic presentation of the gold nanoparticles surface coating commonly employed in delivery applications. Reproduced from ref.51.

Polymers, both synthetic and natural, have provided researchers with a great platform to design a variety of ligands to modify various nanocrystals.\textsuperscript{50, 52, 53} Novel polymerization techniques have remarkable controls over the chemical structure and architecture of polymer materials. These approaches have been exploited to develop effective surface functionalization strategies applicable to a wide range of nanoparticles. The synthetic routes for creating polymer functionalized gold nanoparticles include either the use of pre-synthesized polymers or surface-initiated polymerization from particles which are referred to
as ‘grafting to’ and ‘grafting from’ strategies, respectively.\textsuperscript{54} The latter requires a polymerization initiator to be first attached to the surface of gold nanoparticles and then polymer chains are grown from this attachment point, yielding dense and long coronal polymer chains. Using pre-synthesized polymers, which have a reactive ‘handle’ capable of binding to the particle surface, one can either reduce gold salts in the presence of sulfur end-capped polymers or graft such polymers onto pre-formed gold nanoparticles via ligand exchange. The latter option offers a higher degree of versatility regarding the size and shape of the gold nanoparticles.

Various polymers have been reported to protect gold nanoparticles from aggregation, including poly(ethylene glycol) (PEG),\textsuperscript{55, 56} poly(vinyl alcohol) (PVA),\textsuperscript{57, 58} poly(\acr{N}-vinylpyrrolidone) (PVP),\textsuperscript{59, 60} poly(4-vinylpyridine) (P4VP),\textsuperscript{61, 62} poly(\acr{N}-isopropylacrylamide) (polyNIPAm),\textsuperscript{63, 64} polystyrene-block polymers,\textsuperscript{65, 66} etc... The advantage of using polymers as stabilizers is not only the enhanced stability, adjusted solubility and hydrophilicity of gold nanoparticles, but also the functionalization of gold nanoparticles with polymers to promote compatibility and processibility of gold nanoparticles.

Among the existing polymerization techniques, Reversible Addition-Fragmentation chain Transfer (RAFT; \textbf{Figure 5}) polymerization has gained increasing popularity.\textsuperscript{67} RAFT is tolerant to a wide variety of reaction conditions and functionalities and enables control over a large variety of monomers.\textsuperscript{68} RAFT polymerization was first reported in 1998 by CSIRO\textsuperscript{69} and proceeds via a degenerative transfer processes. Well defined polymers synthesized via RAFT polymerization can be anchored onto the surface of gold nanoparticles based on the use of chain transfer agents (CTAs), bearing a di- or tri-thio carbonate or xanthate groups. Due to the strong covalent bond between gold and sulfur, all of these groups can either adsorb as such onto metallic gold or are reduced into thiols at the gold surface.\textsuperscript{41} Functionality in RAFT polymers is not limited to the choice of monomers, it can also be introduced at polymeric chain end groups and serve for further conjugation, thereby widening the scope of applications.
Temperature-responsive polymer coated gold nanoparticles

Polymers that can respond to an applied external stimulus, such as pH, ionic strength, and light, et al. have attracted significant attention, in particular materials that are responsive to temperature changes are attractive. Most temperature-responsive polymers display lower critical solution temperature (LCST) behavior: upon heating above this critical temperature the polymers expel water and undergo a coil to globule transition.\(^{70}\) This happens because it is energetically more favorable. The reason phase separation is more favorable when increasing the temperature is mostly due to the entropy of the system.\(^{71}\) Considering the free energy of the system based on the Gibbs equation:

\[
\Delta G = \Delta H - T\Delta S; \quad (G: \text{Gibbs free energy}, \ H: \text{enthalpy and } S: \text{entropy});
\]
the main driving force is the entropy of the water. And, when the polymer is not in solution, water molecules are less ordered and thus have a higher entropy. The LCST is defined as the intersection of the binodal and spinodal curves of the phase diagram and is the minimum temperature where all phases are soluble. Experimentally, the phase separation can be followed by turbidimetry, and the cloud point can be defined as the temperature at the onset of the cloudiness, or the temperature at a defined transmittance (e.g. 50%).

Thermosensitive gold nanoparticles have been studied for a decade, by coating gold nanoparticles with temperature-responsive polymers such as poly(2-(2-methoxyethoxy)ethyl methacrylate) (PMDM), poly(poly(ethylene glycol) methacrylate) (PPEGMA), and poly(2-(n-propyl)-2-oxazoline) (polynPropOx). Among these temperature-responsive polymers, poly(N-isopropylacrylamide) (polyNIPAm) and its derivatives are the most studied polymers for the modification of gold nanoparticles to render these thermosensitive. The phase transition behavior of the obtained gold nanoparticles were explored in several studies (Figure 6), but few has been reported on the influence of the solvent quality on the temperature-responsive properties of such polyNIPAm coated goldNP.

Figure 6. Schematic representation of the gold nanoparticles aggregation as function of temperature changes.

APPLICATIONS

Synthetic polymers and their hybrids with gold nanoparticles have increasingly been used in biotechnology, biomedical, and pharmaceutical technology. Although numerous applications have been developed using nature-derived macromolecules, such as proteins and DNAs, we will focus in the next paragraphs on gold nanoparticles functionalized with synthetic polymers for biomedical use.
**Colorimetric assays**

When a polymer layer adsorbs onto the metallic gold surface, the increase in refractive index around the gold nanoparticle will cause a red SPR peak shift smaller than 20nm. However, the aggregation of gold nanoparticles of appropriate sizes (d> 3.5nm) results in a visible color change from red to blue (Figure 6) at nanomolar concentrations, due to inter-particle surface plasmon coupling. The color change provides a practical platform for absorption-based colorimetric sensing of any target analyte that triggers the gold nanoparticles aggregation or redispersion.

The use of gold nanoparticles for colorimetric sensing was pioneered in 1980\(^8\) and has been developed for the detection of nucleic acids, proteins, carbohydrates and the influenza virus.\(^9\) Gold nanoparticle decorated with glycopolymers have been developed for the detection of carbohydrate binding proteins. For example, the dispersion of mannosylated polymer modified gold nanoparticles showed a red color with an SPR peak of 524 nm.\(^10\) Addition of Con A induced aggregation and a red shift of SPR peak to 563 nm. A key advantage of using polymers is that the multivalency effect can offer an increase in avidity, which is attractive in view of the generally weak individual protein-carbohydrate interactions.\(^11\)

**Fluorescence-based sensing**

Gold nanoparticles can serve as ultra-efficient quenchers of molecular excitation energy in chromophore-gold nanoparticle composites, outranging the quenching efficiency of organic acceptor molecules, due to their extraordinary high molar extinction coefficients and broad energy bandwidth.\(^12\) Several important parameters such as the size of particles and the distance between the gold nanoparticles and the chromophores have been studied recently, showing that fluorescence is less quenched when chromophores and gold nanoparticles are separated by bulky spacers. Based on this phenomena, a ‘chemical nose’ strategy (Figure 7) provides a useful alternative to identify analytes or changes in complex mixtures.\(^13\) The electrostatic complexation of gold nanoparticles and a fluorescent polymer (poly(ρ-phenyleneethynylene), PPE) resulted in fluorescence quenching of the polymer through energy transfer. The subsequent binding of protein analytes displaces the dyes via...
competitive binding, thereby causing fluorescence recovery of the polymer. By modulating the protein-nanoparticles and dye-nanoparticles association, distinct signal response patterns can then be used to differentiate between different proteins, leading to a fingerprint for a given protein.

Figure 7. Protein detection using a ‘chemical nose’ strategy developed by the Rotello group. (a) Illustration of the sensing mechanism. Displacement of quenched fluorescent polymer (dark green strips, fluorescence off; light green strips, fluorescence on) by protein analyte (in blue) with concomitant restoration of fluorescence. The particle monolayers feature a hydrophobic core for stability, an oligo(ethylene glycol) layer for biocompatibility, and surface charged residues for interaction with proteins. (b) Fluorescence pattern generation through differential release of fluorescent polymers from gold nanoparticles. Reproduced from ref. 87.

Using a similar strategy, the initially quenched assemblies of gold-nanoparticle-poly(para-phenyleneethynylene) were disrupted in the presence of bacteria, thereby regenerating fluorescence.88 From the distinct fluorescence response patterns, 12 bacteria including both Gram-positive and Gram-negative species as well as three different strains of *E. coli* were rapid and efficient identified by this sensor array. Analogous systems have been used to distinguish between (1) different cell types; (2) normal, cancerous, and metastatic human breast cells; and (3) isogenic normal, cancerous, and metastatic murine epithelial cell lines.

*Localized Surface Plasmon Resonant-based sensing*

When gold nanoparticles approach each other and aggregate, the color of the colloidal solution changes from red to blue. However, colorimetric assays are limited to a certain concentration range of gold nanoparticles. Furthermore, the detection of colorized analytes can be problematic due to overlap of the UV-VIS adsorption spectrum of the analyte and the SPR peak of the gold nanoparticles. Sensors based on the localized surface plasmon
CHAPTER 1 - GENERAL INTRODUCTION AND AIM OF THE THESIS

resonance of gold nanoparticles can exploit interactions between an analyte in solution and a recognition element immobilized on the SPR sensor, depending on the refractive index of the interfacial region. At specific incident wavelengths, gold nanoparticles generate strong light scattering and intense surface plasmon absorption bands appear. Exploiting this principle, robust gold nanorods substrates were developed for refractive index sensing. In those works, gold nanorods were PEGylated with a thiol terminated PEG and then deposited on the surface of APTES coated glass microscopy slides. Interaction with a specific secondary antibody was subsequently monitored through the shift in the SPR spectral extinction peak, through careful control of the surface chemistry of the nanorods. Additionally, binding kinetics could be measured, demonstrating that gold nanorod based SPR sensors can monitor real-time dynamic interactions.

**Surface enhanced Raman scattering (SERS)-based sensing**

Colloidal gold nanoparticles are able to amplify the Raman scattering efficiency of adsorbed molecules as much as $10^{14}$- to $10^{15}$-fold, attributed to a local electromagnetic field enhancement induced by the enhanced electrical field around the nanoparticles as a result of the oscillating electron upon light irradiation. The surface enhanced Raman scattering is dependent on the size, shape, orientation and aggregation of the nanoparticles, allowing spectroscopic detection and identification of single molecules under ambient conditions. For example, based on the use of PEGylated colloid gold and surface enhanced Raman scattering, a class of nontoxic nanoparticles for *in vivo* tumor targeting and spectroscopic detection was reported. Large optical enhancements can be achieved for tumor detection in live animals. The presence of a PEG coating was reported to stabilize gold nanoparticles under very harsh conditions, including strong acids (0.1M HCl), strong bases (0.1-1M NaOH), concentrated salts (1-2M NaCl) and organic solvents (methanol, ethanol and DMSO), without displacing the small-molecule Raman reporters and changing their SERS signals.

**Computed tomography contrast agents**

X-ray computed tomography (CT) is one of the most useful diagnostic tools in clinic. Due to the high X-ray absorption coefficient, gold nanoparticles are widely used as CT contrast agents. Plasma proteins and salts in blood can adsorb specifically onto the surface of bare
gold nanoparticles, leading to the rapid clearance of the particles from the bloodstream. To be used \textit{in vivo}, gold nanoparticles have been modified with antifouling agents to prolong their circulation time. PEG was reported for the synthesis of antifouling gold nanoparticles, allowing the particles to act as a CT contrast agent for angiography and hepatoma detection \textit{in vivo}.

\textbf{Photothermal therapy}

Gold nanoparticles have an absorption maximum in the visible or near-IR region. Upon light irradiation with the corresponding wavelength into the localized SPR bands of gold nanoparticles, the excited conduction band electrons decay to the ground state by releasing their energy as heat to the surrounding medium,\textsuperscript{99} inducing cell death if the particles are located inside or around target cells.\textsuperscript{77, 99, 100} Based on this transfer from light to heat, photothermal therapy using gold nanoparticles is currently a promising research area in the treatment of cancer and infectious diseases, and the effectiveness is dependent on the particles size, shape, structure and aggregation, as well as irradiation power.\textsuperscript{101, 102} Among these particles, gold nanorods enjoy the advantages of easy preparation, with tunable plasmonic absorption, and have been used to ablate tumors in mouse model of colon cancer. Upon NIR irradiation, which allows for high-depth photothermal therapy in tissue due to the higher penetration of light at these wavelength, PEG coated gold nanorods injected in the tail veins of nude mice resulted in a local temperature to 70°C in tumor tissues after 5 min of illumination, which fully destroyed all irradiated tumors.\textsuperscript{103} Combining the intense SERS effect with photothermal heating, gold nanoparticles can be a platform for multiplexed NIR detection and remote-controlled therapy.\textsuperscript{104}

\textbf{Drug & Gene delivery}

Efficient delivery and release of therapeutic agents is crucial for effective therapy. Gold nanoparticles have provided a novel platform for targeted-specific delivery of various payloads, including small drug molecules, proteins, and nucleic acids like DNA or RNA.\textsuperscript{105-107} Tunable size and functionalization allows them to be used as a useful scaffold for efficient recognition and delivery. The high surface area of nanoparticles provides sites for drug loading and enhances solubility and stability of loaded drugs. For example, a highly efficient
drug vector for photodynamic therapy (PDT) drug delivery was developed by synthesizing PEGylated gold nanoparticles with reversible PDT drug adsorption properties.\textsuperscript{108} The drug loaded onto the PEGylated gold nanoparticles could be shielded from serum proteins and could be delivered to the target tumor efficiently. Besides solid nanoparticles, hollow gold nanostructures are also excellent candidates for drug delivery applications.\textsuperscript{2} Gold nanocubes covered with responsive polymers were used to develop a ‘smart’ controlled release delivery system with NIR light (Figure 8).\textsuperscript{109} Drug molecules encapsulated in the hollow interior could be released upon collapse of the thermo-responsive polymers, which resulted from the light conversion to heat.

Gold nanoparticles provide attractive candidates for gene delivery.\textsuperscript{110-112} By tuning the charge and hydrophobicity of monolayer coverage on gold nanoparticles, the transfection efficiency has been reported to increase with decreasing toxicity. Polyelectrolyte coated gold nanorods, prepared by a layer-by-layer deposition method,\textsuperscript{113} demonstrated excellent long-term optical stability even after 4 weeks of storage and higher transfection efficiency.

**Cytotoxicity of Gold Nanoparticles**

Gold nanoparticles have considerable potential for use in the biomedical field, especially for imaging, diagnostics and therapy, which requires their toxicity to be thoroughly examined.
with maximum care and accuracy. Following internalization by cells, gold nanoparticles are typically localized inside highly acidic endosomes and/or lysosomes. It appears that gold nanoparticles usually show rather low toxicity, and those reports that do claim cytotoxicity of ligand-stabilized goldNP often attribute this to the use of toxic ligands such as cationic ones. However, systematic toxicity studies remain required for each specific system, before it could enter any clinical phase.

**Conclusions**

The successful synthesis of polymer coated gold nanoparticles has allowed researchers worldwide to obtain core-shell structures of a new type of material, which combines the properties of inorganic gold nanoparticles and organic polymeric macromolecules. The development of a practical approach for assembling polymers onto gold nanoparticles is crucial in the realization of basic applications, but still remains challenging. The use of a ‘grafting from’ approach to prepare polymer shell covered gold nanoparticles has the ability to control and achieve higher surface graft density of polymer brush by anchoring initiators on the particle surface. However, it should be taken into account that protective ligands could be cleaved from the gold nanoparticles surface under reaction conditions. The tolerance of the polymerization agents to the basic or acidic media is another issue that should receive attention. Via a ‘grafting to’ approach, gold nanoparticles protected with polymers can be prepared directly and facilely. However, the steric hindrance imposed by the grafted polymer chains may be a problem in case of the ‘grafting to’ approach to prepare polymer brush of a high graft density. Further attempts should be directed to establish correlations between the nanoparticle parameters and the observed biological effects for better understanding of the structure-property relationship of polymer decorated gold nanoparticles in a biological context.
**CHAPTER 1 - GENERAL INTRODUCTION AND AIM OF THE THESIS**

**Aim of the Thesis**

The overall aim of this thesis is to investigate the engineering of interactive properties into gold nanoparticles by decorating them with RAFT-based polymers. More specifically, we investigated in detail how the interaction can be engineered between gold nanoparticles mutually, between gold nanoparticles and solid surfaces, living cells and viruses.

In **CHAPTER 2**, we aimed to investigate the effect of salt on the temperature induced reversible aggregation of polymer coated gold nanoparticles, as well as on hydrogen bonding based layer-by-layer assembly with tannic acid. Although the preparation of temperature-responsive gold nanoparticles has been reported via a variety of methods, not much has been reported on how the solvent quality influences the thermosensitive properties of these particles. Therefore, there is still a need to understand the influence of salt on the self-assembly of polymer-gold nanoparticles hybrid structures. The obtained knowhow was then used in **CHAPTER 3** to develop a salt-driven deposition method, allowing for depositing metal nanoparticles on a wide variety of solid surface under all aqueous conditions.

It is known that size, shape and surface chemistry of gold nanoparticles can affect their uptake by living cells. In **CHAPTER 4**, we aimed to explore how the polymer coating with different hydrophilic-to-hydrophobic ratios affects the cellular uptake of gold nanoparticles. In addition, a novel flow cytometry method was developed for label-free investigation of gold nanoparticles-cell interaction and then used to elucidate in more depth the parameters that play a role in the cellular internalization of gold nanoparticles coated with polymers containing various hydrophilic-to-hydrophobic monomer ratios.

The aim of the **CHAPTER 5** was to develop a straightforward strategy to fabricate bioactive glycosylated gold nanoparticles, allowing for rapid and sensitive detection of influenza virus. A combination of RAFT polymerization, carbohydrate ligation via reductive amination and thiol-gold self-assembly will be explored to design glycolpolymer decorated gold nanoparticles that exhibit multivalent interaction with hemagglutinin on the surface of the influenza virus.

In **CHAPTER 6**, the results obtained in this thesis were discussed in view of the broader international context and the future developments to be expected in this field of research.


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CHAPTER 2

SALT PLAYS A PIVOTAL ROLE IN THE TEMPERATURE-RESPONSIVE AGGREGATION AND LAYER-BY-LAYER ASSEMBLY OF POLYMER-DECORATED GOLD NANOPARTICLES

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Abstract

We report that the self-assembly behavior in aqueous medium of citrate based gold nanoparticles decorated with the temperature responsive RAFT-based polymer poly(N-isopropylacrylamide) critically depends on the presence of salt in the medium. Both for temperature induced reversible agglomeration and for hydrogen bonding based layer-by-layer assembly with tannic acid, salt dramatically promotes the assembly behavior. We attribute this to a combination of ionic screening of remaining citrate groups on the nanoparticle surface and a salting out effect which increases the contribution of hydrophobic interactions in the self-assembly process. These findings provide new insights into an attractive class of polymer/gold hybrid nanomaterials that can find application in biotechnology, catalysis and biomedicine.
Introduction

Nanomaterials hold great promise for a wide variety of applications including in the biomedical field, e.g. as imaging tools, phototherapy agents or as drug carriers. In this regard, their surface properties play a critical role in determining the outcome of their interactions with the physiological environment. Amongst many different classes of nanomaterials, gold nanoparticles (goldNP) functionalized with a responsive polymeric coating that is sensitive to external stimuli, including biological cues, light irradiation and changes in pH or temperature, have gained considerable interest for applications in a diverse range of areas embracing catalysis, imaging, diagnostics and therapeutic purposes. Envisioning biomedical applications, gold nanoparticles have the advantage of being chemically inert, having a track record in clinical use for the treatment of rheumatoid arthritis and the versatile option to modify the goldNP surface by virtue of the quasi covalent interaction between gold and (functional) thiols or, more generic, sulfur-based compounds (Scheme 1). Importantly, goldNP exhibit interesting optical properties, owing to surface plasmon resonance of the collective oscillation of electrons, that can be fine-tuned by altering the size and the shape of the goldNP.

The synthesis routes towards polymer coated gold nanoparticles include either the use of pre-synthesized polymers or surface-initiated polymerization from gold nanoparticles. The use of pre-formed polymers is more versatile as it allows a thorough characterization of the polymers combined with a higher degree of freedom regarding reaction conditions, introduction of functionalities or using mixtures of polymers. Using pre-synthesized polymers, one can either reduce gold salts in the presence of sulfur end-capped polymers or graft such polymers onto preformed goldNP via ligand exchange. The latter option offering a higher degree of versatility regarding the size and shape of the gold nanoparticles. Well-defined polymers synthesized via reversible addition-fragmentation chain transfer (RAFT) polymerization have inherent gold binding abilities originating from the use of chain transfer agents (CTA) bearing a di- or trithio carbonate or xanthate group.
These sulfur containing compounds can either adsorb as such onto metallic gold or are reduced into thiols at the gold surface; both mechanisms have been suggested in literature. Additionally, hetero-functional polymers can be easily designed via the judicious selection of RAFT chain transfer agents, which could be used for further conjugation thereby widening the scope of applications.

The preparation of temperature-responsive gold nanoparticles has been previously reported via a variety of methods. Ligand exchange with the sulfur-containing end group of RAFT polymers, including temperature-responsive polymers (Scheme 2), has definitely emerged as one of the most simple routes to obtain well defined hybrid polymer@goldNP constructs. Surprisingly, not much has been reported on how the solvent quality influences the temperature-responsive properties of such polymer@goldNP while salting in and salting out following the Hofmeister series has been reported to strongly influence the phase behavior of temperature-responsive polymers. Up to our knowledge there is one paper describing the influence of salt in aqueous medium on the phase transition behavior of gold nanoparticles coated with a temperature responsive polymer. However, in this study, the aqueous medium contained an excess of free polymer which will induce important cooperative effects with the goldNP grafted polymers, as described by Gibson and co-workers. Therefore, there is still a need to fully understand the influence of salt on the assembly behaviour of pure polymer-coated goldNP into higher order structures. In this chapter we investigate the effect of salt on goldNP coated with a temperature-responsive polymer with respect to temperature-triggered phase transition and hydrogen bonded Layer-by-Layer assembly.
Results and Discussion

*Synthesis and characterization of polymer coated goldNP*

Poly(N-isopropylacrylamide) (polyNIPAm) was chosen as model temperature-responsive polymer in this study. PolyNIPAm is one of the most documented temperature-responsive polymers with a cloud point temperature ($T_{CP}$, i.e. the temperature at which the polymer precipitates from solution due to entropic reasons) of around 32°C which remains, although being slightly affected by molecular weight and polymer end-group, relatively constant over a wide concentration range. Well defined polyNIPAm with a molecular weight of $M_{n,th}$ of 6.9 kDa and a dispersity (Ð) of 1.07 was synthesized via RAFT polymerization according to the reaction scheme depicted in **Scheme 2**. To avoid pH effects caused by the end group of the RAFT chain transfer agent (CTA), a CTA was synthesized with a methyl ester end group. Turbidity measurements on 5 mg/mL aqueous solutions of the synthesized polyNIPAm revealed a $T_{CP}$ of 29.7°C, which is slightly lower than the typical value of 32°C found in literature and can be attributed to the influence of the hydrophobic end groups on a relatively low molecular weight polyNIPAm.

**Scheme 2.** Reaction scheme of the RAFT polymerization of polyNIPAm. Note that the RAFT chain transfer agent (CTA) contains a methyl ester end group to avoid pH effects on the temperature responsive behavior.

Citrate stabilized goldNP were synthesized following the Turkevich method by direct reduction of the HAuCl₄ salt with citrate in aqueous medium under reflux. A deep red solution was obtained containing goldNP with a mean diameter of 13 nm ± 3 nm as verified by TEM (processed by automated image analysis via ImageJ) and a surface plasmon peak of 525 nm as measured by UV-VIS spectrophotometry.
Mixing of aqueous solutions of citrate goldNP and polyNIPAm was subsequently performed in absence and presence of n-propylamine. n-Propylamine aminolyses the trithiocarbonate group of the RAFT agent into a free thiol which could facilitate ligand exchange with citrate. On the other hand also un-cleaved RAFT agents have been reported to efficiently adsorb on gold. In this regard, the issue whether the RAFT agent is cleaved on the gold surface or adsorbs as such, is still a matter of debate. In our hands we observed no influence of the addition of propylamine. In both cases stable colloidal solutions were obtained that could be centrifuged and redispersed in pure water without colour change. This is a first indication that the goldNP were successfully decorated with polyNIPAm as bare citrate stabilized goldNP irreversibly aggregate during centrifugation and removal of the excess citrate. In total the polyNIPAm@goldNP were centrifuged and redispersed three times to completely remove the excess of unbound polyNIPAm. We also verified that the supernatant after the second centrifugation and washing step did not exhibit any phase transition anymore upon heating, indicating effective removal of the polyNIPAm.

Clear proof that the goldNP were indeed coated with polyNIPAm was gained by transmission electron microscopy (TEM). As shown in Figure 1A, bare goldNP are aggregated and form piled assemblies – due to drying upon sample preparation – on the TEM grid. By contrast, the goldNP that were coated with polyNIPAm are well dispersed and clearly show the presence of an approximately 3 nm thick corona surrounding each individual particle (Figure 1B). Next we analysed the goldNP with and without polyNIPAm coating by dynamic light scattering (DLS) and UV-VIS spectroscopy. Uncoated goldNP show a mean (volume average) diameter of 15 nm on DLS measurements while after coating a hydrodynamic diameter of 49 nm is measured (Figure 1C). This increase in diameter can be attributed to tethering of the polyNIPAm to their surface as the length of a 6.9 kDa polymer is estimated to be around 12 nm in the fully stretched state. However, from these data the presence of a minor fraction of aggregates that are formed due to the repetitive centrifugation steps cannot be completely ruled out. The UV-VIS spectra recorded of the particles in solution reveal a slight red shift of the plasmon peak from 525 nm to 529 nm upon polyNIPAm coating (Figure 1D). The observation of a slight red shift of the plasmon peak is in accordance with earlier reports by Li and Gibson stating that red shifts smaller than 20 nm are attributed to polymer grafting rather than the formation of
agglomerates. In this regard it is also noteworthy that TEM analysis of over 1000 polyNIPAm@goldNP did not reveal the presence of aggregates.

Figure 1. Transmission electron microscopy (TEM) images of (A) uncoated and (B) polyNIPAm coated goldNP. The zoomed image in panel B2 clearly indicates the presence of a polymer coating surrounding the goldNP. Size distribution (C) and UV-VIS spectra (D) of uncoated and polyNIPAm coated goldNP obtained via DLS and UV-VIS spectroscopy, respectively.

Figure 2. Photographs of plastic cuvettes containing polyNIPAm@goldNP in water (A) and in 0.1 M NaCl (B). The numbers (1 -> 3) in the upper left corner refer to polyNIPAm@gold solutions (1) at room temperature, (2) heated to 40°C and (3) cooled back to room temperature.
Temperature-responsive properties of polymer coated goldNP

In a next series of experiments we aimed at investigating the temperature responsive properties of the polyNIPAm coated goldNP, at first in deionized water. We found that simply heating a polyNIPAm@goldNP solution (0.18 mg/mL on gold base) above the $T_{CP}$ of the polyNIPAm did not induce any colour change of the goldNP solutions, even up to the boiling temperature of the aqueous medium. Also no change in turbidity of the polyNIPAm@goldNP solutions was observed. Even very concentrated (1 mg/mL on gold base; achieved via centrifugation) polyNIPAm@goldNP solutions did not exhibit any temperature-responsive behavior. When these experiments were repeated with polyNIPAm that was treated with propylamine to assure the presence of a thiol end group on the polyNIPAm, still no temperature responsive behaviour could be observed. These findings already rule out the influence of the polymer end group, i.e. thiol vs. trithiocarbonate, on the synthesis of temperature responsive polyNIPAm@goldNP. These observations further add to the unresponsive behaviour of the nanoparticles themselves. DLS and UV-VIS measurements confirmed these findings, yielding constant values of particle size and position of the plasmon peak as function of temperature. Control experiments with freely soluble polyNIPAm (1 mg/mL) added to goldNP do show phase transition above the $T_{CP}$ of the polyNIPAm which is expressed as an increase in turbidity with a slight shift in plasmon peak of the goldNP.

However, when the polyNIPAm@goldNP were dispersed in 0.1 M sodium chloride (NaCl), rather than deionized water, the nanoparticles did exhibit temperature-responsive behavior. Firstly, visual proof was gained by simply heating the polyNIPAm@goldNP solutions until 40°C which induced a colour change from red to bluish of the polyNIPAm@goldNP solution. After cooling back to room temperature the solution regained its red colour. Figure 2 shows a series of snapshots taken from polyNIPAm@goldNP at different temperatures with (A) and without (B) salt added to the medium. From literature it is also known that salt affects the $T_{CP}$ of polyNIPAm. We confirmed this (Figure 3) for the polyNIPAm used in our present study and observed in presence of 0.1 M NaCl, a decrease in $T_{CP}$ and a broadening of the temperature interval over which the phase transition takes place.
To investigate the phase transition behavior of the polymer coated gold nanoparticles and the effect on their optical properties in detail, we monitored their size and plasmon peak as function of temperature by DLS and UV-VIS, respectively. Figure 4A shows that in pure water uncoated citrate stabilized goldNP remain invariant in size and the plasmonic properties also remain constant (Figure 4B1 and 4C1) over the temperature range from 25 °C to 40 °C, which is in agreement with our earlier visual observations in this chapter. However, in presence of 0.1 M NaCl, temperature-responsive behavior emerges. Both DLS and UV-VIS indicate the onset of nanoparticle aggregation to be 29 °C, followed by a steady increase in size of the aggregates (Figure 4). At this transition temperature of 29°C, also the plasmon peak undergoes a red shift from 530 nm at 25 °C to 565 nm at 40°C accompanied by a decrease in absolute value of the absorbance (Figure 4B-C).28, 29 Figure 5 depicts a zoomed view on the interval of the phase transition. Note that data of uncoated goldNP in 0.1 M NaCl are not shown because of immediate precipitation from solution of the goldNP. In salt solutions, the electrostatic charges on the surface of the citrate@goldNP are screened and do not provide sufficient repulsion to keep the citrate@goldNP colloidally stable. The substantial red shift in plasmon peak of the polyNIPAm@goldNP in salt above the T_{CP} of the polyNIPAm can

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**Figure 3.** Transmittance of polyNIPAm (5 mg/mL) measured in water and in 0.1 M NaCl. Note that the increase in transmittance of the polyNIPAm in 0.1 M NaCl above 33 °C is due to the formation of large aggregates which decrease the overall turbidity of the solution. This formation of larger aggregates can be attributed to the effect of salt which enhances hydrophobic interaction between the collapsed polyNIPAm chains.
unambiguously be attributed to electronic dipole-dipole interaction between plasmons of goldNP that come into close proximity with each other.\textsuperscript{30}

\textbf{Figure 4.} (A) Evolution of particle size as function of temperature measured by DLS. (B) UV-VIS absorbance as function of temperature of polyNIPA@goldNP in (B1) water and (B2) 0.1 M NaCl. (C) Evolution of the wavelength of maximum absorbance (red curve) and the absorbance at this wavelength (blue curve) as function of temperature of polyNIPA@goldNP in (C1) water and (C2) 0.1 M NaCl.
Next, we measured the ζ-potential values (Figure 6) of the polyNIPAm@goldNP in presence and absence of salt. A strongly negative value of -40 mV was measured for polyNIPAm@goldNP in absence of salt which is only slightly less negative than the ζ-potential of -47 mV measured for bare citrate@goldNP. This suggests that a certain fraction of citrate is still bound to the goldNP surface, providing the negative ζ-potential. However, when we measure the ζ-potential in presence of 0.1 M of NaCl, a near neutral value of -1 mV is observed, which we attribute to screening of the negative citrate charges by Na⁺ ions. Thus, in absence of salt, when the polyNIPAm chains collapse above the T<sub>CP</sub>, there is still electrostatic repulsion between the gold nanoparticles due to the remaining citrate groups. By contrast, in presence of salt, the charges are screened, thereby strongly reducing electrostatic repulsion and allowing above the T<sub>CP</sub> the dehydrated polyNIPAm chains to agglomerate. Additionally, taking into consideration the substantial effect of salt on the T<sub>CP</sub> of polyNIPAm (Figure 3; 0.1 M NaCl induces a shift of the T<sub>CP</sub> to lower temperature and a broadening of the phase transition interval) also partial dehydration of the polyNIPAm chains in presence of NaCl (i.e. salting out effect) can contribute to enhanced inter-particle hydrophobic interactions by enhancing dehydration of the polyNIPAm chains.

Figure 5. Zoomed image on the interval in which the phase transition occurs. Note that data of uncoated goldNP in 0.1M NaCl are not shown as citrate@goldNP precipitate immediately from solution of the goldNP upon addition to salt solution.
CHAPTER 2 – SALT PLAYS A PIVOTAL ROLE IN THE TEMPERATURE-RESPONSIVE AGGREGATION AND LAYER-BY-LAYER ASSEMBLY OF POLYMER-DECORATED GOLD NANOPARTICLES

Figure 6. ζ-potential values of goldNPs. In absence of NaCl, goldNPs with and without polyNIPAm coating display a pronounced negative ζ-potential. However, near neutral ζ-potential values are measured in presence of 0.1 M NaCl.

Hydrogen bonded Layer-by-Layer assembly with polymer coated goldNP

In a final series of experiments we aimed at elucidating whether salt also plays a crucial role in the assembly of higher order structures containing polyNIPAm@goldNP. As water-soluble amide-containing polymers, such as polyNIPAm, are known to form donor-acceptor complexes with hydrogen bond donors such as tannic acid (TA), we attempted to assemble the polyNIPAm@goldNP together with TA in a Layer-by-Layer (LbL) fashion. This means that TA and polyNIPAm@goldNP will be alternately adsorbed on a substrate while hydrogen bonding between the phenol groups of TA and the amide bonds of polyNIPAm, likely aided by hydrophobic interactions as well, will act as driving force for multilayer formation. Scheme 3 schematically represents the interaction between the amide groups of the polyNIPAm and the phenol groups of TA. In the framework of the present research we aimed at elucidating the effect of salt on the formation of TA/polyNIPAm@goldNP multilayers.
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Scheme 3. Schematic representation of the hydrogen bonding interaction between TA and polyNIPAm@goldNP.

Figure 7A shows UV-VIS spectra recorded from TA/polyNIPAm@goldNP multilayers absorbed on quartz slides in absence and presence of 0.1 M NaCl salt. Additionally also the evolution of the absorbance at 279 nm (i.e. adsorption maximum of TA) and 560 nm (i.e. the plasmon peak of the polyNIPAm@goldNP in the multilayer film) is represented as function of the number of deposition cycles (Figure 7B). In both conditions, i.e. with and without salt, alternating deposition of TA and polyNIPAm@goldNP onto quartz slides leads to an increase in absorbance. However, in presence of 0.1 M of NaCl salt, a dramatic increase in amount of adsorbed material is observed. The same trend is also witnessed by real time quartz crystal microbalance (QCM) monitoring of the assembly of TA and polyNIPAm@goldNP. Indeed, as shown in Figure 7C2, depicting a larger drop in resonance frequency of the quartz chip when TA and polyNIPAm@goldNP were alternately flown over the quartz chip in presence of salt versus in absence of salt. The influence of salt concentration on Layer-by-Layer assembly of oppositely charged polymers via electrostatic interaction is well known.36 Salt ions screen the electrostatic charges along the polymer chains thereby inducing a rod to coil conformational change which allows a higher mass to be deposited during each deposition step. However, case of hydrogen bonded Layer-by-Layer assembly, electrostatic interaction does not play a role and the influence of salt on the assembly behavior is, contrary to the influence of pH,37,38 unexplored. In accordance to our findings, the role of salt will be a combination of reducing the electrostatic repulsion between the polyNIPAm@goldNP and enhancing hydrophobic interactions. From both the UV-VIS and QCM measurements the most significant growth in layer thickness is witnessed for the first deposition cycle of polyNIPAm@goldNP. This suggests that likely no distinct strata of TA and nanoparticles are obtained.
To further characterize the film growth, we performed atomic force microscopy (AFM) to characterize the TA/polyNIPAm@goldNP multilayers formed in 0.1 M aqueous NaCl solution. Figure 8, shows 1x1 µm scans of silicon substrates that were coated with respectively 1, 5 and 10 cycles of TA/polyNIPAm@goldNP. After the first deposition cycle of TA/polyNIPAm@goldNP, the substrate is clearly coated with a layer of nanoparticles. From this AFM image and taking into account a diameter of the goldNP of 13 nm (measured by TEM), we calculated a surface coverage of 9.3 % (on gold base). We also calculated the surface coverage based on the shift in resonance frequency $\Delta f = 253$ Hz measured by QCM upon adsorption of the polyNIPAm@goldNP onto a TA layer (see the Methods section for the detailed calculation) and obtained a value of 6.7 %, which is in fairly good agreement with the values derived from the
AFM data. The AFM images corresponding to 5 and 10 deposition cycles reveal features beyond the dimensions of a single particle and correspond to piled assemblies of particles. These observations confirm the UV-VIS and QCM data suggesting a slighter film growth after the first TA/polyNIPAm@goldNP deposition cycle.

**Figure 8.** AFM images in tapping mode (topography channel) of TA/polyNIPAm@goldNP films after 1 (A), 5 (B) and 10 (C) assembly steps. The height profile was measured along the X axis in the middle of the image, as indicated by the dotted line.
Conclusions

In summary, we have demonstrated that salt plays a crucial role regarding the temperature responsive behavior of purified citrate stabilized goldNP that were grafted through ligand exchange with thiol-functional polyNIPAm synthesized via RAFT polymerization. Whereas in pure water these particles were found insensitive to temperature, the polyNIPAm@goldNP did show temperature-responsive behavior in aqueous 0.1 M sodium chloride solutions. Additionally, the presence of salt also strongly enhances Layer-by-Layer build-up of polyNIPAm@goldNP with tannic acid. We attribute these observations to a combination of ionic screening of remaining citrate groups on the nanoparticle surface and a salting out effect on the polyNIPAm, causing a stronger dehydration of the polyNIPAm chains, thereby enhancing hydrophobic interactions. We believe our findings will contribute to a better understanding of the self-assembly behavior of polymer@goldNP hybrid nanostructures. In forthcoming work we will explore multiple types of temperature-responsive systems which also appear to yield salt-dependent responsive metal nanoparticles. This is important in view of the design of functional higher order structure for a wide variety of applications ranging diagnostics to catalytically and optically active surfaces.
Materials and Methods

Materials

All chemical were purchased from Sigma-Aldrich unless otherwise stated. The CTA with methyl ester group was synthesized by esterification of methanol with 3-(((butylthio)carbonothioylthio)-2-methylpropanoic acid. Purified Milli-Q grade water was used for all experiments.

Polymerization of NIPAm (performed by Dr. Samarendra Maji (Prof. Hoogenboom group))

The RAFT polymerization of N-isopropyl acrylamide (NIPAm) was performed in a 25 mL Schlenk tube under nitrogen atmosphere. In a typical run, NIPAm (2 g, 17.67 mmol), methyl 2-(butylthiocarbonothioylthio)propanoate (MBTTC) (44.61 mg, 0.18 mmol), 2,2'-azobisisobutyronitrile (AIBN) (14.51 mg, 0.09 mmol) and DMF (8.8 mL) were charged in the 25 mL Schlenk tube at a molar ratio of 100:1:0.5. The solution was deoxygenated by three freeze-pump-thaw cycles. Then the reaction mixture was placed in a preheated oil bath at 60°C to initiate the polymerization. After 50 min of polymerization, the reaction vessel was removed from oil bath and opened to the air to stop the polymerization. Polymer was first precipitated by dropping the polymer solution into large amount of a 50:50 mixture of hexane and diethyl ether. After decantation of the solvent the polymer was dissolved in tetrahydrofuran (THF) and precipitated again in diethyl ether. This precipitation procedure was repeated three times. The powdery light yellow polymer was dried overnight under vacuum at 50°C.

By determining the conversion via gas chromatography (GC), a molecular weight of $M_{n,th}$ of 6.9 kDa was measured. $^1$H-NMR analysis indicated a $M_{n,NMR} = 7.6$ kDa. Size exclusion chromatography (SEC) in dimethylacetamide (DMA) showed $M_{n,SEC} = 15.4$ kDa against PMMA standards and a dispersity (D) of 1.07.

Cloud points were measured on a Cary 300 Bio UV-Visible spectrophotometer at a wavelength of 600 nm with a temperature controller. Aqueous polymer solutions (5mg/mL) were heated from 10 to 40°C with a heating rate of 1.0°C/min followed by cooling to 10°C at a cooling rate.
of 1.0°C/min. This cycle was repeated two times. The cloud points are reported as the 50% transmittance temperature in the second heating run.

**Synthesis of citrate stabilized gold nanoparticles**

Citrate stabilized gold nanoparticles were synthesized according to the reported literatures. All glassware was first washed with aqua regia and then rinsed with MilliQ water several times prior to synthesis. Briefly, 20 mL of 1mM HAuCl₄ was refluxed for 30 min. Then 2 mL of 1wt. % sodium citrate was quickly added and the color of solution changed from yellow to wine red within 5 min. After cooling, the reaction solution was stored at 4°C for further research.

**PolyNIPAm coating of gold nanoparticles**

9 mL of a citrate stabilized gold nanoparticles solution was mixed with 200 µL of an aqueous solution containing 8 mg polyNIPAm, and stirred overnight at room temperature. The resulting conjugates were three times purified by centrifugation at 4°C with 10 000 g for 30 min followed by re-dispersion in pure water.

**Characterization**

*Transmission Electron Microscopy (TEM)*

A drop of gold nanoparticle solution was allowed to air-dry onto a Formvar-carbon-coated 200 mesh copper grid, and visualized using 80 keV TEM (Jeol 1010, Japan). TEM images of the goldNPs were processed via ImageJ to determine the number average size distribution in dry state.

*UV-VIS spectroscopy*

UV-VIS spectra were acquired with a Shimadzu UV-1650PC spectrophotometer. Gold nanoparticles with different coatings were placed in plastic cuvettes and spectral analysis was performed in the 200 to 800nm range at room temperature. In the case of temperature-dependent measures (gold concentration of 0.09 mg/mL), a Cary 300 Bio UV-Visible spectrophotometer was utilized to monitor the absorbance via changing the temperatures from 20 to 40°C.
Dynamic Light Scattering (DLS)

The size and ζ-potential of the particles (gold concentration of 0.18 mg/mL) were measured by photon correlation spectroscopy using a Malvern Zetasizer Nano Series operating a 4mW He-Ne laser at 633nm. All samples were filtered through a 0.2µm filter prior to analysis. Analysis was performed at an angle of 90° and at constantly changing temperatures from 20 to 40°C at a heating rate of 1.0°C/min.

Layer-by-Layer assembly of polyNIPAm@goldNP and TA

QCM monitoring of LbL assembly

Quartz crystal microbalance monitoring of the Layer-by-Layer assembly was performed using a Gamry eQCM 10M quartz crystal microbalance equipped with an ALS flow cell. Gold coated quartz chips with a nominal resonance frequency of 10 MHz were first pre-coated by 1h immersion in an aqueous solution of mercaptosuccinic acid (2 mg/mL) followed by extensive rinsing with water. Secondly, the quartz chip was immersed into an aqueous poly(ethyleneimine) PEI solution (2 mg/mL) for 1h and again extensively washed with water and dried under a gentle nitrogen stream. Next, the chip was mounted into the flow cell and water was injected and the measurement was started in continued until a flat baseline was obtained. Then the measurement was restarted and after 100s, 0.3 mL of a 2 mg/mL TA solution was injected into the flow cell. After 100s, when the signal leveled off, indicating that saturation of the surface was obtained and longer incubation times would not lead to further adsorption of material. The flow cell was then rinsed by injection of 0.7 mL water. Next 0.3 mL of polyNIPAm@goldNP solution was injected followed a after 100 s by 0.7 mL of water. This procedure was repeated for 5 TA/polyNIPAm@goldNP deposition cycles.

The coverage of the surface upon deposition of the first layer of polyNIPA@goldNP was calculated from the Sauerbrey equation.\(^40,41\)

\[
\Delta f = -\frac{2f_0^2}{A\sqrt{\rho_q \mu_q}} \Delta m
\]

With \(f_0=10 \text{ MHz}\) the fundamental resonance frequency of the quartz crystal, \(A= 0.2047 \text{ cm}^2\) the electrode area, \(\rho_q (\sim 2.65\text{g.cm}^{-2})\) and \(\mu_q (\sim 2.95\text{g}\cdot\text{s}^{11}\cdot\text{cm}^{-2})\) are the shear modulus and
density of quartz, respectively. From the obtained $\Delta f$ of 253 Hz, $\Delta m/A =1124 \text{ ng.cm}^{-2}$ was calculated as the mass change per area. Considering the diameter of the goldNP to be 13 nm (as measured by TEM) and the density of gold to be 19.3 g.cm$^{-3}$, the number of goldNP per cm$^2$ was calculated to be $5.066 \times 10^{10}$ which corresponds to a surface coverage of 6.72 %.

**Atomic Force Microscopy (AFM)**

The morphology of the polyNIPAm@goldNP /TA hybrid films was investigated by tapping mode AFM. Experiments were performed on air-dried films deposited onto silicon wafers. Images were obtained in tapping mode under ambient conditions in air with Bruker Innova equipped with a 100 µm scanner. Antimony doped silicon AFM probes were used with a nominal spring constant of 3 N/m and a resonance frequency of 75 KHz. From the number of particles on a 1 µm$^2$ image and a mean particle diameter of 13 nm (as determined by TEM) a surface coverage of 9.32 % which is in fairly good agreement with the value obtained by QCM.

**UV-VIS monitoring of LbL assembly**

Quartz slides (Hellma Optics) were cleaned in piranha solution (a 3:1 mixture of sulfuric acid and hydrogen peroxide. Caution, piranha is extremely corrosive and should be handled with extreme care and not be stored in closed containers!) for 10 min, followed by rinsing with water. After thorough cleaning, the quartz substrate was immersed in a 2 mg/mL of polyethylene imine (22 kDa; PEI) solution for 10min to facilitate deposition of the subsequent layer. After exhaustive rinsing with water, the slide was immersed in a tannic acid (2 mg/mL) solution for 5 min. After rinsing with water, the substrate was transferred to 2 mg/mL (gold content) polyNIPAm@goldNP solution for 5 min. This procedure was repeated for 10 TA/polyNIPAm@goldNP deposition cycles. After the deposition of each layer, the quartz slide was dried and the UV-VIS absorption spectrum was measured using a Shimadzu UV-1650PC spectrophotometer.
References

CHAPTER 2 – SALT PLAYS A PIVOTAL ROLE IN THE TEMPERATURE-RESPONSIVE AGGREGATION AND LAYER-BY-LAYER ASSEMBLY OF POLYMER-DECORATED GOLD NANOPARTICLES
CHAPTER 3

SALT-DRIVEN DEPOSITION OF THERMO-RESPONSIVE POLYMER-COATED METAL NANOPARTICLES ON SOLID SUBSTRATES

Parts of this chapter were published in:

Salt-Driven Deposition of Thermoresponsive Polymer-Coated Metal Nanoparticles on Solid Substrates

Zhang, Z.; Maji, S.; Antunes, A.B.D.; De Rycke, R.; Hoogenboom, R.; De Geest, B.G.

ANGEWANDTE CHEMIE INTERNATIONAL EDITION 2016, DOI: 10.1002/anie.201601037
Abstract

Thin films are used in a wide range of applications including biotechnology, medicine, catalysis, microelectronics etc. Whereas bulk materials have size-independent properties, nanomaterial properties strongly depend on size and shape. This offers the opportunity to engineer solid surfaces with very specific properties by a thin layer of nanomaterials. However, there is a currently unmet need for easy and scalable methods for substrate independent deposition of nanomaterials under benign all aqueous conditions. Here we report on a simple, generally applicable method for depositing metal nanoparticles on a wide variety of solid surfaces under all aqueous conditions. Noble metal nanoparticles (i.e. gold and silver nanoparticles) obtained by citrate reduction followed by coating with temperature-responsive polymers (such as amongst others polyNIPAm) spontaneously form a monolayer-like structure on a wide variety of substrates in presence of sodium chloride whereas this phenomenon does not occur in salt free medium. We hypothesize that salt ion-induced screening of electrostatic charges on the nanoparticle surface entropically favors hydrophobic association between the polymer coated nanoparticles and a hydrophobic substrate. The attractiveness of our approach is that it allows for extremely easy engineering of bulk materials with ultrathin coatings to introduce specific properties (exemplified by plasmonic and catalytic functionality) under all aqueous conditions.
CHAPTER 3 – SALT-DRIVEN DEPOSITION OF THERMO-RESPONSIVE POLYMER-COATED METAL NANOPARTICLES ON SOLID SUBSTRATES

Introduction

Nanoparticles and nanostructured films have attracted major interest for a wide variety of applications, including biotechnology,\textsuperscript{1-3} medicine,\textsuperscript{4} photonics,\textsuperscript{5-8} microelectronics,\textsuperscript{9} catalysis, etc.\textsuperscript{10} In contrast to bulk materials, whose physicochemical properties are size-independent, many nanoparticle properties strongly depend on particle size and shape.\textsuperscript{11,12} Combining bulk materials with a thin coating of a specific nanomaterial therefore allows to engineer the bulk material with unique high added-value properties through the coating.\textsuperscript{13} The currently available toolbox for surface modification of solid materials comprises solvent casting,\textsuperscript{14} chemical vapor deposition\textsuperscript{15} and a wide variety of self-assembly approaches such as Langmuir-Blodgett,\textsuperscript{16} layer-by-layer self-assembly\textsuperscript{17-20} and self-assembled monolayer formation.\textsuperscript{21,22} In recent years, nature-inspired compounds such as polyphenols and mussel-derived polydopamine that spontaneously deposit on solid substrates have fueled the interest for simple and straightforward surface coating techniques under all aqueous conditions.\textsuperscript{23-26} Whereas these methods provide access to all-organic films, there remains an unmet need for simple assembly methods for inorganic nanoparticle coatings under aqueous conditions.

Here we report on a simple, broadly applicable method for depositing metal nanoparticle films on a wide variety of solid surfaces under all aqueous conditions. Our approach is demonstrated for gold (goldNP) and silver (silverNP) nanoparticles and allows for extremely easy engineering of bulk materials with plasmonic and catalytic functionality.

Results and Discussion

GoldNP were synthesized by direct reduction of HAuCl\textsubscript{4} in aqueous medium in presence of sodium citrate, which acts both as reducing and stabilizing agent.\textsuperscript{27,28} Subsequently, the nanoparticles were coated via ligand-exchange with defined triothiocarbonate end-functionalized poly(N-isopropylacrylamide) (polyNIPAm (PN-3, Table 1); Mn: 4.9 kDa, Φ: 1.08; Figure 1A), yielding polyNIPAm@goldNP.\textsuperscript{29} PolyNIPAm is a temperature responsive polymer that undergoes an entropy driven coil-to-globule transition upon heating in aqueous solution with a phase transition temperature (T\textsubscript{cp}) around 32 °C. Synthesis of this polymer by RAFT (Reversible Addition-Fragmentation chain Transfer) polymerization\textsuperscript{30} provides direct access to
polymers with sulfur-containing end-groups that can form a quasi-covalent bond with metallic gold and silver.\textsuperscript{31} These polyNIPAm@goldNP only exhibit temperature-triggered reversible aggregation and disaggregation when salt (e.g. NaCl) is added to the aqueous medium while remaining fully stable over a broad temperature range in absence of salt.\textsuperscript{29} The reason for this behavior is that in absence of salt, the remaining citrate ions on the goldNP surface (after polyNIPAm coating) provide the nanoparticles with a negative ζ-potential (-40 mV) providing colloidal stability even when the polyNIPAm chains are collapsed. Only when these charges are screened by counter ions, the polyNIPAm@goldNP exhibited reversible temperature-responsive behavior. Physicochemical properties of the polyNIPAm@goldNP are listed in Table 2 and the presence of the polymer coating was also evidenced by TEM (Figure 1B).

Table 1. Polymerization conditions and results for the synthesis of polyNIPAm samples in DMF.

<table>
<thead>
<tr>
<th>Sample</th>
<th>[NIPAm]:[CTA]:[AIBN]</th>
<th>Temperature (°C)</th>
<th>Reaction time (min)</th>
<th>Conv. (%)</th>
<th>$M_n$th (g/mol)</th>
<th>SEC $M_n$,SEC (g/mol)</th>
<th>$\theta$</th>
<th>$D^\theta$</th>
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<tr>
<td>PN-1</td>
<td>[100]:[1]:[0.5]</td>
<td>60</td>
<td>30</td>
<td>24</td>
<td>3000</td>
<td>7900</td>
<td>1.10</td>
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<tr>
<td>PN-2</td>
<td>[100]:[1]:[0.5]</td>
<td>60</td>
<td>40</td>
<td>35</td>
<td>4200</td>
<td>10700</td>
<td>1.08</td>
<td></td>
</tr>
<tr>
<td>PN-3</td>
<td>[100]:[1]:[0.5]</td>
<td>60</td>
<td>50</td>
<td>41</td>
<td>4900</td>
<td>11700</td>
<td>1.08</td>
<td></td>
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<tr>
<td>PN-4</td>
<td>[250]:[1]:[0.25]</td>
<td>70</td>
<td>55</td>
<td>42</td>
<td>12100</td>
<td>20400</td>
<td>1.11</td>
<td></td>
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<tr>
<td>PN-5</td>
<td>[250]:[1]:[0.25]</td>
<td>70</td>
<td>95</td>
<td>60</td>
<td>17200</td>
<td>24400</td>
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<td></td>
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<tr>
<td>PN-6</td>
<td>[250]:[1]:[0.25]</td>
<td>70</td>
<td>190</td>
<td>81</td>
<td>23100</td>
<td>41700</td>
<td>1.23</td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a} Relative against PMMA standards
Figure 1. Characterization of polyNIPAm@goldNP and deposited polyNIPAm@goldNP films. (A) Molecular structure of polyNIPAm synthesized via RAFT polymerization. (B) TEM image of polyNIPAm@goldNP. Scale bar represents 10 nm. (C) Photographs of plastic cuvettes, polymethylmethacrylate and glass slides immersed in a polyNIPAm@goldNP solution in presence (left) or absence (right) of 0.1 M NaCl followed by extensive washing with deionized water. (D) AFM images of polyNIPAm@goldNP deposited on silicon wafers.

Table 2. Physicochemical properties of polyNIPAm@goldNP (polyNIPAm properties; Mn: 4.9 kDa, D: 1.08)

<table>
<thead>
<tr>
<th>goldNP</th>
<th>Size (TEM) $^{[1]}$ [nm]</th>
<th>Size (DLS, water) [nm]</th>
<th>Size (DLS, NaCl) [nm]</th>
<th>ζ-pot (water) [mV]</th>
<th>ζ-pot (NaCl) [mV]</th>
</tr>
</thead>
<tbody>
<tr>
<td>citrate@goldNP</td>
<td>13 ± 3</td>
<td>50.1 ± 13.4</td>
<td>/ $^{[2]}$</td>
<td>-48</td>
<td>/ $^{[2]}$</td>
</tr>
<tr>
<td>polyNIPAm@goldNP</td>
<td>19 ± 3</td>
<td>101.1 ± 2.5</td>
<td>84.9 ± 8.1</td>
<td>-40</td>
<td>-0.2</td>
</tr>
</tbody>
</table>

$^{[1]}$ Processed by automated image analysis via ImageJ. Note that the discrepancy in size measured by DLS and TEM is due to the hydrodynamic radius in hydrated state that is measured by DLS. $^{[2]}$ Citrate@goldNP immediately aggregated in presence of NaCl.
We observed that irrespective of the temperature of the aqueous medium, upon addition of sodium chloride (NaCl) to an aqueous polyNIPAm@goldNP solution, in time a colored deposition spontaneously formed on the walls of the recipient, i.e. both glass sample vials and PMMA cuvettes, in which the experiment was performed. Moreover, we also observed that solid substrates that were immersed into solutions that contained 0.1 M NaCl and polyNIPAm@goldNP also became stained, thereby indicating nanoparticle deposition. Figure 1C shows photographs of plastic cuvettes as well as polymethylmethacrylate and glass slides that were coated in this way. Importantly, control experiments with polyNIPAm@goldNP in absence of salt or citrate stabilized goldNP in presence or absence salt do not lead to nanoparticle deposition clearly indicating the importance of both the thermo-responsive polymer coating as well as the presence of salt (Figure 1C). Importantly, these deposited polyNIPAm@goldNP films remain fully stable upon repeated washing with water and organic solvents demonstrating that robust stable nanoparticle coatings are obtained that can be used for further applications. Atomic force microscopy (AFM) analysis revealed that the deposited film is composed of densely packed polyNIPAm@goldNP that appear to form quasi monolayer-like structure (Figure 1D). Besides goldNP, also silverNP synthesized by sodium citrate reduction of AgNO₃ followed by partial ligand exchange with polyNIPAm, exhibited similar monolayer-forming properties (Figure 2). Such polyNIPAm@silverNP coatings could find applications for engineering anti-microbial properties onto a wide variety of substrates, including surgical rubber gloves (see Figure 2 panel b3).
To investigate whether our findings are unique for polyNIPAm-based systems, we also coated goldNP with other polar polymers that exhibit temperature-responsive behavior (i.e. poly(diethylene glycol acrylate) (polyDEGA; \(T_{cp} \sim 25 ^\circ C\)) and poly(\(n\)-propyl-2-oxazoline) (poly\(^n\)PropOx; \(T_{cp} \sim 20 ^\circ C\))^32 and polar polymers that are fully water-soluble over a broad temperature range (i.e. poly(N-vinylpyrrolidone) (polyNVP)). Interestingly, only the temperature-responsive polymers polyDEGA and poly\(^n\)PropOx yielded, in presence of 0.1 M NaCl salt, nanoparticle film coatings, whereas in absence of salt no nanoparticle deposition was observed. In case of PVP coated goldNP, no nanoparticle deposition is observed both in presence or absence of salt, independent of temperature (Figure 3). The unsuccessful deposition of polyNVP@goldNP hints to a hydrophobic driving force for the deposition process as the thermo-responsive polymers have more hydrophobic character.
To gain further insights into the driving forces for this salt-induced NP deposition process, we investigated the parameters that play a role in polyNIPAm@goldNP adsorption process. Plastic cuvettes were immersed into solutions of polyNIPAm coated goldNP containing 0.1 M NaCl and the effect on the nanoparticle deposition process of goldNP concentration, deposition time, molecular weight of the polyNIPAm and deposition temperature was studied by UV-VIS spectrophotometry. Table 1 lists the properties of the different polyNIPAm used for the investigation of the effect of polymer molecular weight. Due to the surface plasmon resonance (SPR) band of the goldNP,\(^3\) UV-VIS spectroscopy is well suited to measure deposition of the nanoparticles as the absorption intensity provides a measure for the amount of deposition while the maximum absorption wavelength provides information on the hydrophobicity of the local environment of the goldNP.\(^4\) Upon deposition of the
polyNIPAm@goldNP on the surface of the cuvettes, a shift in the absorption maximum of the SPR band from 526 nm to 584 nm is observed (Figure 4) indicating that the local environment of the goldNP has become less polar, which attributes to the hypothesis that hydrophobic interactions are the driving force for nanoparticle deposition.

![Normalized UV-VIS spectra](image)

**Figure 4.** Normalized UV-VIS spectra of citrate@goldNP, polyNIPAm@goldNP in solution and polyNIPAm@goldNP deposited onto plastic cuvettes in presence of 0.1 M NaCl. Note that data of uncoated goldNP are not shown as citrate@goldNP precipitate immediately upon addition to 0.1M NaCl solution and thus cannot deposit on the walls of the plastic cuvette.

As shown in Figure 5A, the nanoparticle deposition increases, as function of the nanoparticle concentration until a plateau is reached at a concentration of 0.1 mg/mL and upwards. A similar trend is observed for the influence of the deposition time on the nanoparticle deposition (at a fixed nanoparticle concentration of 0.1 mg/mL), where the amount of adsorbed material increases as function of incubation time until a plateau is reached at 30 min of incubation (Figure 5B). In both cases the plateau has a very similar absorption value of 0.5, which in combination with the very smooth surfaces that were observed with AFM further supports that a near-ideal monolayer of polyNIPAm@goldNP is deposited. The similar trend observed for the plasmon peak of the deposited film in Figure 5A-B, suggests that increased nanoparticle deposition is accompanied with the nanoparticles being more densely
packed. The molecular weight of the polyNIPAm negatively influences the amount of deposited material with a higher molecular weight leading to less adsorbed nanoparticles (Figure 5C). This is again in agreement with the formation of a monolayer of nanoparticles as the larger polymer coatings will decrease the maximum number of deposited nanoparticles per monolayer and is confirmed by the decrease in plasmon peak of the nanoparticle film that can be attributed to the lower packing density of the nanoparticle film. When varying the deposition temperature (Figure 5D), nanoparticle deposition does not significantly alters below the T_{cp} of the polyNIPAm, but strongly decreases above the T_{cp}. The latter can be attributed to macroscopic precipitation of the polyNIPAm@goldNP in 0.1 M NaCl at a temperature above the T_{cp} of polyNIPAm as previously reported.\textsuperscript{29} The NaCl concentration was found to increase nanoparticle deposition up to a maximum value of 0.1 M whereas higher salt concentrations prevent nanoparticle deposition (Figure 5E). The latter is again due to macroscopic precipitation of polyNIPAm@goldNP from solution at higher salt concentrations. When changing the type of salt from the kosmotropic NaCl to the chaotropic sodium thiocyanate (NaSCN), a slight decrease in nanoparticle deposition occurs up to salt concentration of 0.1 M. Interestingly, whereas the kosmotrope NaCl induces nanoparticle aggregation in solution above the T_{cp} of the polymer coating preventing nanoparticle deposition, the chaotropic NaSCN alleviates the temperature-induced aggregation of the nanoparticles, suggesting that its salting in effect renders the polymer coating less hydrophobic. This lower nanoparticle hydrophobicity could explain the slightly lower extent of nanoparticle deposition in presence of NaSCN relative to NaCl. Importantly, both salts induce nanoparticle deposition indicating that the deposition is not driven by the salting in or salting out of the polymer chains. As such, the deposition process must be related to the charge screening effect, i.e. suppression of the electrostatic colloidal stabilization. To investigate the influence of the substrate hydrophobicity we used quartz slides instead of plastic cuvettes. The quartz slides were treated with piranha solution to render them more hydrophilic or treated with piranha plus chlorotrimethylsilane (CTMS) to render them more hydrophobic. As depicted in Figure 5F-G, the nanoparticle deposition is not only more efficient on more hydrophobic surfaces, but also an outspoken blue shift of the SPR band is observed. The latter indicates that CTMS treated quartz slides provide a more hydrophobic environment to the nanoparticles, which implies close proximity of the goldNP and the hydrophobic surface.
Figure 5. Influence of parameters on polyNIPAm@goldNP deposition. Influence of the (A) concentration of polyNIPAm@goldNP, (B) deposition time, (C) number average molecular weight (Mn) of polyNIPAm, (D) deposition temperature, (E) salt type and concentration and (F) substrate properties on nanoparticle deposition (red data points) and the plasmon peak ($\lambda_{\text{max}}$; blue data points in panels A-C) of the deposited nanoparticle film, measured by UV-VIS spectrophotometry. (*: $p<0.05$) All experiments were run in triplicate. Data shown in panels A-E were recorded on plastic cuvettes, the data shown in panel F was recorded on quartz slides. (G) Photographs of quartz slides: (G1) untreated, (G2) treated with piranha and polyNIPAm@goldNP, (G3) treated with piranha, chlorotrimethylsilane (CTMS) and polyNIPAm@goldNP. Unless specifically noted in the respective panels, the conditions for nanoparticle deposition were: polyNIPAm Mn of 4.9 kDa, 0.1 mg/mL, 1 h deposition time, polyNIPAm Mn of 4.9 kDa, 0.1 M NaCl, 20 °C deposition temperature.
Based on these findings, we postulate a hypothesis for the spontaneous deposition of the polymer-coated nanoparticles in presence of salt. The polyNIPAm@goldNP have colloidal stability due to both steric repulsion caused by the polymer chains and electrostatic repulsion. When both are suppressed by heating above the $T_C$ of the polymer and addition of salt, nanoparticle agglomeration occurs in solution. However, below $T_C$ in presence of salt the surface charges are screened while the steric stabilization remains, suppressing interparticle interactions. The polyNIPAm@goldNP in presence of salt are, thus, stabilized by polyNIPAm-water interactions that compete with polymer-polymer interactions. In presence of a hydrophobic substrate, the hydrophobic polyNIPAm-surface interactions come into play and these hydrophobic interactions in combination with the entropic gain of releasing the hydrating water-molecules is postulated to be the driven force for the polyNIPAm@goldNP deposition process. This process is irreversible because multiple monomeric repeating units per polymer chain and multiple polymer chains adsorb on the surface as schematically illustrated in Figure 6. This proposed mechanism is in good agreement with our experimental observations that the more hydrophilic polyNVP@goldNP do not show deposition and that deposition of polyNIPAm@goldNP is more efficient on more hydrophobic surfaces.

**Figure 6.** Proposed mechanism for salt-induced deposition of polymer@goldNP on solid surfaces. (A) At low ionic strength, polyNIPAm@goldNP remain stable in solution, owing to electrostatic repulsion between the nanoparticles residing from residual citrate anions on the metal nanoparticle surface. At higher ionic strength, the repulsive electrostatic charges are screened and the hydrophobicity of the polymer chains increases due to the salting out effect. This increase in hydrophobicity causes an irreversible adsorption to a more hydrophobic surface. (B) Multiple monomeric repeating units per polymer chain and multiple polymer chains per nanoparticle adsorb to the underlying hydrophobic surface, thereby attributing to the irreversible nature of the nanoparticle deposition.

Besides engineering a surface with plasmonic properties owing to the plasmon peak of the goldNP, we aimed as a proof-of-concept at elucidating whether the adsorbed goldNP coating
could render a substrate catalytically active. For this purpose we took poly(ethylene-co-vinylacetate) granular beads and immersed these into a 0.1 M NaCl solution of polyNIPAm@goldNP. As shown in Figure 7A, the beads become distinctly purple colored, indicating successful deposition of a nanoparticle coating. Calculation of the deposited amount of polyNIPAm@goldNP yielded an amount of 0.1 mg of gold per gram of beads. Subsequently, these beads were packed into a column and percolated with a solution of 4-nitrophenol. Metallic gold is able to catalyze the conversion of 4-nitrophenol to 4-aminophenol in presence of sodium borohydride (NaBH₄) (Figure 7B), which can be monitored spectrophotometrically by a decrease of the adsorption at 400 nm (Figure 7C). Samples were collected after different residence time in the column and the conversion is plotted as function of time (Figure 7D). These data show that, when adsorbed onto the bead surface, the goldNP are still capable of catalyzing a chemical reaction. Control experiments with bare, uncoated, plastic beads did not show any conversion of 4-nitrophenol.

Figure 7. Catalytic activity of the polyNIPAm@goldNP coating. (A) Photograph of poly(ethyl-co-vinylacetate) beads before (left) and after (right) coating with polyNIPAm@goldNP. (B) Reaction scheme for the conversion of 4-nitrophenol to 4-aminophenol. (C) Change in UV-VIS spectrum of the reaction solution catalyzed by polyNIPAm@goldNP. (D) Conversion of 4-nitrophenol to 4-aminophenol as function of time, monitored by UV-VIS absorbance at 400 nm. Experiments were run in triplicate.
Conclusions

Summarizing, we have demonstrated that metal nanoparticles coated with a temperature-responsive polymer can undergo salt-induced adsorption onto solid substrates. We attribute this behavior mainly to hydrophobic interaction between the metal surface and the substrate, which is facilitated by screening residual charges on the metal nanoparticle surface by addition of salt ions. Furthermore, we provide a proof-of-concept that this method allows engineering surfaces with specific functionalities attributed to the presence of the adsorbed nanoparticles. We foresee this approach to be broader applicable to different kinds of inorganic or organic nanoparticles, with specific functions. Moreover, our approach is not limited to planar substrates only, but is capable of coating substrates with a complex morphology as demonstrated by coating of a rubber glove. Also the role of polymers that exhibit coil-to-globule transition in response to other stimuli than temperature and the use of other metallic and non-metallic nanoparticles remain attractive tracks for future research endeavors.
Materials and Methods

Materials

All chemicals and solvents were commercially available and use as received unless otherwise noted. \(N\)-isopropylacrylamide (NIPAm) was obtained from Scientific Polymer Products INC. and recrystallized twice in \(n\)-hexane prior to use. The RAFT chain transfer agent (CTA) methyl 2-(butylthiocarbonothioylthio)propanoate (MBTTC) was prepared as previously reported.\(^{36}\) 2,2’-Azobis(isobutyronitrile) (AIBN) was recrystallized twice from methanol and stored at -20°C. Poly(ethylene-co-vinylacetate) (EVA) beads (Elvax\textsuperscript® 40w) were obtained from DuPont. Poly(diethyleneglycolacrylate),\(^{37}\) poly(\(N\)-vinylpyrrolidone)\(^{38}\) and poly(\(n\)-propyl-2-oxazoline)\(^{32}\) were synthesized according to literature.

Polymer synthesis (polyNIPAm) (synthesized by Dr. Samarendra Maji (Prof. Hoogenboom group))

A typical procedure for the synthesis of poly(\(N\)-isopropylacrylamide) (polyNIPAm) via RAFT polymerization is as follows: NIPAm (2.00 g, 17.67 mmol), MBTTC (44.61 mg, 0.18 mmol), AIBN (14.51 mg, 0.09 mmol) and DMF (8.8 mL) were charged in a 25 mL Schlenk tube. Residual oxygen was removed from the solution by three freeze and thaw cycles. Then the reaction mixture was placed in a preheated oil bath at 60 °C to run the polymerization. After 30 minutes of polymerization, the reaction vessel was removed from the oil bath and opened to the air to stop the polymerization. The polymer was first precipitated by dropping the polymer solution into a large amount of a 50:50 mixture of hexane and diethyl ether. After decanting the solvent, the polymer was dissolved in THF and precipitated in diethyl ether. This precipitation procedure was repeated three times. The polymer was dried overnight under reduced pressure at 50 °C, yielding a yellowish powder. The monomer conversion was determined via gas chromatography (GC) and \(^1\)H NMR spectroscopy. Size exclusion chromatography (SEC) in N,N-dimethylacetamide (DMA) was used to calculate \(M_{n,SEC}\) and the dispersity \(D\) relative to polymethylmethacrylate (PMMA) standards. To prepare polyNIPAm samples of different molecular weights [NIPAm]/[CTA] ratio was varied in addition to stopping the reaction at different reaction times. Polymerization conditions and results for the synthesis of polyNIPAm samples in DMF are given in Table 1.
**Polymer characterization**

**Gas Chromatography (GC)**

GC was performed on a 7890A from Agilent Technologies with an Agilent J&W Advanced Capillary GC column (30 m, 0.320 mm, and 0.25 mm). Injections were performed with an Agilent Technologies 7693 auto sampler. Detection was done with a FID detector. Injector and detector temperatures were kept constant at 250 and 280 °C, respectively. The column was initially set at 50 °C, followed by two heating stages: from 50 °C to 120 °C with a rate of 20 °C /min and from 100 °C to 300 °C with a rate of 50 °C /min, and then held at this temperature for 0.5 minutes. Conversions of the monomers were determined based on the integration of monomer peaks using the polymerization solvent, DMF, as an internal standard.

**Size exclusion chromatography (SEC)**

SEC was performed on a Agilent 1260-series HPLC system equipped with a 1260 online degasser, a 1260 ISO-pump, a 1260 automatic liquid sampler (ALS), a thermostatted column compartment (TCC) at 50 °C equipped with two PLgel 5 µm mixed-D columns and guard column in series, a 1260 diode array detector (DAD) and a 1260 refractive index detector (RID). The used eluent was DMA containing 50mM of LiCl at a low rate of 0.593 mL/min. The spectra were analysed using the Agilent Chemstation software with the GPC add on. \( M_n,SEC \) and \( Đ \) values were calculated against PMMA standards from polymer labs.

**Nuclear magnetic resonance spectroscopy (NMR)**

Proton nuclear magnetic resonance spectra (\(^1\)H-NMR) of the polymers were recorded on a Bruker Avance 300 MHz spectrometer at room temperature using CDCl\(_3\) as solvent.

**Turbidimetry**

The cloud point temperatures (\( T_{cp} \)) were measured on a Cary 300 Bio UV-Visible spectrophotometer at a wavelength of 600 nm with a temperature controller. Aqueous polymer solution (5 mg/mL) was heated from 10 to 40 °C with a heating rate of 1.0 °C/min followed by cooling to 10 °C at a cooling rate of 1.0 °C /min. This cycle was repeated two times. The \( T_{cp} \) is reported as the 50% transmittance temperature in the 2\(^{nd}\) heating run.
Nanoparticle synthesis

Gold nanoparticles

The synthesis and characterization details for the citrate stabilized goldNP used in this work can be found in Chapter 2. Briefly, 20 mL of an aqueous 1 mM HAuCl$_4$ solution was refluxed in a 50 mL round-bottom flask for 30 minutes. Then 2 mL of an aqueous 1 wt.% sodium citrate solution was quickly added and the color of solution changed from yellow to wine red within 30 seconds. The reaction solution was stirred for another 5 min under reflux and cooled to room temperature. After cooling, the reaction solution was stored at 4 °C until further use.

Silver nanoparticles

Silver nanoparticles were synthesized according to a slightly modified literature procedure. 50 mL of 1 mM silver nitrate aqueous solution was refluxed in a 100 mL round-bottom flask for 30 minutes. Then 1 mL of a 1 wt.% of sodium citrate solution was added and the color of the solution changed from yellow to turbid gray within 5 minutes. The reaction solution was refluxed for another 1 hour and cooled to room temperature under stirring and then stored at 4°C until further use.

Polymer coated nanoparticles

Both gold and silver nanoparticles were modified by polymers via a ‘grafting to’ approach. Briefly, 8 mg of polymers were dissolved in 0.2 mL of cold MilliQ water, prior to be added to 8 mL of citrate coated gold or silverNP. After stirring for 24 h, the excess of unbound polymer was removed by 3 cycles of centrifugation at 4 °C with 15000 g for 20 min followed by re-dispersion in MilliQ water.

Nanoparticles characterization

Transmission Electron Microscopy (TEM)

Carbon-coated Cu grids (200-mesh) were used in all experiments. For TEM, A drop of gold nanoparticle solution was allowed to air-dry onto a grid, and visualized using 80 keV TEM (Jeol 1010, Japan). TEM images of the goldNP were processed via Image J to determine the number average size distribution in dry state.
**Dynamic Light Scattering (DLS)**

The size of particles (gold concentration of 0.18 mg/mL) was measured by DLS which was performed using a Malvern Zetasizer Nano Series running DTS software and operating a 4 mW He-Ne laser at 633 nm. Detection was performed at an angle of 173°.

**UV-Vis Spectroscopy (UV-Vis)**

UV-Vis spectra were acquired on a Shimadzu UV-1650PC spectrophotometer. In the case of temperature-dependent measurements, a Cary 300 Bio UV-Vis spectrophotometer was utilized to check absorbance via changing the temperatures from 20 to 40 °C.

**Atomic Force Microscopy (AFM)**

The morphology of the polyNIPAm@goldNP films was investigated by tapping mode AFM. Experiments were performed on air-dried films deposited onto silicon wafers. Images were obtained in tapping mode under ambient conditions in air with Bruker Innova equipped with a 100 µm scanner. Antimony doped silicon AFM probes were used with a nominal spring constant of 3 N/m and a resonance frequency of 75 KHz.

**Catalytic activity of the deposited polyNIPAm@goldNP**

**Deposition of polyNIPAm@goldNP on Beads**

3 mL of polyNIPAm@goldNP with a concentration of 1 mg/mL was mixed with 12 mL of a 0.1 M NaCl aqueous solution in a 100 mL glass flask. Next, EVA polymer beads were immersed into the mixture and incubated overnight. Afterwards the polyNIPAm@goldNP deposited beads were rinsed extensively several times with deionized water and subsequently dried in air. To determine the amount of adsorbed nanoparticles, UV-Vis was used to measure the residual concentration of non-deposited nanoparticles in the 100 mL flask with and without EVA polymer beads after overnight incubation. Since the nanoparticles can also deposit on the surface of glass flask, a glass flask containing the same amount of nanoparticles without beads was used as a control. The amount of adsorbed nanoparticles was calculated as follows:

\[
\text{Amount of adsorbed nanoparticles on beads (mg/g)} = \left( \frac{A_{\text{without}} - A_{\text{with}}}{A_{\text{original}}} \right) \times CV
\]
Where, $A_{\text{without}}$ is the absorbance of nanoparticles in the flask without EVA polymer beads, $A_{\text{with}}$ is the absorbance of nanoparticles in the flask with EVA polymer beads, $A_{\text{original}}$ is the absorbance of original nanoparticles in the flask, $C$ and $V$ are the original concentration and volume of nanoparticles added into the flask, respectively. $W_{\text{beads}}$ is the weight of EVA polymer beads.

*Catalytic activity of polyNIPAm@goldNP deposited beads*

15 µL of an aqueous 4-nitrophenol solution with concentration of 2 mg/mL was added to 10 mL of deionized water. Next 40 µL of an aqueous solution of NaBH$_4$ with a concentration of 3 mg/mL was added and vigorously mixed. 20 mL of the above solution was added into a tube filled with 30 g of polyNIPAm@goldNP coated beads. At regular time intervals, 1 mL sample of the reaction mixture was collected and analysed by UV-VIS spectroscopy.
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CHAPTER 4

TAILORING CELLULAR UPTAKE OF GOLD NANOPARTICLES VIA THE HYDROPHILIC-TO-HYDROPHOBIC RATIO OF THEIR (CO)POLYMER COATING

Parts of this chapter were published in:
Tailoring Cellular Uptake of Gold Nanoparticles via The Hydrophillic-to-Hydrophobic Ratio of Their (co)Polymer Coating
ADVANCED FUNCTIONAL MATERIALS 2015, 25, 3433-3439
Abstract

It is demonstrated how cellular uptake and protein corona of (co)polymer-coated gold nanoparticles can be altered by the hydrophilic-to-hydrophobic co-monomer ratio. A novel, label-free flow cytometry strategy was developed to investigate particle uptake. These findings offer insight in the design and analysis of hybrid nanomaterials for interfacing with biological systems.
Introduction

The design of engineered nanoparticles that can interact with living cells and tissues is important for many biomedical applications, including imaging, diagnostics and drug delivery.\textsuperscript{1-6} Gold nanoparticles (goldNP) have attracted major interest due to their chemical stability, cytocompatibility and their tunable optical and electronic properties.\textsuperscript{7-10} Furthermore, (quasi) covalent surface functionalization of metallic gold is straightforward using sulfur-containing compounds that form self-assembled monolayers.\textsuperscript{11, 12} Polymer-decoration of goldNP is attractive to modulate the goldNP properties and to render them colloidally stable in complex media. To produce polymer-decorated goldNP, reversible addition fragment chain-transfer polymerization (RAFT) is particularly well suited to synthesize sulfur-end-functionalized polymers due to the use of thiocarbonate RAFT chain transfer agents (CTAs).\textsuperscript{13}

It is known that size, shape and surface chemistry of goldNP affects their uptake by living cells.\textsuperscript{14-18} However, few has been reported on whether altering the hydrophilic-to-hydrophobic ratio of a polymer coating can affect cellular interaction. In this chapter we report on goldNP decorated with a series of polymers that differ in their hydrophilic-to-hydrophobic comonomer ratio and investigate how the latter affect cellular uptake. In addition, we developed a novel flow cytometry method for label-free investigation of goldNP-cell interaction. This methodology is then used to investigate the different parameters that affect the interaction of the goldNP and \textit{in vitro} cultured cells.

Results and discussion

Copolymers composed of the hydrophilic 2-hydroxyethylacrylate (HEA) and the hydrophobic methoxyethylacrylate (MEA) were obtained by RAFT in an automated parallel synthesis robot to minimize batch-to-batch variation.\textsuperscript{19, 20} Polymers with a theoretical degree of polymerization (DP; monomer to CTA ratio) of 100 and HEA:MEA ratios of 100:0, 80:20, 60:40, 50:50, 40:60 and 20:80 respectively, were synthesized according to Scheme 1. For clarity of presentation these polymers will further on be denoted as $\text{HEA}_x\text{MEA}_y$ ($x$ and $y$ represent HEA and MEA to CTA ratio, respectively). Polymerization was stopped at a
conversion of approximately 65% to ensure good chain end-fidelity of the polymers and Table 1 summarizes their properties. In all cases linear first order polymerization kinetics (Figure 1) were observed and good control over the polymerizations was further evidenced by dispersities below 1.3. As reported earlier by Hoogenboom et al., copolymers of HEA and MEA have an ideal random monomer distribution \(^{21,22}\) which was confirmed in our present study. The polymers HEA\(_{40}\)MEA\(_{60}\) and HEA\(_{20}\)MEA\(_{80}\) appeared to be insoluble in water at room temperature or above and were not included in further experiments.

![Scheme 1](image)

**Scheme 1.** Reaction scheme of the RAFT copolymerization of HEA and MEA.

![Figure 1](image)

**Figure 1.** First order kinetic plots for the copolymerization of HEA and MEA, indicating good control over the polymerization.
Table 1. Characterization of the HEAₓMEAᵧ copolymers synthesized via RAFT process

<table>
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<th>Polymer composition</th>
<th>Conversion (%)⁽¹⁾</th>
<th>Number average Molecular weight (SEC)⁽²⁾ (kDa)</th>
<th>Number average Molecular Weight⁽³⁾ (kDa)</th>
<th>Dispersity⁽²⁾</th>
<th>Amount of HEA(%)⁽³⁾</th>
<th>Amount of MEA(%)⁽³⁾</th>
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</table>

⁽¹⁾ calculated by GC using DMA as internal standard; ⁽²⁾ data collected by THF SEC; ⁽³⁾ data calculated based on the conversion measured by GC.

13 nm (size measured by TEM) citrate-stabilized goldNP were synthesized by the Turkevich-Frens method via direct reduction of the HAuCl₄ with trisodium citrate in aqueous medium under boiling.⁵³, ⁵⁴ Characterization of the goldNP is reported in Figure 2A. Surface functionalization with the respective HEAₓMEAᵧ copolymers was performed by simply mixing aqueous solutions of the goldNP and copolymers (in large excess) followed by several centrifugation and washing steps to remove non-adsorbed polymer. Successful functionalization is evidenced by the ability of the polymer@goldNP solution to remain colloidally stable after removal of the access citrate. Indeed, when citrate-stabilized goldNP are centrifuged and re-dispersed in deionized water or buffer solution, irreversible aggregation occurs. However, this was not the case in any of the HEAₓMEAᵧ@goldNP samples and DLS and TEM gave further proof of the formation of colloidally stable dispersions, as shown in Figure 2B. DLS and UV-Vis further confirmed that the HEAₓMEAᵧ@goldNP are stable at room temperature and at 37°C in physiologically relevant media, including phosphate buffered saline (PBS; 50 mM phosphate salts, 150 mM NaCl, pH 7.4) and PBS supplemented
with 10% fetal calf serum (FBS; typically used in cell culture experiments and is composed of a complex mixture of serum proteins). (Figure 2C and Table 2)

Figure 2. (A) Characterization of citrate stabilized goldNP. (A1) TEM image; (A2) Size distribution measured by DLS in deionized water; (A3) UV-Vis spectrum. (B) TEM images and size distribution (measured by DLS in deionized water) of the HEA,MEA modified goldNP. The Z-ave diameters shown in the inserts were measured by DLS. (C) Size distribution measured by DLS and UV-Vis spectra of HEA,MEA,@goldNP in PBS and PBS supplemented with 10% FBS. Note that the appearance of a secondary maximum in the UV-Vis spectrum at 407 nm is due to the presence of porphyrins in the FBS.
The method of choice to measure cellular association (i.e. uptake or cell surface binding) of a specific component is flow cytometry (FCM). FCM involves the simultaneous measurements of light scattering and fluorescence properties of single cells as they move in a liquid stream through a laser/light beam past a sensing area. For this purpose, the components of interest are typically labelled with a fluorescent dye to allow, upon excitation by a laser beam, detection of fluorescence emission by a photomultiplier tube (PMT). Besides PMTs, flow cytometers are typically also equipped with diode detectors to measure scattered light in both axial (forward scatter; FSC) and perpendicular (side scatter; SSC) direction with respect to the fluid stream. The FSC signal is proportional to the cell size whereas the SSC signal is proportional to the granularity of the cell. We hypothesized that cellular uptake of goldNP could, owing to the light scattering properties of goldNP, lead to an alteration in the SSC signal of the cells. If so, this method would afford label-free detection of goldNP uptake by cells in a facile and high throughput fashion. Indeed, fluorescent labelling of a component of interest might significantly alter its physicochemical properties, and thus influence its interaction with cells. Additionally, metal nanoparticles are known to strongly quench fluorescence.\textsuperscript{25-27} Thus, conjugating a fluorophore to the goldNP surface is very likely a sub-optimal choice. Therefore, cellular uptake of goldNP is typically assessed by atomic absorption or emission spectroscopy. However, with regard to measuring metal content in cells, drawbacks of these methods include: (1) Careful sample preparation to know the exact cell number in each sample. (2) The non-high throughput nature of these techniques. (3)
impossibility to measure different parameters, as is the case with FCM using additional immunostains. (4) The lack of availability of these systems in common life sciences laboratories, whereas FCM is a well-established technique in this field.

Figure 3. (A) FCM gating strategy to assess cell uptake of goldNP by the shift in SSC. (B) Histograms (B1) and quantitative analysis (B2) of DC2.4 cells pulsed with different concentrations of HEA50MEAS0@goldNP. (n=3) (C) Uptake HEAxEYMEAx@goldNP by DC2.4 cells measured by FCM (C1) and ICP-MS (C2). (n=3).
As a proof-of-concept to validate whether indeed the SSC properties of cells change upon uptake of goldNP we incubated DC2.4 cells for 12 h with different concentrations of HEA_{50}MEA_{50}@goldNP. DC2.4 is an immortalized mouse dendritic cell (DC) line. DCs are the most potent class of professional antigen presenting cells of the immune system and a premier target cell population for the delivery of vaccine antigens and immune-modulating compounds. The FCM gating strategy that we applied to investigate the uptake of HEA_{x}MEA_{y}@goldNP in DC2.4 cells is shown in Figure 3A. Note that for the sake of clarity, these data correspond to blank untreated cells. The first two gates serve to discard multiplets (i.e. two or more cells that stick together) and cell debris. The two remaining cell populations marked by the red dashed ellipses represent dead cells (population (1)) and living cells (population (2)). We deliberately do not gate out the dead cell population based on the SSC vs. FSC plot as is typically done in flow cytometry analysis. The reason for this is that as the SSC would increase upon internalization of goldNP, population (1) and (2) would start to overlap. Thus, a priori gating out population (1) would mistakenly discard living cells that have internalized goldNP. Therefore, to gate out dead cells, we performed a co-staining with propidium iodide (PI). This fluorescent dye is impermeable to alive cells but is permeable to dead cells and stains their nucleus. As a third gate, we selected those cells that were negative for PI staining. By counting the number of living cells per volume, we also verified that the concentration range of goldNP that was used in these experiments did not strongly affect cell viability (see Figure 4). Finally, a gate was set to mark the onset of what will be considered as cells that are associated to goldNP (i.e. goldNP+ cells). As shown by the histograms in Figure 3B1, a shift in SSC can be clearly distinguished with increasing goldNP concentration in the cell culture medium. The quantitative results in Figure 3B2 give further proof that both the number of events in the goldNP+ cells gate and the mean SSC intensity in the living cells gate increases as a function of the goldNP concentration.
To enlarge our experimental setting, we incubated DC2.4 cells with increasing amounts of goldNP decorated with the different HEAₓMEAᵧ copolymers. As shown in Figure 3C1, a dose-dependent increase in SSC is witnessed for all HEAₓMEAᵧ@goldNP samples. Interestingly, also a strong influence of the hydrophilic-to-hydrophobic ratio of the polymer coating is observed, with increasing hydrophobicity of the polymer coating leading to increased goldNP-cell association. To validate our FCM strategy and to confirm whether the shift in SSC intensity as a function of goldNP concentration in the cell culture medium and polymer hydrophobicity is truly due to an increase in goldNP-cell association, we measured in parallel the goldNP content of the cells by inductive coupled mass spectroscopy (ICP-MS) for the lowest and highest goldNP concentrations in the cell culture medium. In these experiments we carefully assured that the number of cells in each sample was equal by counting cell numbers by FCM. As depicted in Figure 3C2, similar trends as observed by FCM are found, thereby confirming the high potential of this method for straightforward and high throughput screening of cellular interaction with goldNP.

To investigate whether the HEAₓMEAᵧ@goldNP are effectively taken up by the DCs, or merely surface-bound, and to elucidate the intracellular fate of the nanoparticles, we used optical microscopy and transmission electron microscopy (TEM). The series of fluorescence
microscopy images depicted in Figure 5 indeed confirms the presence of nanoparticles observed as black dots inside the cells. Furthermore, DCs pulsed with HEA_{100}MEA_{0}@goldNP barely show presence of nanoparticles whereas this is increasingly more pronounced for the other nanoparticle samples with increasing MEA content. This again confirms the FCM and ICP-MS data. Further insight into the intracellular localization of the goldNP was obtained by TEM on ultrathin microtomed sections taken from DCs that were pulsed with goldNP in a similar fashion as for the optical microscopy experiments. The corresponding images (Figure 6) show that the nanoparticles are predominantly located inside vesicular organelles, likely endosomes, lysosomes and/or phagosomes. Besides this, the zoomed images also show the presence of a minor fraction of the nanoparticles being localized outside vesicular organelles in the cytoplasm. No influence of the hydrophilic-to-hydrophobic ratio on the extent of cytoplasmic localization of the nanoparticles was observed. Although a minority, cytoplasm-located nanoparticles might play an important role. Indeed, as recently reported by Leong and co-workers, nanoparticles located in the cytosol can interfere with the microtubule assembly and, by doing so, strongly affect cellular migration capability. Whether this is also the case for DCs, remains to be investigated. But in view of the crucial role of this highly motile nature of this cell type, a decrease in migratory properties could strongly affect the way DCs respond to an infectious thread or their ability to transport therapeutic nanoparticles (e.g. vaccines) to the immune-inducing sites in the draining lymph nodes.

Figure 5. Fluorescence microscopy images of DC2.4 cells incubated with HEA_{MEA}@goldNP. Cell nuclei were stained blue with Hoechst and the cell membrane was stained green with AlexaFluor488 conjugated cholera toxin subunit B. The images are an overlay with the DIC channel.
Next we aimed at elucidating in more depth those parameters that play a role in the cellular internalization of the HEA$_x$MEA$_y$@goldNP. To investigate whether particle uptake proceeds via passive or active endocytosis, DC2.4 cells were incubated with the different HEA$_x$MEA$_y$@goldNP for 2 h and 12 h at 37°C and 4°C, respectively. The latter is used to block active, energy-dependent, cell uptake. As shown in Figure 7, a vast difference in particle uptake is observed dependent on the incubation time and temperature. Firstly, after 2h, the amount of goldNP+ cells is still fairly low relative to the values measured after 12 h. Secondly, incubation at 4°C does not lead to significant particle uptake of any of the HEA$_x$MEA$_y$@goldNP samples, thereby suggesting that the cellular uptake of the particles proceeds via active receptor-mediated phagocytosis or macropinocytosis. This corresponds well with the TEM observations that show the nanoparticles being predominantly present in intracellular vesicles, which is a clear sign of an active uptake mechanism.\textsuperscript{28}
CHAPTER 4 — TAILORING CELLULAR UPTAKE OF GOLD NANOPARTICLES VIA THE HYDROPHILIC-TO-HYDROPHOBIC RATIO OF THEIR (CO)POLYMER COATING

When a nanomaterial enters a biological fluid (for example cell culture medium), proteins rapidly absorb to its surface and, as a result, the surface properties, charges, and hydrodynamic radius of nanoparticles are reshaped. The protein corona constitutes a primary nano-bio interface and determines the fate of nanoparticles in a biological environment. To investigate the role of serum proteins on the cellular uptake of the HEAₓMEAᵧ@goldNP, DC2.4 cells were pulsed for 2 h with the different HEAₓMEAᵧ@goldNP samples either in presence or absence of 10 % FBS in the cell culture medium. Note that the 2 h time window was chosen because a period of 12 h in absence of serum was severely affecting cell viability. Depleting the cell culture medium from serum strongly reduces cellular association of all HEAₓMEAᵧ@goldNP samples. These findings suggest that the presence of serum plays an important role in governing cellular interaction with the HEAₓMEAᵧ@goldNP.

The effect of the protein corona on the cellular uptake of the HEAₓMEAᵧ@goldNP was further elucidated by incubating the nanoparticles overnight in PBS supplemented with 10 % FBS. Triple washing/centrifugation was used to remove the excess of proteins and the samples were analyzed by ζ-potential measurement and liquid chromatography combined with tandem mass spectrometry/mass spectrometry for detection (LC-MS/MS). The ζ-potential
values of the respective nanoparticles are listed in Table 2 and do not show major differences as a function of polymer coating before and after exposure to FBS. Therefore, it is likely that the ζ-potential values cannot be accounted for the differences in cellular interaction of the nanoparticles. Prior to LC-MS/MS analysis, the FBS-exposed HEAₓMEAᵧ@goldNP were loaded on polyacrylamide gel (PAGE) and subjected to electrophoresis followed by in-gel digestion with trypsin. Three biological replicates were analysed per polymer. Quantification of the protein amount by optical integration of the PAGE gel (Figure 8A1) does show a higher amount of proteins absorbed to the HEA100 polymer. However, the difference was not significant.

Semi-quantitative analyses based on the number of identified proteins (Figure 8A2), and the number of spectra showed similar results. We also performed quartz crystal microbalance (QCM) studies on planar gold films on which HEAₓMEAᵧ polymers were adsorbed followed by flowing a PBS + 10 % FBS solution over the chips. These experiments (Figure 9 and Table 3) also did not indicate a difference in the absolute amount of proteins that are adsorbed onto the different HEAₓMEAᵧ coated chips, confirming the optical integration and LC-MS/MS data. These findings are contrary to earlier reports on planar gold surfaces and goldNP functionalized with low Mw compound of varying hydrophobicity. The Rotello group reported that hydrophobic goldNP show a higher tendency in binding serum proteins, which in turn decreases their cellular uptake. This inhibition effect was attributed to the repulsive interaction between anionic BSA and negatively-charged cell membrane. However, differences in nanoparticle surface chemistry, cell type, culture protocol, and uptake pathway may account for this discrepancy. For example, direct reaction between the HEAₓMEAᵧ chains hydrogen bonding with the hydroxyl groups or the methoxy groups, and the cell surface could contribute to the increase in cellular uptake.
Figure 8. Proteomic analysis of HEA,MEA, @goldNP incubated in PBS + 10% FBS. (A) Quantification of the total protein content by optical density integration (A1) of the PAGE gel and LC-MS analysis (A2). (B) Finger printing of the protein corona. (B1) Schematic overview of the number of proteins that are found in the respective samples in all triplicate runs. (B2) Heat map of the relative abundance of the 32 proteins that were found in all triplicate runs in all HEA,MEA, samples. (B3) Spectral counts of the 10 most abundant proteins found in all triplicate runs in all HEA,MEA, samples. (n=3)
When analysing the exact content of the protein corona further in depth, clear differences between the respective HEA$_x$MEA$_y$@goldNP were found. To process the LC-MS/MS data, we selected those proteins that were found in all 3 biological replicates and clustered these as a function of their appearance in the respective HEA$_x$MEA$_y$@goldNP samples (Figure 8B1). These data show that of a total of 65 proteins, 32 proteins are found in each of the samples. Other proteins are found exclusively in one of the samples, or in discrete combinations. The 32 proteins that were found in all of the samples are presented in Figure 8B2 in a heat map, representing the relative abundances of these proteins based on spectracount. Spectral count is a label-free, semi-quantitative measure for proteins abundance. HEA$_{50}$MEA$_{50}$, HEA$_{60}$MEA$_{40}$ and HEA$_{80}$MEA$_{20}$ show similar results. HEA$_{100}$MEA$_{0}$, however, shows a discrepancy with the other polymers: some proteins are clearly upregulated in HEA$_{100}$MEA$_{0}$ such as Complement
C3, others are downregulated such as alpha-S1-casein. Binding of complement C3 to hydroxyl bearing polymers (as is the case for HEA\textsubscript{100}) has been reported by the Hubbell group for purely organic nanoparticles\textsuperscript{35} and is thus confirmed in our present studies for such polymers grafted on goldNP. Furthermore, the observation that HEA\textsubscript{100}MEA\textsubscript{0} leads to the highest diversity of adsorbed proteins corresponds with earlier reports by the Whitesides group\textsuperscript{36} that to avoid protein adsorption, a polymer should ideally have only hydrogen bond accepting groups and no hydrogen bond donating groups like HEA\textsubscript{100}MEA\textsubscript{0} solely has. The full list of adsorbed proteins is included in Appendix, and Figure 8B3 shows the spectral counts of the 10 most abundant proteins that were found in all HEA\textsubscript{x}MEA\textsubscript{y}@goldNP samples. GRAVY (grand average of hydropathy) analysis of the hydrophobicity of the absorbed proteins did not show difference in hydrophobicity of the absorbed proteins/peptides in function of the polymer coating. These observations suggest that either the subtle differences in the composition of the protein corona or the interaction between the polymer coating itself and the cell membrane might account for the increased particle uptake at increasing hydrophobicity of the polymer coating.

Conclusions

Summarizing, in this chapter we have shown using a novel label-free FCM approach that tailoring the hydrophilic-to-hydrophobic ratio of the polymer coating of goldNP strongly influences cellular uptake. Serum proteins play a role in governing nanoparticle uptake, but its exact contribution needs to be further unravelled. Our current research efforts focus on investigation on the applicability of the FCM approach to measure cellular interaction with other crystalline and inorganic materials and its applicability to complex cell mixtures derived from live tissues. Furthermore, our findings can offer a rational base to design materials with varying cell interacting tendency, which might be useful for controlling residence time of nanoparticles in the blood stream or lymphatic vessels, both for therapeutic or research purpose.
Materials and Methods

Materials

All chemicals were purchased from Sigma-Aldrich unless otherwise stated. Cell culture media and additives and AlexaFluor488-conjugated cholera toxin subunit B and propidium iodide were purchased from Life Technologies. The RAFT CTA (PABTC) was synthesized according to literature. 37

Synthesis of HEAx MEAy copolymers (synthesized by Glenn Vanluchene (Prof. Hoogenboom group))

Stock solutions of 320 mg PABTC in 10.3 mL DMF, 22 mg AIBN in 10.3 mL DMF, 7.80 g HEA in 9.08 mL DMF and 8.75 g MEA in 8.16 mL DMF were prepared. The stock solution were de-oxygenized by bubbling through nitrogen for 30 min. In a ChemSpeed synthesis robot, each feed mixture was prepared twice, with feed ratios of 100/0, 80/20, 60/40, and 50/50 of HEA to MEA. To these mixtures were added 0.613 mL of PABTC stock solution and 0.613 mL of AIBN stock solution. Polymerizations were conducted at 70°C under a nitrogen atmosphere while the liquid handling system was programmed to automatically take samples from the polymerization mixtures to follow the kinetics by GC and SEC. Based on the observed reaction kinetics, the polymerization were repeated at a larger scale and the polymerization were then stopped after 60 minutes by reducing temperature and bubbling with air. Polymers were then isolated by manual precipitation in a cold 50/50 mixture of diethyl ether and hexane.

Polymer characterization

Gas Chromatography (GC)

Gas chromatography was performed on a GC8000 from CE instruments with a DB-SMS column (60 m x 0.249 mm x 0.25 m) from J&W scientific. Injections were performed with a CTC A200S auto sampler and detection was done with a flame ionization detector (FID) which burned a H2/air mixture. The carrier gas (He) was pushed through the column with a pressure of 100 bar. Injector and detector were kept at a constant temperature of 300 °C.
Size Exclusion Chromatography (SEC)

Size-exclusion chromatography or Gel permeation chromatography (GPC) was performed on a Agilent 1260-series HPLC system equipped with a 1260 online degasser, a 1260 ISO-pump, a 1260 automatic liquid sampler (ALS), a thermostated column compartment (TCC) at 50°C equipped with two PLgel 5 m mixed-D columns in series, a 1260 diode array detector (DAD) and a 1260 refractive index detector (RID). The used eluent was DMA containing 50mM of LiCl at a flow rate of 0.593 mL/min. The spectra were analyzed using the Agilent Chemstation software with the GPC add on. Molar mass and PDI values were calculated against PMMA standards from polymer labs.

Cloud point temperature ($T_{cp}$) measurement

Cloud point temperatures were determined on a Cary 300 Bio UV-Visible spectrophotometer at a wavelength of 600 nm with a temperature controller. Aqueous polymer solution at 5 mg/mL were heated from 10 to 65°C at a rate of 1K/min followed by cooling to 10 at same rate of 1°C/min. this cycle was repeated two times. The cloud point was reported as the 50 % transmittance temperature in the 2nd heating run.

Synthesis of citrate stabilized goldNP

Citrate stabilized goldNP were synthesized according to literature. All glassware was first washed with aqua regia and then rinsed with MilliQ water several times prior to synthesis. Briefly, 20 mL of an aqueous 1 mM HAuCl$_4$ solution was refluxed for 30 min. Then 2 mL of 1 wt.% sodium citrate solution was quickly added and the color of solution changed from yellow to wine red within 5 min. After cooling , the reaction solution was stored at 4°C until further use.

Polymer functionalization of goldNP

A total of 8 mL of a citrate stabilized gold nanoparticles solution was mixed with 200 mL of an aqueous solution containing 8 mg HEA$_n$MEA$_y$, and stirred overnight at room temperature. The polymer coated goldNP were purified three times by centrifugation at 4°C with 15 000 g for 30 min followed by re-dispersion in MilliQ water.
**Physicochemical characterization of HEAx-MEAy@goldNP**

*Transmission Electron Microscopy (TEM)*

Formvar/Carbon-coated Cu grids (200-mesh) were used in all experiments. For TEM, a drop of gold nanoparticle solution was allowed to air-dry onto a grid, and was subsequently visualized using 80 keV TEM (Jeol 1010, Japan). TEM images of the goldNP were processed via Image J to determine the number average size distribution in dry state.

*Dynamic Light Scattering (DLS)*

The size of particles (gold concentration of 0.18 mg/mL) was measured by DLS which was performed using a Malvern Zetasizer Nano Series running DTS software and operating a 4 mW He-Ne laser at 633 nm. Analysis was performed at an angle of 173°.

*UV-Vis spectroscopy*

UV-Vis spectra were acquired on a Shimadzu UV-1650PC spectrophotometer. In the case of temperature-dependent measurements, a Cary 300 Bio UV-Visible spectrophotometer was utilized to check absorbance via changing the temperatures from 20 to 40°C.

**Cellular uptake of HEAxMEAy@goldNP**

The DC2.4 cell line is a kind gift from Dr. Ken Rock (Dana Farber Cancer Institute and presently University of Massachusetts Medical School). DC2.4 cells were cultured in DMEM supplemented with 10% Fetal Bovine Serum (FBS) and antibiotics (100 units/mL penicillin and 100 µg/mL streptomycin). Cells were grown at 37°C in humidified air containing 5% CO₂ and passaged every 3-4 days. The cells were plated in a density of 250 000 per well (1 mL per well) on 24-well plates 24 h before addition of goldNP.

*Flow cytometry (FCM)*

All samples were measured on a Accuri (BD) flow cytometer and the data thus obtained were analyzed using FlowJo software.
**Cell viability assay**

DC2.4 cells were seeded in 24-well plates at 250,000 cells/well in medium with 10% fetal bovine serum. After 24 h incubation, various concentrations of goldNP were added into cells. After another 12 h incubation, cell viability was measured by FCM via propidium iodide staining.

**Effect of incubation temperature on goldNP cellular uptake**

DC2.4 cells were seeded in 24-well plates at 250,000 cells/well. After 24 h incubation, the cells were placed in fridge at 4°C or in incubation culture at 37°C for half an hour prior to pulsing with goldNP at varying concentrations. After another 12 h incubation, cells were washed with cold PBS and detached from the plates by adding 500 µl of EDTA solution. Centrifugation at 300 g for 5 min was used to collect cells and then cells were treated with PI solution in PBS containing 1% BSA. After staining for 1 h at room temperature, the cells were measured via FCM.

**Effect of incubation time on goldNP cellular uptake**

DC2.4 cells were seeded in 24-well plates at 250,000 cells/well. After 24 h incubation, cells were pulsed with goldNP at varying concentrations for another 2 h or 12 h. Cells were stained with PI solution prior to FCM analysis.

**Effect of serum effect on goldNP cellular uptake by DC2.4**

DC2.4 cells were seeded in 24-well plates at 250,000 cells/well and cultured for 24 h at 37°C in a medium with 10% fetal bovine serum. To investigate the serum effect in culture medium on goldNP cellular uptake, prior to addition of goldNP, cells were washed with PBS and exposed to a medium containing various concentrations of goldNP with and without serum. After another 2 h incubation, the cells were treated with PI and analysed by FCM.

**Fluorescence microscopy**

Fluorescence microscopy was performed on a Leica DM2500P microscope equipped with a 40x objective. DC2.4 cells were plated a density of 15,000 per well in a 8-well Ibidi chamber and incubated overnight with 5µL of a 2.50mg/mL poly(HEA-MEA) coated goldNP suspension. AlexaFluor488 conjugated cholera toxin subunit B (cell membrane) and Hoechst (cell nuclei) staining was performed according to the manufactures’ instructions.
**Inductively Coupled Plasma-Mass Spectrometry (ICP-MS)**

ICP-MS was used to measure the amount of goldNP that were internalized by DC2.4 cells. DC2.4 cells were plated at a density of 250 000 cells/well and cultured for 24 h. Then cells were pulsed with different concentrations of goldNP. After 12 h incubation, cells were washed with PBS three times, detached from plates by EDTA, collected and digested with aqua regia on a hot plate (100°C) for 10 hours. After appropriate dilution of the digested samples, the concentration of gold was measured using ICP-MS (Element XR, ThermoScientific, Germany), and the average number of goldNP internalized by one cell was determined taking into account the size of the goldNP and the atomic weight of gold. The experiments were run in triplicate.

**Transmission Electron Microscopy (TEM)**

Dendritic Cells from culture with HEA,MEA@goldNP were fixed in 4% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M NaCacodylate buffer, pH 7.2 and centrifuged at 500 rpm. Low melting point-agarose was used to keep the cells concentrated for the further processing. Cells were fixed for 4 hours at RT followed by fixation O/N at 4 °C after replacing with fresh fixative. After washing in buffer, they were post fixed in 1% OsO4 with 1.5% K3Fe(CN)6 in 0.1 M NaCacodylate buffer at room temperature for 1 hour. After washing, cells were subsequently dehydrated through a graded ethanol series, including a bulk staining with 1% uranyl acetate at the 50% ethanol step followed by embedding in Spurr’s resin.

Ultrathin sections of a gold interference color were cut using an ultra-microtome (Leica EM UC6), followed by a post-staining in a Leica EM AC20 for 40 min in uranyl acetate at 20 °C and for 10 min in lead stain at 20 °C. Sections were collected on formvar-coated copper slot grids. Grids were viewed with a JEM1010 transmission electron microscope (JEOL, Tokyo, Japan) operating at 80 kV using Image Plate Technology from Ditabis (Pforzheim, Germany).

**Liquid chromatography–mass spectrometry / mass spectrometry (LC-MS/MS)**

Five hundred microliters of goldNP coupled to polymer were spun down (14000g, 4°C, 10 minutes) and 400 microliter of supernatant was removed. 24µL of Laemmli buffer (50 mM TrisHCl, pH 6.8, 2% SDS, 10% glycerol, bromophenol blue) and 12µL of 500mM DTT was added. Subsequently, the goldNP were heated to 70°C for 1 hour and centrifuged for 15
 minutes, 14000g, 4°C. Thirty-five µL of supernatant was loaded on a 10% TrisHCl polyacrylamide gel (PAGE) and proteins were separated for 30 min at 150V and 60 minutes at 200V in running buffer (2.5mM Tris, 0.1% SDS, 192 mM glycine). Proteins were visualised by Sypro Ruby staining (Invitrogen). Peak intensities were quantified by Quantity one software (Intxmm) (v4.1, Bio-Rad, Hercules, CA, USA). Extraction and digestion of the proteins was performed as previously described.39

Dried peptides were dissolved in 0.1 % formic acid (FA) in water (buffer A) and half of the sample was injected on reversed phase nano-HPLC column (Pepmap C18 column 15 cm, particle size 3 µm, 0.3 mm internal diameter by 150 mm; Dionex, Sunnyvale, CA,USA) using a linear gradient of 97:3 buffer A/bufferB to 20:80 buffer A/buffer B at 300 nL/min over 70 min(buffer B: 80 % ACN/0.1 % FA). The different peptides were analyzed on a TripleTOF 5600 (ABSciex, Framingham, MA, USA) in a data dependent mode. Data-analysis was performed with Mascot Daemon (Matrix Science, London, UK) (peptide mass tolerance: 15ppm; fragment mass tolerance: 0.3Da; fixed modification: carbamidomethyl (C); variable modifications: carbamidomethyl (N-term), oxidation (M), deamidation (NQ); database: Bovine, 5984 entries). Hydrophobicity of the identified proteins and peptides were determined by means of GRAVY index.

**Quartz crystal microbalance (QCM)**

QCM traces were recorded in a Gamry eQCM 10M equipped with an ALS flow cell. 10 MHz gold coated quartz chips were used.
References

38. Shen, Z.H., et al., Cloned dendritic cells can present exogenous antigens on both MHC class I and class II molecules. Journal of Immunology 1997, 158, 2723-2730
## Appendix

Full list of proteins found in triplicate in the protein corona of polyHEA$_x$MEA$_y$@goldNP

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## Chapter 4 — Tailoring Cellular Uptake of Gold Nanoparticles via the Hydrophilic-to-Hydrophobic Ratio of Their (Co)Polymer Coating

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CHAPTER 4 – TAILORING CELLULAR UPTAKE OF GOLD NANOPARTICLES VIA THE HYDROPHILIC-TO-HYDROPHOBIC RATIO OF THEIR (CO)POLYMER COATING
CHAPTER 5

INFLUENZA-BINDING SIALYLATED POLYMER COATED GOLD NANOPARTICLES PREPARED VIA RAFT POLYMERIZATION AND REDUCTIVE AMINATION

Parts of this chapter were published in:

Influenza-Binding Sialylated Polymer Coated Gold Nanoparticles Prepared via RAFT Polymerization and Reductive Amination

CHEMICAL COMMUNICATIONS 2016, 52, 3352-3355.
Abstract

We report on a straightforward strategy to fabricate bioactive glycosylated gold nanoparticles via a combination of RAFT polymerization, carbohydrate ligation through reductive amination and thiol-gold self-assembly. This approach is used for the design of gold nanoparticles decorated with the complex sialylated glycan Neu5Ac-α-2-6-Gal, and we demonstrate multivalent and specific recognition between the nanoparticles, lectins and hemagglutinin on the surface of the influenza virus.
**Introduction**

The first hallmark in triggering cellular internalization of the influenza virus is binding of hemagglutinin (HA) to sialylated proteins on the host cell surface.\(^1\)\(^-\)\(^4\) This proceeds via docking of Neu5Ac-α-2-6-Gal motifs (i.e. sialic acid connected to galactose via a α-2-6 linkage) to the central grove of HA (Scheme 1A).\(^5\)\(^-\)\(^8\) Nanoparticles that present multiple Neu5Ac-α-2-6-Gal motifs are capable of multivalent ligand-receptor recognition\(^9\),\(^10\) and could be useful for therapeutic application or diagnostic application. With regards to therapeutic application, such nanoparticles could serve to assemble HA on the surface of nanoparticles as synthetic vaccine carriers that expose multiple antigen copies, inhibit host-pathogen interaction, or carry an anti-viral drug payload. With regard to diagnostics, equipping particles with a simple, e.g. plasmonic, readout system would allow for rapid and sensitive detection of viral lectins.

Gold nanoparticles (goldNP) are attractive templates\(^11\)\(^-\)\(^14\) for the design of glycosylated nanoparticles\(^15\)\(^-\)\(^17\) that form synthetic nature-inspired biomolecular assemblies.\(^18\)\(^-\)\(^20\),\(^19\),\(^21\)\(^-\)\(^25\) GoldNP can easily be functionalized with thiolated molecules that form self-assembled monolayers\(^26\) and they exhibit a plasmon peak in the visible region that depends on particle size and aggregation state.\(^27\)\(^-\)\(^34\) Two approaches that have intensively been explored involve either coupling of thiol-modified carbohydrates to goldNP via ligand exchange,\(^35\) or conjugation of carbohydrates to thiol-containing polymers (i.e. so-called glycopolymers) that are then ligated to goldNP.\(^14\),\(^26\) For the latter, reversible addition-fragmentation chain transfer (RAFT) polymerization is highly suited due to the inherent presence of thiocarbonate end-groups. Both approaches commonly require recombinant, chemo-enzymatic or carbohydrate chemistry routes to obtain functionalized carbohydrates, most desirably modified at the anomeric carbon of the outermost carbohydrate unit for the sake of bioactivity. Although conceptually attractive, a major drawback is the labour-intensiveness of these routes, especially the purification.\(^36\) For example, pioneering work from the Whitesides group reported on multistep synthesis of sialic acid for subsequent polymer post-modification.\(^37\)\(^-\)\(^39\) Routes that do not require prior modifications of the respective carbohydrates could be a viable alternative. Bertozzi and co-workers have reported on the synthesis of hydrazide containing polymers that can be coupled
to carbohydrates via their reducing end.\textsuperscript{40} This was highly efficient for monosaccharide, however, conjugation efficiency dropped to approximately 20 \% for more complex glycans.

\textbf{Scheme 1.} (A) Schematic representation of the recognition between glyco-goldNP and influenza virus. (B) Synthesis route for the ligation of \(\alpha\)-2,6-sialyllactose to RAFT-based poly(HPMA-co-APMA) through reductive amination.
CHAPTER 5 – INFLUENZA-BINDING SIALYLATED POLYMER COATED GOLD NANOPARTICLES PREPARED VIA RAFT POLYMERIZATION AND REDUCTIVE AMINATION

Table 1. Characterization of poly(HPMA-co-APMA) and derived glycopolymers.

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[1] Data collected by HFIP SEC; [2] data calculated based on <sup>1</sup>H-NMR spectra;

Results and Discussion

In this chapter, we report on a simple and straightforward approach to design complex glycopolymer decorated goldNP by a combination of RAFT polymerization and reductive amination. The trisaccharide α-2,6-sialyllactose is conjugated to a polymer containing primary amines in the side chain. (Scheme 1B) α-2,6-sialyllactose is a trisaccharide comprising of a sialic acid, galactose and glucose unit, thus possessing the Neu5Ac-α-2-6-Gal motif that can be recognized by hemagglutinin. Random copolymers (targeted degree of polymerization
(DP) of 100 and 25, respectively) of N-(2-hydroxypropyl)methacrylamide (HPMA) and N-(3-aminopropyl) methacrylamide (APMA) (molar ratio of 75/25 and 90/10, respectively) were synthesized via RAFT as earlier reported.\textsuperscript{42, 43} Poly(HPMA) was chosen due to its non-immunogenic and hydrophilic nature,\textsuperscript{44} while APMA provides the required primary amine moieties for carbohydrate-ligation via reductive amination. We reasoned that 100 % APMA would lead to residual primary amine groups that would impart the stability of the goldNP and/or promote unwanted protein adsorption at a later stage. SEC (size exclusion chromatography) and $^1$H-NMR spectroscopic analysis (Table 1, Figure 1) indicated for all polymers a low dispersity a DP corresponding well to the theoretical DP, which is indicative of good control over the polymerization and is in accordance to literature.\textsuperscript{43}

![Figure 1](image)

**Figure 1.** (A) SEC traces of the different poly(HPMA-co-APMA) copolymers and their α-2,6-sialyllactose (abbreviated as SiLac) derivatives. (B) SEC traces of poly(HPMA$_{75\%}$-APMA$_{25\%}$)$^{DP100}$ and its lactose and α-2,6-sialyllactose derivatives.
Next, the primary amines of the APMA repeating units were ligated to α-2,6-sialyllactose by reductive amination. The terminal glucose unit has a hydroxyl substituent on the anomeric carbon and equilibrates between a cyclic hemiacetal and an aldehyde. The latter can react with the primary amines of the APMA repeating units, forming an iminium ion which is reduced by triacetoxyborohydride leading to a stable secondary amine (Scheme 1B). This synthesis strategy sacrifices the terminal glucose unit, but retains the essential Neu5Ac-α-2-6-Gal motif. For further use in control experiments we also prepared a lactose glycopolymer using a similar procedure. Successful modification of the polymers was confirmed by $^1$H-NMR spectroscopy and ninhydrin assay (Figure 2-3) indicating nearly full conversion of the amine moieties. SEC indicates for all polymers a counterintuitive slight decrease in molecular weight upon α-2,6-sialyllactose-ligation which we attribute to the difference in hydrodynamic behaviour, whereas the dispersity remains low (1.10-1.20) for all polymers.
CHAPTER 5 – INFLUENZA-BINDING SIALYLATED POLYMER COATED GOLD NANOPARTICLES PREPARED VIA RAFT POLYMERIZATION AND REDUCTIVE AMINATION

DP 25 25%

DP 100 10%
**Figure 2.** $^1$H-NMR analysis of the different poly(HPMA-co-APMA) copolymers and their lactose, respectively α-2,6-sialyllactose derivatives. Note that only for the DP 100 25% APMA copolymer the lactose derivative was synthesized whereas for all polymers the α-2,6-sialyllactose derivative was synthesized.

**Figure 3.** UV-VIS spectra of poly(HPMA-co-APMA) of sialyllactose modified poly(HPMA-co-APMA) after addition on the ninhydrin reagent, showing nearly full conversion of the primary amines.
In a next step, 17 nm (measured by TEM) goldNP were synthesized via direct reduction of HAuCl₄ by citrate in aqueous medium under reflux according to the Turkevich-Frens method. Decoration of the goldNP with the glycopolymers was done by simple mixing in aqueous medium followed by several centrifugation and washing steps to remove the unbound polymer. Quartz crystal microbalance (QCM) measurements on planar gold films confirmed that the polymers can adsorb onto gold (Figure 4). A first indication that the glycopolymers can also adsorb onto goldNP via ligand exchange with citrate ions was provided by the observation that the nanoparticles could be centrifuged and redispersed in deionized water, which is not possible for pure citrate goldNP that irreversibly aggregate after removal of the excess of citrate. Note that a control experiment for coating of the goldNP with unmodified poly(HPMA-co-APMA) leads to irreversible aggregation, likely due to electrostatic interactions between the cationic poly(HPMA-co-APMA) with the negatively charged citrate stabilized goldNP. Thermogravimetric analysis (TGA) (Figure 5) indicated that 5.7 % of organic materials are degraded before 750 °C for α-2,6-sialyllactose (DP100 – HPMA/APMA:75/25) glyco-goldNP, showing the packing density of polymer coating in 0.04 chain/nm² and 42 chains per particle.

Figure 4. QCM traces of the respective α-2,6-sialyllactose containing polymer followed by SNA or MAA adsorption on gold coated quartz chips. (B) quantification of the frequency shift upon adsorption of the α-2,6-sialyllactose containing polymer and SNA or MAA. n=3.
To assess whether ligation of α-2,6-sialyllactose to poly(HPMA-co-APMA) and subsequent goldNP decoration yields biologically active Neu5Ac-α-2-6-Gal moieties, we investigated the interaction between the glyco-goldNP and sialic acid binding lectins. Glyco-goldNP were mixed with either Sambucus nigra agglutinin (SNA) or Maackia amurensis agglutinin (MAA). These lectins bind preferentially to Neu5Ac-α-2-6-Gal in case of SNA, and to Neu5Ac-α-2-3-Gal in case of MAA.\textsuperscript{48, 49} Subsequent agglutination of the glyco-goldNP was investigated by ultraviolet-visible spectroscopy (UV-VIS) and dynamic light scattering (DLS) (Figure 6 A&C). The size of goldNP measured by DLS increases when SNA is added to sialyllactose glyco-goldNP, but not when MAA is added. Interestingly, a higher number of Neu5Ac-α-2-6-Gal units per polymer chain (i.e. DP100, 75/25 HPMA:APMA ratio) resulted in the most pronounced agglutination between the SNA and the sialyllactose glyco-goldNP, which points at an important contribution of multivalency in promoting nanoparticle agglutination.
Figure 6. Interaction between glyco-goldNP and the lectins Sambucus nigra agglutinin (SNA) and Maackia amurensis agglutinin (MAA) measured by DLS (A1&C1) and UV-VIS spectroscopy (A2&C2). In panel A1, different types of sialyllactose glycopolymers are used (with varying sialyllactose content and polymeric chain length). For all other experiments, only the polymer (i.e. DP100 – HPMA/APMA:75/25) that was most potent in agglutinating SNA was used. (B) Inhibition of glyco-goldNP-lectin interaction by soluble α-2,6-sialyllactose-polymer measured by UV-VIS spectroscopy (B1) and DLS (B2).

In contrast, neither SNA, nor MAA, induced aggregation of the control lactose glyco-goldNP, which rules out specific effects such as electrostatic interaction or hydrogen bonding. These findings were confirmed by UV-VIS measurements showing a shift in surface plasmon resonance peak of the glyco-goldNP from 529 nm to 540 nm and a strong decrease in
absorbance due to macroscopic precipitation. Furthermore, when soluble α-2,6-sialyllactose-polymer was added prior to the addition of SNA, inhibition of glyco-goldNP agglutination was observed (Figure 6B). QCM measurements (Figure 4) also confirmed these findings. Taken together, these observations clearly show that the Neu5Ac-α-2-6-Gal moieties retain their selective lectin-binding properties upon polymer ligation of α-2,6-sialyllactose via reductive amination.

Next, we aimed at investigating whether the glyco-goldNP can specifically bind to influenza virus by docking of the Neu5Ac-α-2-6-Gal units into the binding grooves on HA. PR8 H1N1 influenza virions were mixed with α-2,6-sialyllactose glyco-goldNP and lactose glyco-goldNP, respectively, followed by DLS and transmission electron microscopy (TEM). DLS (Figure 7A1) of bare virions shows a peak at 250 nm. Addition of α-2,6-sialyllactose glyco-goldNP led to the formation of two peaks, that we assign to individual α-2,6-sialyllactose glyco-goldNP (left peak, green curve) and aggregates of virus with the sialyllactose glyco-goldNP (right peak, green curve).

To prove the specificity of the interaction, two control experiments were performed. Firstly, when lactose glyco-goldNP were added to virus (Figure 7A3), no sign of goldNP-virus aggregation was observed. Note that in this case, the peak of the virus was no longer visible due to the excess of the lactose glyco-goldNP. Secondly, when mixing α-2,6-sialyllactose glyco-goldNP with virus is performed in presence of an excess of soluble α-2,6-sialyllactose-polymer (Figure 7A2), no goldNP-virus aggregation occurred as we expect all binding sites were occupied with soluble ligand preventing the multivalent interaction between α-2,6-sialyllactose glyco-goldNP and virus. Visual proof of the selectivity of the α-2,6-sialyllactose glyco-goldNP toward binding influenza virus was gained by TEM (Figure 7B). α-2,6-sialyllactose glyco-goldNP do show binding to influenza virus whereas lactose glyco-goldNP do not.
Finally, we aimed at investigating whether the Neu5Ac-α-2-6-Gal is prone to enzymatic cleavage, which would further contribute to the bioactive properties of the glyco-goldNP presented in this work. For this purpose, Arthrobacter ureafaciens sialidase was added to the α-2,6-sialyllactose glyco-goldNP followed by addition of either SNA or influenza virus. As shown in Figure 7C, prior treatment of the sialyllactose glyco-goldNP with Arthrobacter ureafaciens sialidase inhibits SNA lectin induced aggregation of the goldNP. This is evidenced by both DLS and UV-VIS showing return of the size and plasmon peak to the values of the original sialyllactose glyco-goldNP. Also binding to influenza virus was found, based on DLS results, to be inhibited by treatment of the sialyllactose glyco-goldNP with Arthrobacter ureafaciens sialidase.
Conclusions

Summarizing, we have demonstrated a straightforward route to design complex nanohybrid materials composed of sialylated carbohydrates, polymers and inorganic nanoparticles. These material are biologically active and interact with receptor molecules on the surface of the influenza virus. We believe our approach further extends the ‘glyco’-armory for the development of therapeutic or diagnostic applications or for fundamental studies looking at carbohydrates interactions.
Materials and Methods

Materials

N-(2-Hydroxypropyl)methacrylamide (HPMA) and N-(3-aminopropyl)methacrylamide) (APMA) were purchased from Polysciences. α-2,6-Sialylactose was purchased from Carbosynth. All other chemicals were obtained from Sigma-Aldrich. Milli-Q grade water was used for all experiments.

RAFT copolymerization of HPMA and APMA

Four types of copolymers of HPMA and APMA with different degrees of polymerization and APMA contents were synthesized by aqueous RAFT polymerization as previously reported.\textsuperscript{41,42} In brief, CTP (4-cyanopentanoic acid dithiobenzoate, 97%) and ACVA (4,4'-azobis-4-cyanopentanoic acid, >98%) were weighed with molar ratio of 2:1 and added to a round-bottomed flask equipped with a stir bar. HPMA and APMA with two proportions of 3:1 and 9:1 ([M]: [CTA] = 100:1 and 25:1) were then added to the flask and dissolved in a mixture of water and dioxane (2:1 in volume). After complete dissolution of monomer and CTP, the final solution was degassed by nitrogen bubbling for 30 min. The round bottomed flask was then placed in a preheated oil bath at 70°C. After reaction for overnight, the reaction was quenched by cooling the reaction vessel in an ice bath followed by exposing to air. The copolymer was obtained as pink powder after precipitation in pre-cooled acetone and drying under vacuum.

Synthesis of glycopolymers

α-2,6-sialyllactose was conjugated to the primary amino group of poly(HPMA-co-APMA) by reductive amination using NaBH(OAc)\textsubscript{3} as a reducing agent. The NaBH(OAc)\textsubscript{3} was added to the mixture of polymer and α-2,6-sialyllactose in 2 mL of DMSO. The reaction mixture was incubated at 50 °C for 4 days while it was constantly stirred, followed by dialysis against distilled water for 3 days using a Spectra membrane (molecular weight cutoff of 8000) to remove unreacted saccharide. Then the polymer was recovered in dry state by freeze-drying. Lactose modified polymers were synthesized in the same way, except using lactose instead of α-2,6-sialyllactose as reagent.
Physicochemical characterization of glycopolymers

$^1$H-NMR spectroscopy

$^1$H-NMR spectra were recorded on a Bruker Avance DRX 300 MHz spectrophotometer in deuterated water solution at room temperature. Chemical shifts are reported relative to tetramethylsilane (TMS).

Size exclusion chromatography (SEC)

For analytical SEC measurements in hexafluoroisopropanol (HFIP) (containing 3.0 g/L of potassium trifluoroacetate), a PU 2080+ pump, an auto sampler AS1555, a UV-detector UV 1575 (detection at 230 nm) and an RI-detector RI2080+ from JASCO were used. Columns packed with modified silica were obtained from MZ-Analysentechnik: PFG columns, particle size 7 µm, porosity 100 Å and 1000 Å. Calibration was carried out with poly(methyl methacrylate) standards purchased from PSS.

Ninhydrin assay

The ratio of substitution of primary amino group in polymers with saccharide was determined by the ninhydrin assay. Solutions of the glycopolymers were prepared in 0.1 M NaHCO$_3$ buffer. Ninhydrin was added and tubes were placed in a boiling water bath for 10mins. The solutions were cooled and the absorbance value at 570 nm was recorded. Poly(HPMA-co-APMA) copolymer solution were used to generate a standard curve.

Preparation of glycopolymer decorated goldNP

Citrate stabilized gold nanoparticle was synthesized via classic Turkevich and Frens method by direct reduction of gold salt in the presence of sodium citrate as reducing agent under reflux. Briefly, 24.4 mg of HAuCl$_4$ was dissolved in 50 mL of milliQ water in a round bottom flask pre-cleaned with aqua regia solution (mixture of HCl and HNO$_3$ at a ratio of 3:1) and heated to reflux. Then, 5ml of a sodium citrate stock solution with a concentration of 10 mg/mL was added to the gold salt solution and kept stirring for another 5 min under reflux. The obtained red colored solution depicted the formation of gold nanoparticles. After cooling to room temperature, the colloidal gold can be stored in fridge stably for a couple of years.

α-2,6-Sialyllactose-polymer decorated gold nanoparticles were prepared via “grafting-to” strategy by treating citrate stabilized goldNP with an aqueous solution of α-2,6-sialyllactose.
conjugated glycopolymers. Briefly, 8 mg of α-2,6-sialyllactose-polymer were dissolved in 200 µL of milliQ water and then added into 8 mL of citrate stabilized goldNP under constant stirring at room temperature for overnight. The glycoconjugate was purified using centrifugation at speed of 15000 g and washed with milliQ water for 3 times. After redispersion in water, α-2,6-sialyllactose-polymer decorated goldNP were stored in fridge prior to usage. Lactose-polymer modified goldNP were obtained via exchange of citrate stabilized goldNP with lactose functionalized polymers in the same way.

**Physicochemical characterization of glycopolymer decorated goldNP**

**Dynamic light scattering (DLS)**

DLS was performed at 25°C using a scattering angle of 173° with a Malvern Zetasizer Nanos S instrument equipped with a 4 mW He-Ne laser operating at 633 nm, an avalanche photodiode detector with high quantum efficiency, and an ALV-LSE-5003 multiple digital correlator electronics system. The intensity-average diameter and polydispersity of the goldNP and viral particles were calculated by cumulants analysis of the experimental correlation function using Dispersion Technology software version 7.20.

**Ultraviolet-Visible spectroscopy (UV-VIS)**

The UV-VIS spectra were obtained with a Shimadzu UV-1650PC spectrophotometer. The mixture of different glycopolymer coated gold nanoparticles with two types of lectins were placed in plastic cuvettes, and spectra were recorded in the 350-800 nm wavelength range at room temperature.

**Transmission electron microscopy (TEM)**

TEM images were recorded on a Jeol 1010 transmission electron microscope. GoldNP were incubated with formalin inactivated PR8 influenza virus for overnight at 4°C prior to TEM measurement and placed onto an electron microscopical grids followed by negative staining.

**Thermogravimetric analysis (TGA)**

The grafting efficiency on the GoldNP was determined by thermogravimetric analysis using a TGA Q 5000 from TA Instruments in a modulated mode, with a heating linear ramp of 20°C
/min from room temperature to 1000 °C and under N₂ atmosphere. The graft density of polymer chains could be calculated as follows:

Surface density of polymer (chains/nm²) = \( \frac{(W_{\text{polymer}} - W_{\text{polymer}})}{100} \cdot \rho \cdot V_{\text{particle}} \cdot N_A \cdot M_{\text{polymer}} \cdot S_{\text{particle}} \)

Where, \( W_{\text{polymer}} \) is the percent weight loss corresponding to the decomposition of polymer, \( \rho \) is the density of gold (19.32 g/cm³), \( V_{\text{particle}} \) is the volume of one goldNP calculated from the size measured by TEM (2572 nm³), \( N_A \) is Avogadro constant (6.02×10²³ mol⁻¹), \( M_{\text{polymer}} \) is the molecular weight of polymer (42716 g/mol), and \( S_{\text{particle}} \) is the surface area of one goldNP (940 nm²).⁴⁴

**Binding assay of glycopolymer decorated goldNP**

To investigate the binding properties of glycopolymer decorated goldNP with lectins, 10 µL of α-2,6-sialyllactose-polymer decorated goldNP (2.5 mg/mL in gold content) was diluted in 950 µL of PBS solution prior to the addition of 40 µL of SNA stock solution in PBS with concentration of 1 mg/ml and incubated at 4°C for overnight with lectin. MAA was used as a control for binding to the goldNP. The change in size of the goldNP was determined via DLS measurements. The surface plasmon resonance peak of goldNP before and after incubation with lectin was obtained via UV-VIS spectroscopy. To investigate the specific lectin-binding property of α-2,6-sialyllactose-polymer decorated goldNPs, lactose-polymer decorated goldNPs were also treated with lectins as control.

To investigate the binding properties of glycopolymer decorated goldNP with influenza virus, all binding studies were performed by incubating aliquots of the goldNP with formalin inactivated PR8 influenza virus suspension for overnight at 4°C. Briefly, 2 µL of goldNP decorated with α-2,6-sialyllactose-polymer were dispersed in 98 µL of PBS and then added to 100 µl of virus suspension which was prepared via dilution of virus (512 hemagglutinating units/µl) in PBS (1 to 10). After overnight culture, the change in size was performed via DLS measurement at 25°C. Lactose-conjugated goldNP were used as control to investigate the specific virus-binding property with virus.
Formalin inactivation of PR8 influenza virus

PR8 influenza virus (A/Puerto Rico/8/1934) was grown on MDCK cells in serum-free medium in the presence of TPCK-treated trypsin. Seven days after inoculation, culture medium was collected and centrifuged twice for 10 min at 450 g to remove cellular debris. Virions were then pelleted by centrifugation (20,000 g, 4°C, 16 h). Virus was inactivated by dissolving the pellets in 0.05% formaldehyde (prepared from formalin stock) in PBS followed by continuous shaking for 7 days at 4 °C. Formalin-containing buffer was exchanged for PBS >10,000-fold (volume / volume) by sequential ultrafiltration with Vivaspin filtration columns (Sartorius Stedim Biotech, Aubagne Cedex, France) with 100 kDa cutoff. X47-WIV was stored at 4 °C in the dark until used.
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CHAPTER 5 – INFLUENZA-BINDING SIALYLATED POLYMER COATED GOLD NANOPARTICLES PREPARED VIA RAFT POLYMERIZATION AND REDUCTIVE AMINATION
CHAPTER 6

BROADER INTERNATIONAL CONTEXT, RELEVANCE AND FUTURE PERSPECTIVES
Gold nanoparticles have been explored by many different groups for a wide variety of applications, ranging from biomedical applications including imaging,\textsuperscript{1, 2} drug delivery,\textsuperscript{3, 4} diagnostic\textsuperscript{5, 6} to catalysis,\textsuperscript{7, 8} optics\textsuperscript{9, 10} and microelectronics\textsuperscript{11, 12} to name only a few. For many of these applications, surface functionalization plays a critical role. Different routes have been explored for this purpose and require the use of ligands that stably bind to the metallic gold surface.\textsuperscript{7, 13} This offers the opportunity to directly introduce the functionality of choice using functional ligands during the nucleation of the gold nanoparticles. However, this route offers poor control of nanoparticle size and geometry as these parameters are typically determined by the choice of ligand and are thus fixed when using a specific functionalized ligand. To offer the freedom of using pre-formed gold nanoparticles as template with desired size and geometry, a ligand-exchange approach is a highly attractive approach to functionalize gold nanoparticles.\textsuperscript{14, 15} In this regard, the use of thiol-containing ligands is the most widespread strategy to obtain functionalized gold nanoparticles.\textsuperscript{16-18} The most commonly used ligands used to prepare gold nanoparticles that can easily be post-functionalized through ligand exchange are citrate,\textsuperscript{19} DMAP\textsuperscript{20} and CTAB.\textsuperscript{21} Citrate acts as both reducing agent – to obtain metallic gold from gold ions – and stabilizing agent. The thus obtained colloidal gold suspensions bear a negative surface charge and lose their colloidal stability upon removal of the excess of citrate ligand, which attributes to the dynamic behavior of this kind of ligand stabilization.

In this thesis work, citrate based gold nanoparticles were used in all experiments and were found in Chapter 2 (vide supra) to confer particular properties to polymer coated gold nanoparticles. One of the advantages of using citrate-stabilized gold nanoparticles is that the typical synthesis procedure reported by Turkevich\textsuperscript{22} yields gold nanoparticles with a size of roughly 15-25 nm, which have plasmonic properties\textsuperscript{23} that can be utilized for sensing purposes, by monitoring the red to blue shift of a colloidal gold suspension upon agglomeration of the nanoparticles.\textsuperscript{2} DMAP-stabilized gold nanoparticles\textsuperscript{24} bear a positive surface charge due to the presence of basic amino groups. Generally DMAP-stabilized gold nanoparticles are much smaller and have a size from 1-5 nm and don’t exhibit the plasmonic properties of their larger counterparts. Although potentially interesting for several
applications explored in this thesis, we did not explore the use the DMAP-stabilized gold nanoparticles. This remains an avenue for further research. The same accounts for CTAB stabilized gold nanoparticles. The cationic surfactant CTAB has been reported to grant access to anisotropic gold nanoparticles,\textsuperscript{25,26} which have attractive plasmonic properties in the near infrared region.\textsuperscript{27,28} It is certainly interesting to investigate in future endeavors the influence of anisotropy on the interactive properties of polymer-functionalized gold nanoparticles.

Amongst the different types of functional molecules that can be ligated to the surface of gold nanoparticles, polymers are of particular interest for several reasons.\textsuperscript{29,30} Firstly, polymer, unlike small molecules, can provide steric stabilization which is of interest to confer colloidal stability in complex physiological fluids. Secondly, polymers can possess properties that are uncommon for small molecules. A good example of this, which is elaborated on as well in this PhD thesis, are temperature-responsive properties.\textsuperscript{31} Third, as polymers exists of many different copies of the same (or a combination of a few) monomeric repeating units, polymers are able to undergo multivalent ligand-receptor recognition.\textsuperscript{32} This feature has also been explored in this thesis.

To obtain polymer-functionalized gold nanoparticles, polymers bearing a sulfur-containing end-group are most explored.\textsuperscript{33,34} In this regard, the advent of controlled radical polymerization has strongly boosted research endeavors in polymer-functionalized gold nanoparticles. Indeed, controlled radical polymerization techniques afford the synthesis of well-defined polymers with specific functional end-groups. As free thiols are known transfer agents that interfere with the radical polymerization process,\textsuperscript{35} typically ‘protected’ thiols are used to synthesize polymers with sulfur-containing end-groups. These typically include disulfides, than can be present in linear or cyclic (i.e. lipoic acid derivatives)\textsuperscript{36,37} form or as pyridyl-disulfides. These routes have been explored to synthesize sulfur-containing polymers for gold nanoparticle conjugation via Atom Transfer Radical Polymerization (ATRP).\textsuperscript{38} Reversible Addition-Fragmentation chain Transfer (RAFT) polymerization\textsuperscript{39,40} is particularly well suited for the design of polymer that can adsorb onto metallic gold.\textsuperscript{41} RAFT requires the use of thiocarbonate or xanthate chain transfer agents that contain sulfur and thus have inherent gold-binding properties. In this thesis we almost uniquely make use of RAFT-based polymers, but similar findings could be obtained using sulfur-end-functionalized obtained via
ATRP – as briefly touched upon in Chapter 3 – as well as by polymers obtained via cationic ring-opening polymerization that are subsequently capped with a xanthate moiety. The latter was demonstrated in a jointly published manuscript of our research group and the Hoogenboom group.42

In Chapter 2 we report on the temperature-responsive behavior of citrate based gold nanoparticles functionalized with polyNIPAm. In this work we make use of two very well-known building blocks, i.e. gold nanoparticles prepared via citrate reduction of gold ions and poly(N-isopropylacrylamide) (polyNIPAm). PolyNIPAm is the most studied temperature-responsive polymer that is soluble below a critical temperature of 32 °C (often referred to as the phase transition temperature \(T_{CP}\)) whereas it precipitates out of solution above this temperature due to entropic reasons.43 Although there are several reports on the temperature-responsive behavior of polyNIPAm-functionalized gold nanoparticles44, 45 we found to our surprise that when adsorbing RAFT-based polyNIPAm onto citrate-stabilized gold nanoparticles, followed by removal of the excess of citrate and non-adsorbed polymer, no temperature-responsive behavior of the resulting colloidal solution could be observed. When carefully analyzing the different parameters that could play a role in this process we found that the remaining citrate groups on the gold nanoparticles – which conferred them with a negative \(\zeta\)-potential – provided electrostatic stabilization of the colloidal solution above the \(T_{CP}\) of the polyNIPAm. However, when salt was added to the solution, the electrostatic interaction were screened and a temperature-responsive behavior of the polyNIPAm-functionalized gold nanoparticle solution was observed within the same temperature-regime as for soluble polyNIPAm. The crucial contribution of salt to achieve such behavior has been overlooked so far in literature and provides to our opinion an important contribution to the understanding of the behavior of polymer-coated gold nanoparticles in aqueous medium. Together with the Hoogenboom group, we have further explored different kinds of polymers that also exhibited temperature responsive behavior, including poly(2-oxazolines)42 and copolymers of \(N\)-vinylpyrrolidone and \(N\)-vinylcaprolactam.46 Practical applications for the dual salt- and temperature-responsive behavior of polymer-coated gold nanoparticles have also been developed in collaboration with the Hoogenboom group to design colloidal logic gates and sensors.47
During several of our experiments with polyNIPAm-functionalized gold nanoparticles we observed that upon addition of salt to a colloidal polyNIPAm@goldNP solution, a colored film was deposited on the surface of the recipient. Interestingly, this phenomenon also took place below the T_{CP} of polyNIPAm. Atomic force microscopy (AFM) analysis revealed that a quasi-monolayer of gold nanoparticles was deposited. Similar observations were made for other temperature-responsive polymers, but not for fully hydrophilic polymers. In Chapter 3, careful exploration of the different parameters that played a role in this process prompted us to postulate the hypothesis that screening of the remaining electrostatic interactions by salt ions induced irreversible adsorption of gold nanoparticles onto hydrophobic surface, likely through hydrophobic interactions. Colloidal lattices of gold and other nanoparticles onto a solid support have been reported earlier, but were never assembled by mere addition of salt to an aqueous solution. In this regard our observation afford an extremely easy way to functionalize surfaces with a coating of metal nanoparticles, and holds some similarity with the work of the Messersmith and Caruso groups that reported on the spontaneous growth of nano-thin films based on dopamine and polyphenols. The versatility of our approach was further demonstrated for polyNIPAm-coated silver nanoparticles. As colloidal silver has anti-bacterial properties, such films could hold potential to confer antibacterial properties to solid surfaces through adsorption of a polyNIPAm@silverNP film. However, the suitability of such films for real applications still needs to be investigated. Further applicability of our approach to coat solid surfaces with a metal nanoparticle film was investigated to confer catalytic properties to an otherwise catalytically inter material. This was demonstrated for model chemical reaction – i.e. the conversion of 4-nitrophenol to 4-aminophenol – but showed that despite the presence of the polyNIPAm coating on the gold nanoparticles they still exhibited catalytic activity even when adsorbed onto a solid surface. This approach holds potential to design larger solid-supported catalysts for industrial applications. Without doubt such approach circumvents environmental issues related to homogeneous catalysts, but the performance against other more-established types of heterogeneous solid-supported catalysts still needs to be investigated.

In Chapter 4, we prepared a series of random RAFT-based copolymers that had varying ratios of a hydrophilic and a hydrophobic co-monomers. These polymers were used to functionalize colloidal gold nanoparticles leading to a series of nanoparticles with varying surface
hydrophobicity. As it is well recognized that particle size, shape and surface chemistry strongly influence the efficiency at which particles are internalized by living cells, we investigated whether nanoparticle-cell interaction could be influenced by the hydrophobicity of a polymer coating. This was found to be indeed the case with increasing hydrophobicity of the polymer coating leading to increased goldNP-cell association. For these research endeavors we devised a flow cytometry strategy to assess the uptake of gold nanoparticles by living cells based on the shift in SSC intensity that occurs when nanoparticles that efficiently scatter light are taken up by cells. This method was validated by ICP-MS and could hold potential for straightforward and high throughput screening of cellular interaction with other metallic crystalline and inorganic materials.

Our findings suggest that either the subtle differences in the composition of the protein corona or the interaction between the polymer coating itself and the cell membrane might account for this phenomena. We have demonstrated that the presence of serum plays an important role in governing cellular interaction with polymer coated goldNP, but its exact contribution needs to be further studied. Earlier findings by the Rotello group\textsuperscript{53} using gold nanoparticles coated with small molecule ligands with different hydrophobicity have shown that surface hydrophobicity alters the cytokine expression profile by immune cells. Such investigation would be relevant as well for polymer-coated gold nanoparticles and could be the subject of future research. The Stellacci\textsuperscript{54,55} and Irvine\textsuperscript{56} groups have shown that gold nanoparticles – although in the lower nanometer range – coated with ligands of mixed hydrophobicity exhibit striped ordering of the ligands and resulted in particular biological behavior, including energy-independent cell uptake. Such behavior was not observed for our systems, but this could be attributed to the larger size of the gold templates that were used as well as to the specific nature of the ligands (polymer in our case versus small molecule thiols by the Stellacci group). Furthermore, our investigation on the biological interaction between polymer-functionalized gold nanoparticles and living cells were limited to \textit{in vitro} settings. The behavior of these systems in vivo still needs to be explored and recent findings by the Parak group have suggested that the presence of enzymes can strongly alter the \textit{in vivo} fate of polymer coated gold nanoparticles.\textsuperscript{57}
In **CHAPTER 5** we reported on the design of multivalent glycosylated gold nanoparticles by a combination of RAFT polymerization and reductive amination. This methodology was applied for the ligation of di- and tri-saccharides to primary amine moieties in a RAFT-based polymer, followed by grafting of these polymers on the surface of goldNP via ligand exchange. Despite the sacrifice of the terminal monosaccharide unit to form an aldehyde for the further conjugation with a primary amine, our method offers a simple and straightforward strategy to obtain complex glyco-conjugates that circumvents the need for complex and labor-intensive recombinant, chemo-enzymatic or carbohydrate chemistry routes.\(^{58}\)

In this thesis we used this approach for the synthesis of sialylated gold nanoparticles that were able to undergo multivalent and specific ligand-receptor binding with sialic acid binding lectins in solution and on the surface of influenza viruses. Sialylated polymers have extensively been reported by the Whitesides group in the 80ties for specific influenza agglutination.\(^{59-61}\) However, even for ligating the sialic acid monosaccharide multi-step synthesis was required, whereas for proper binding to hemagglutinin on the influenza virus surface α-2,6-sialic acid-galactose is needed. Sialylated gold nanoparticles were reported by the Haag group,\(^ {62}\) but were also based on sialic acid monosaccharides. So far, the applicability of our system was only demonstrated *in vitro* for mere agglutination of influenza viruses and practical applicability is still to be demonstrated. Currently, we anticipate that the system would have potential as plasmonic sensor for influenza virus, to inhibit influenza binding or as delivery vehicle. For the later purpose, one of the future research endeavors could focus on the assembly of recombinant hemagglutinin on the surface of sialylated goldNP to serve as vaccine against more conserved antigen epitopes that are situated closer to the virus membrane.
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Gold nanoparticles (goldNP) are immensely popular objects of study for chemists, physicists and biomedical scientists. Beyond their beauty, gold nanoparticles exhibit fundamentally properties that differ them from many other types of materials. Due to their tailorable size, gold nanoparticles can be engineered to enter cells much more rapid than small molecules, and can preferentially accumulate at sites of tumor growth or inflammation. Owing to their Surface Plasmon Resonance, colloidal goldNP solutions exhibit size- and shape-dependent color, a feature that is widely used for the development of diagnostic assays. Due to their ability to enhance surface-enhanced Raman scattering, goldNP have been used as effective substrates for Raman spectroscopy. As goldNP can efficiently promote the conversion of light into heat, they have high potential for use in photothermal therapy. Due to their high X-ray attenuation, goldNP have gained recent attention as an X-ray contrast agent in X-ray imaging and computed tomography. The unique reactivity of metallic gold surface allows goldNP to be coated with polymers, small molecules, and biological (macro)molecules. Moreover, many of the previously discussed properties of goldNP can be combined in a single construct, enabling multifunctional particles to be used in chemical, biological, engineering, and medical applications.

In this thesis, we explore the use of Reversible Addition Fragmentation chain Transfer (RAFT) polymer coated goldNP as interactive materials in a biomedical context. In our work we apply a grafting-to strategy to functionalize goldNP with polymers. This strategy comprises first the synthesis of polymers via RAFT polymerization followed by adsorption (i.e. grafting) of these polymers onto the surface of citrate-stabilized goldNP via ligand exchange. The latter is possible owing to the inherent gold binding abilities of RAFT polymers that contain sulfur atoms at the Z-position of the chain transfer agent. This generic strategy offers a higher degree of versatility regarding the size and shape of the gold nanoparticles to be used. In this thesis, different types of polymers with different types of functional groups are used which
equip goldNP with interactive properties that allow them to interact with themselves, solid surfaces, living cells and viruses.

In Chapter 1, an introduction about the goldNP for biomedical use was given. The synthesis methods, the modification strategies as well as several biomedical application were discussed.

In Chapter 2, we evaluated the effect of salt on (citrate-stabilized) goldNP coated with a temperature-responsive polymer with respect to temperature-triggered (reversible) nanoparticle aggregation and hydrogen bonded Layer-by-Layer assembly. PolyNIPAm modified goldNP were found insensitive to temperature in pure water, but show temperature responsive behavior in aqueous 0.1M sodium chloride solutions. Moreover, the presence of salt can also strongly enhance Layer-by-Layer build-up of polyNIPAm coated goldNP with tannic acid. These findings were attributed to a combination of ionic screening of remaining negatively charged citrate groups on the surface of nanoparticles and a salting-out effect on the polyNIPAm, causing a stronger dehydration of the polymer chains, thereby enhancing hydrophobic interactions.

Temperature-responsive polymer coated noble metal nanoparticles were found to form a monolayer-like structure on a wide variety of substrates spontaneously in presence of sodium chloride. Therefore in Chapter 3, a simple, broadly applicable method for depositing metal nanoparticle films on a wide variety of solid surfaces under all aqueous conditions was reported. Our approach is demonstrated for gold and silver nanoparticles and allows for extremely easy engineering of bulk materials with plasmonic and catalytic functionality.

In Chapter 4, we reported on how cellular uptake and protein corona of (co)polymer-coated goldNP can be altered by the hydrophilic-to-hydrophobic co-monomer ratios. Owing to the strong light scattering properties of goldNP, a novel, label-free flow cytometry strategy was developed to investigate particle uptake by dendritic cells line DC2.4 and different parameters that affect goldNP-cell interaction. The strong influence of the hydrophobic-to-hydrophilic ratio of the polymer coating is observed, with increasing hydrophobicity of the polymer coating leading to increased goldNP-cell association. These phenomena were confirmed by transmission electron microscopy (TEM) images and inductively coupled plasma mass
spectrometry (ICP-MS) data. Additionally, a vast difference in particle uptake is observed dependent on the incubation time and temperature.

In **CHANGERTY 5**, a simple and straightforward strategy was reported to fabricate bioactive glycosylated gold nanoparticles via a combination of RAFT polymerization, carbohydrate ligation through reductive amination and thiol-gold self-assembly. The trisaccharide α-2,6-sialyllactose is conjugated to a pre-synthesized polymer containing primary amines in the side chain. Importantly, this strategy preserves the Neu5Ac-α-2-6-Gal motif that can be recognized by hemagglutinin. The obtained glycopolymers were used to decorate citrate stabilized goldNP via ligand exchange, allowing the goldNP to specifically interact with lectins and with influenza virus. Moreover, a higher number of Neu5Ac-α-2-6-Gal units per polymer chain resulted in more pronounced agglutination between the SNA and the α-2,6-sialyllactose glyco-goldNP, pointing at an important contribution of multivalency in promoting nanoparticle agglutination.

As a general conclusion, one can state that RAFT polymers allow for engineering goldNP with a wide variety of interactive properties. The influence of salt and the ionic strength of the surrounding aqueous medium, on a number of these properties was explicitly demonstrated and allowed for modulating these properties. Furthermore, our findings illustrate the importance of surface chemistry on the final properties of polymer coated goldNP and could be of importance for the rational design of interactive nanoparticles for various applications.

In deze thesis wordt het potentieel van RAFT polymeer gecoate goud nanopartikels onderzocht in een biomedische context. Om dergelijke nanopartikels te verkrijgen werd gestart met het synthetiseren van polymeren via RAFT polymerisatie, die vervolgens op goud nanopartikels geadsorbeerd wordt via ligand exchange. Dit laatste is mogelijk door de inherente goud-bindende eigenschappen van RAFT chain transfer agents die zwavelatomen bevatten. Deze generische strategie geeft een grotere vrijheid m.b.t. het gebruik van goud nanopartikels met verschillende grote en vorm, dan wanneer een polymeerketen rechtstreeks vanop het goud oppervlak zou gesynthetiseerd worden. In deze thesis werden verschillende types polymeer-gecoate goud nanopartikels ontwikkeld met verschillende types functionele
groepen, waardoor interactieve nanopartikels verkregen werden die kunnen interageren met respectievelijk zichzelf, vaste oppervlakken, levende cellen en virussen.

In **Hoofdstuk 1** wordt een introductie gegeven over het gebruik van goud nanopartikels voor biomedisch gebruik. Hierbij worden verschillende synthese en modificatie methodes besproken en wordt dieper ingegaan op enkele biomedische toepassingen.

In **Hoofdstuk 2** wordt de invloed bestudeerd van zout op het gedrag van (citraat gestabiliseerde) goud nanopartikels die gecoat zijn met het temperatuur-responsieve polymeer poly(N-isopropylacrylamide) (polyNIPAm). Meer bepaald wordt nagegaan of de aanwezigheid van zout een invloed heeft op de temperatuur-getriggerde (reversibele) aggregatie van polyNIPAM gecoate goud nanopartikels en wordt de mogelijkheid van deze nanopartikels onderzocht om samen met tanninezuur *Layer-by-Layer* filmen te vormen. We stellen vast dat polyNIPAm-gecoate goud nanopartikels ongevoelig zijn voor temperatuurwijzigingen in gedeioniseerd water. In aanwezigheid van 0.1 M natriumchloride werd echter temperatuur-responsief gedrag waargenomen, waarbij de nanopartikels reversibel konden aggregeren wanneer de fase-transitie temperatuur van het polyNIPAm wordt overschreden. Tevens werd vastgesteld dat de aanwezigheid van zout de *Layer-by-Layer* opbouw van deze nanopartikels met tanninezuur sterk verbetert. Deze observaties worden toegeschreven aan een combinatie van het afschermen van negatieve ladingen op het goudoppervlak van residuele citraatgroepen door zoutionen enerzijds, en anderzijds het uitzoutingseffect dat zorgt voor een sterkere dehydratatie van de polymeerketens en hierbij hydrofobe interacties bevordert.

Goud en zilver nanopartikels gecoat met temperatuur-responsieve polymeren geven aanleiding tot de spontane vorming van een *monolayer*-achtige structuur op verschillende soorten substraten in aanwezigheid van natriumchloride. Deze observatie wordt verder uitgewerkt in **Hoofdstuk 3**, waarbij een eenvoudige een breed toepasbare methode werd ontwikkeld om dunne films bestaande uit metaal nanopartikels af te zetten op vaste substraten onder volledige waterige condities. Deze methode wordt aangetoond voor zilver en goud nanopartikels en laat toe om oppervlakken te voorzien van plasmonische en katalytische eigenschappen.
In **Hoofdstuk 4** wordt beschreven hoe de cellulair opname en de eiwitcorona van (co)polymeer-gecoate goud nanopartikels wordt beïnvloed door de hydrofiele-tot-hydrofoob ratio van de respectievelijke co-monomeren. Het vermogen van goud nanopartikels om licht te verstrooien liet ons toe om op basis van flow cytometrie een *label free* methode te ontwikkelen om de opname van goud nanopartikels door levende cellen te bestuderen. Hiervoor werd de dendritische cel DC2.4 cellijn gebruikt en werden verschillende parameters onderzocht die de interactie tussen de nanopartikels en deze cellen beïnvloedden. Een uitgesproken invloed van de hydrofiele-tot-hydrofoob ratio van de polymeer coating werd waargenomen, waarbij een meer hydrofobe coating aanleiding gaf tot een sterkere associatie tussen nanopartikels en cellen. Deze observaties werden bevestigd door transmissie elektronen microscopie (TEM) en massaspectrometrie (ICP-MS). Tevens werden grote verschillen in nanopartikel opname waargenomen afhankelijk van de incubatietijd en temperatuur.

In **Hoofdstuk 5** wordt een eenvoudige maar efficiënte strategie ontwikkeld om bio-interactieve geglycosyleerde goud nanopartikels te verkrijgen. Hierbij werd gebruik gemaakt van een combinatie van RAFT polymerisatie, *carbohydrate ligation* d.m.v. reductieve aminering en thiol-goud *self-assembly*. Het trisacharide α-2,6-sialyllactose werd gekoppeld aan een polymeer dat primaire amines bevat in de zijketen. Deze strategie laat het Neu5Ac-α-2-6-Gal motief intact, waardoor er binding mogelijk is met hemagglutinine. De verkregen glycopolymeren werden vervolgens gebruikt om goud nanopartikels te coaten via *ligand exchange*. Deze nanopartikels waren in staat om selectief te interageren met lectines en met het influenzavirus. Tevens werd vastgesteld dat een groter aantal Neu5Ac-α-2-6-Gal eenheden per polymeerketen aanleiding gaf tot een meer uitgesproken lectine-binding. Deze observatie duidt op een belangrijke bijdrage van *multivalency* in het promoten van biologische interactie van deze nanopartikels.

Als algemeen besluit kan gesteld worden dat polymeren verkregen via RAFT polymerisatie toelaten om goud nanopartikels te voorzien van een breed scala aan interactieve eigenschappen. Tevens werd vastgesteld dat de ionaire sterkte van het medium een uitgesproken invloed heeft op het gedrag van een aantal types polymeer gecoate goud nanopartikels. De bevindingen vermeld in deze thesis illustreren het belang van
oppervlaktechemie om de finale eigenschappen van polymer gecoat goud nanopartikels te engineeren en kunnen van belang zijn voor het rationeel ontwerpen van interactieve nanopartikels voor diverse toepassingen.
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EDUCATIONS

2016 Faculty of Pharmaceutical Science, Ghent University, Belgium
PhD in Pharmaceutical Science
Thesis Interactive RAFT Polymer-Decorated Gold Nanoparticles for Biomedical Use

2012 School of Pharmaceutical Sciences, Shandong University, P.R. China
Master in Pharmaceutical Science
Thesis The Study of Etoposide Loaded Microspheres for Pulmonary Delivery

2009 School of Marine, Shandong University, P.R. China
Bachelor in Science
PUBLICATIONS


PRESENTATIONS ON CONFERENCES


AWARDS

1. FWO (Fonds Wetenschappelijk Onderzoek) conference travel grant, 2014, UGent, Belgium

2. FWO (Fonds Wetenschappelijk Onderzoek) conference travel grant, 2015, UGent, Belgium