Radiosensitizatioin of cancer cells using gold nanoparticles

Bachelor Thesis

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April 21, 2016

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Abstract

abstract

1 Introduction

Each year 8.2 million people die from cancer, that is an estimated 13% of all deaths worldwide. Moreover the number of new cases is expected to increase by 70% over the next two decades. [**?**] These numbers are a clear indication of the importance of cancer research, which includes the development of new treatments and the enhancement of existing treatments.
Cancer is a general term for a large group of diseases that are all characterised by a rapid creation of abnormal cells that grow beyond their usual boundaries. The cells grow out of control and thereby form tumors, because the part of the DNA responsible for cell death is disturbed.
The three major cancer treatments that exist today are chemotherapy, surgery and radiation therapy. In chemotherapy anti-cancer drugs are used to damage the cancer cells. These drugs only affect rapid dividing cells, which is a typical characteristic of cancer cells. Cancer can also be treated by removing the tumor by surgery. A third important cancer treatment is radiation therapy, where ionizing radiation is used to damage the DNA of the cancer cells. Because in most cases combinations of these treatments are used, it is estimated that 50% of all cancer patients undergo radiation therapy as part of their treatment. [**?**]
The problem with ionizing radiation is, that it does not discriminate between malignant and normal tissues. This means that normal tissue is also irradiated. Therefore radiation therapy has an unintentional toxic effect on the healthy tissue surrounding the tumor. These effects range from those that cause mild discomfort to others that are life-threatening. The radiation dose has to be carefully chosen: it has to be high enough to bring damage to the cancer cells, but it cannot be too high because of the negative effect on the healthy tissue.
The toxic effect of radiation therapy can be reduced in different ways. For example instead of delivering the total dose of radiation at once, the dose can be fractionated in smaller doses that are delivered over a longer period of time. Dose fractionations offers the opportunity for healthy cells to recover from the previous dose before the next dose is delivered. [**?**] Another technique to reduce the effect on healthy tissue is radiosensitization. With this technique it is possible to make the cancer cells more sensitive to ionizing radiation, so that lower radiation doses can be used. This reduces the effect on the surrounding tissue. Here radiosensitization using gold nanoparticles (GNP) is discussed. The absorption of photons is higher for elements with high mass numbers. Therefore because of the high mass number of gold (Z=79), when the GNP can be brought inside the tumor, the ionizing radiation will mainly interact with the nanoparticles. Thereby causing local secondary radiation, which delivers its energy locally, so inside the tumor. [**?**] The reason gold is used instead of other element with high atomic mass, is that gold is biocompatible, which

makes it suitable for medical treatment. [**?**]
The first experimental evidence of the use of GNP to enhance radiation therapy was provided by Hainfeld et al. [**?**]. Mice with cancer tumors were injected with 1.9 nm diameter GNP and then radiated with 250 kVp X-rays. The combination of GNP and radiation resulted in a one-year survival of 86% compared to 20% with radiation therapy alone. Other experiments showed similar evidence of the radiosensitizing effect of GNP. [**?**][**?**] These results provide a motivation for further research within the field of nanoparticle enhanced radiation therapy.
The main goal of this project is to synthesize GNP of different sizes and to characterize them using different methods. Next the GNP are functionalized with a polyethylene glycol (PEG) coating, which increases the probability of delivering the nanoparticles to the cancer cells. The PEG coating also provides stability to the GNP solution, i.e., prevents them from aggregating. Finally a mixture of DNA and GNP is irradiated and the effect on the DNA is analysed.

2 Theoretical background

2.1 Radiation physics

The ionizing radiation type used for radiation therapy can vary ranging from photons and electrons to protons, neutrons and low-mass ions, but photons are by far the most common form of radiation used in cancer treatment. [**?**] There are three possible ways photons can interact with matter: photoelectric absorption, Compton scattering and pair production.
In the photoelectric effect, the energy of an incoming photon is transferred to an electron, which is then ejected. The vacancy left by this electron is then filled with another electron from a higher shell. This electron then gives of its excess energy as an characteristic X-ray photon. In some cases the excess energy may be transferred to an outer-shell electron. As a consequence this electron is ejected and is called an Auger electron. The cross section for photoelectric absorption τ increases for increasing mass number Z and in decreases sharply with the photon energy E_γ:

$$\tau \cong C^{te} \cdot \frac{Z^n}{{E_\gamma}^{3.5}} \tag{1}$$

with n varying between 4 and 5.[**?**]
The Compton effect is an inelastic scattering between a photon and an electron, where part of the energy of the incoming photon is transferred to the recoiling electron. The cross section for Compton scattering grows linearly with Z and falls of gradually with increasing energy.[**?**]
With pair production, a photon creates an electron-positron pair. The cross section for this process varies approximately with Z^2 and increases for increasing energy.[**?**]
Since the cross sections for each of these processes increases with increasing Z, it is clear that gold, with its high atomic mass, is suitable for radiosensitization.

2.2 Biological effects

2.3 Targeting

In order to have a beneficial effect of GNP in radiation therapy, it is important to bring the nanoparticles as close as possible to the DNA of the cancer cells. The uptake of GNP into the nucleus of the cells is only possible below a certain upper size limit.
http://onlinelibrary.wiley.com/doi/10.1002/smll.201000134/full Therefore the size of the nanoparticle is a very important parameter. GNP are known to passively accumulate in cancer cells because of the enhanced permeability and retention (EPR) effect. Because cancer cells are rapid growing cells, tumors have leaky, immature vasculature, so that their blood vessels are more permeable.
http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3473940/ This effect can be enhanced by functionalizing the naked gold nanoparticles with PEG. This PEG coating sterically hinders nonspecific binding of proteins to the surface of the particle and delays the recognition of the particles by the reticuloendothelial system. This increase the circulation time of the GNP in the blood and as a result increases the probability of delivering the nanoparticles to the tumor.
http://onlinelibrary.wiley.com/doi/10.1002/smll.201000134/full The hydrodynamic radius of the

particles increases because of this PEG coating, therefore the optimal size (of the naked GNP) for uptake is smaller when the particle is functionalized. Finally the functionalization also has a positive effect on the stability of the GNP solution.
Besides PEGylation, the GNP can also be coated with antibodies, which actively bind to receptors that are specific for cancer cells. An example of a receptor that is overexpressed in tumors is the epidermal growth factor receptor. Nanoparticles coated with an antibody that corresponds to this receptor are guided to the tumor and bind on its surface. http://www.mdpi.com/2079-4991/1/1/31/htm

3 Theoretical backround

The project consists of three major parts. First the gold nanoparticles are synthesized using the Turkevich [2] method followed by thorough characterization of the particles using several techniques such as TEM, UV-VIS, DLS and ζ-potential. Finally the radiosensitization effect of the GNP is tested by irradiation of samples containing circular DNA and GNP.
This section gives an overview of all techniques and their mechanism used in this project.

3.1 Synthesis GNP

3.1.1 Method of Turkevich

The synthesis of gold nanoparticles is a two step process. First there is the nucleation step. Small seeds of atomic gold are formed after the addition of an reducing agent to a solution of gold ions in an aqueous environment. Secondly there's the growth process where small particles aggregate together to form bigger particles.

As mentioned above the Turkevich method is used. Sodiumcitrate ($NA_3C_6H_5O_7$) is added to an tetrachlorauric acid ($HAuCl_4$) aqueous solution to reduce the Au^{3+} ions (see figure 1),the nucleation process has started. After the reduction the negative citrate ions remain on the surface of the newly synthesized gold seed causing a negative surface potential. At first the electrostatic repulsion is low and the Van der Waals forces cause the seeds to aggregate and form bigger particles. During this growth process more and more citrate ions cover the surface of the particles and eventually establish an sufficiently high electrostatic potential to prevent further growth of the nanoparticles. That way the sodiumcitrate both starts and ends the synthesis of the nanoparticles, controlling the size of the nanoparticles. Adding more citrate to the tetrachlorauric acid solution will stop the growth process sooner creating smaller nanoparticles. For this project and the further medical applications it's interesting to do the experiments (functionalization, characterization and radiosensitization) for particles of different sizes. Following the method described in the appendix particles of size 15nm, 30nm and 45nm are synthesized.

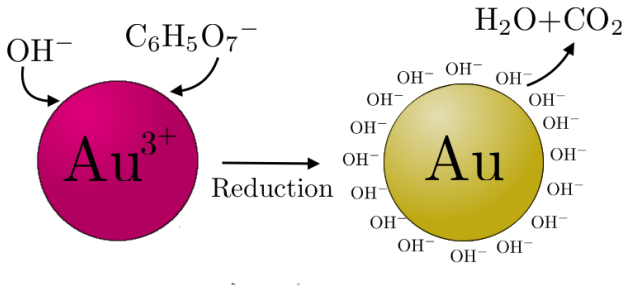


Figure 1: The synthesis of GNP using the sodiumcitrate ($NA_3C_6H_5O_7$) as an reducing agent.

3.1.2 Functionalisation with PEG

In order to have an optimal targeting of the GNP to the tumor the particles are coated with an layer of PEG derivates (Polyethyleen glycol). One of the end hydroxyl groups is substituted by an sulfhydryl group (SH). This substitution ensures an favorable PEG-GNP bound. In an aqueous environment this group deprotonates forming an radical (RS·). The PEG_n thiol is now negatively charged. It will substitute the citrate ions on the surface of the GNP since an thiolategold bound is comparable in strength to that of the gold-gold bound[1]. The second hydroxyl substitution will determine the surface properties of the particles in the colloid.

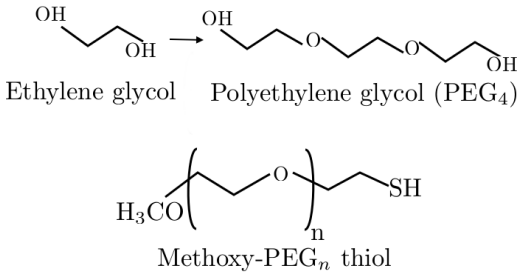


Figure 2: The synthesis of methoxy-PEG_n thiol

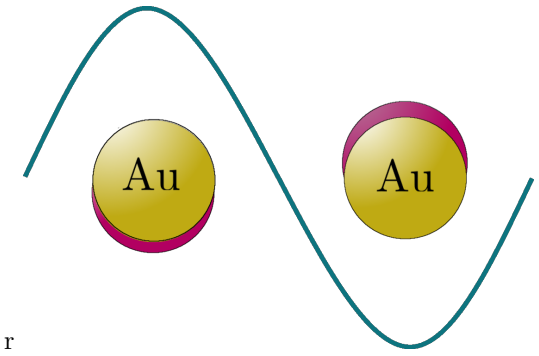


Figure 3: Schematic representation of surface plasmons

In this project we substitute with an methyl-group creating an neutral surface potential. Thus, the GNP are coated with an layer of Methoxy-PEG$_n$ thiol, see figure 2. Furthermore PEG polymers of different sizes 1k, 5k, 10k and 20k[1] are used creating particles of different total radius (GNP+PEG). The total radius is important for the diffusion of the particles through biological membranes.
Besides the targeting to the tumor the PEG-coating also provides steric repulsion to prevent aggregation of the nanoparticles. To find out how much PEG is needed to form a stable colloid an saturated NaCl solution is added to the functionalised GNP. The NaCl causes aggregation of the nanoparticles which can be prevented if there's enough PEG around the particle. Measurements of the size before and after NaCl addition for different proportions of PEG vs. GNP are compared. If (almost) no difference is observed between the two there's enough PEG to provide the necessary steric repulsion forming a stable colloid.

3.2 Characterization

3.2.1 Core Diameter

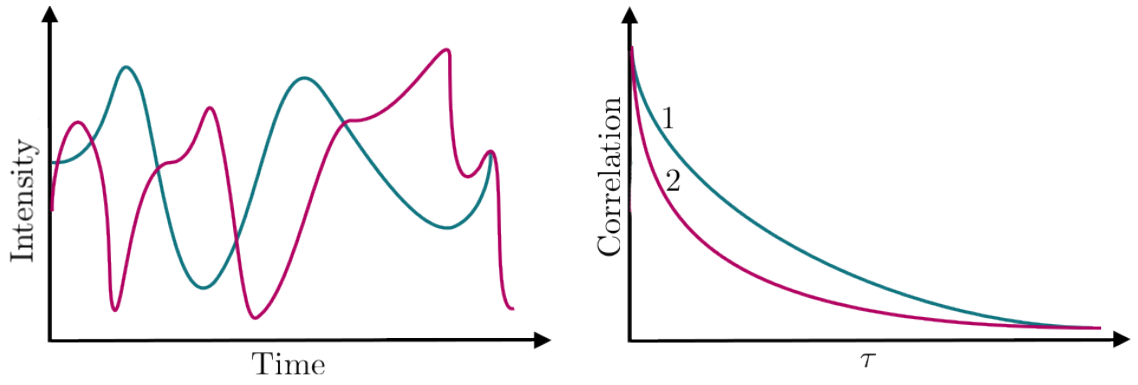
To measure the radius of the gold nanoparticle, images of the colloid are created with an transmission electron microscope (TEM). These images are then analysed to obtain a statistical estimate of the particle radius.. TO BE COMPLETED

3.2.2 Relative size

A typical characterization technique of GNP is a measurement of the abosorbance spectrum in the ultra-violet and visible spectrum; UV-VIS spectroscopy. The peak in the spectrum provides information about the core diameter of the particles.

When electromagnetic radiation falls in on the surface of a particle the oscillating electric field causes the surface electron cloud to oscillate in the opposite direction (see figure 3). One side of the particle becomes negatively charged whereupon the other side becomes positive since the particles are netto neutral. This distribution of charge establishes a restoring force which causes the electron cloud to oscillate back, this time with a frequency depending on the geometrical properties of the particle; the natural surface plasmon frequency. If this frequency is equal to the frequency of the incident radiation the resonance condition is satisfied creating a peak in the absorbance spectrum.
From the quantitative result of the absorbance peak approximate results for the diameter of the particles can be obtained. These are in relatively good agreement with results from TEM images [4]. In this project the absorbance spectra of different particles will be compared to analyze the size of the samples relative to each other. A smaller particle will create a higher restoring force which results in a higher surface plasmon frequency (see figure)
This technique will also be used to determine the necessary proportion PEG vs. GNP to create a stable colloid, see section 3.1.2. If after the addition of NaCl the absorbance peak has shifted to

[1] where k stands for kDa, the molecular weight of the polymer



(a) **(b)**

Figure 5: The total scattered intensity (a) and autocorrelation function (b) for two different particles. Due to faster brownian motion of the smaller particle it's intensity variation in time is higher and consequently have a steeper autocorrelation function.

higher wavelengths (lower frequencies) or a secondary peak arises, aggregation of the nanoparticles has taken place and more PEG is needed.

3.2.3 Hydrodynamic Radius

For the practical applications the GNP will be coated with a PEG layer for targetting to the tumor. It's important to know the total radius of the particle (Gold plus coating) since this will determine the diffusive properties of the particles.
The total radius of the particle plus layer (gold + PEG or gold + H_2O) is called the hydrodynamic radius (R_h), see figure 4. This radius can be measured using the dynamic light scattering (DLS) technique The DLS technique is based on the Rayleigh scattering of incident infrared light by the gold nanoparticles.
The GNP in the colloid perform a brownian motion. Due to this random motion the total intensity of the scattered light will vary over time. If the particles have a smaller R_h they will move faster and the total intensity will vary accordingly. The variation of the total intensity over time is thus a measure of the hydrodynamic radius of the particles.

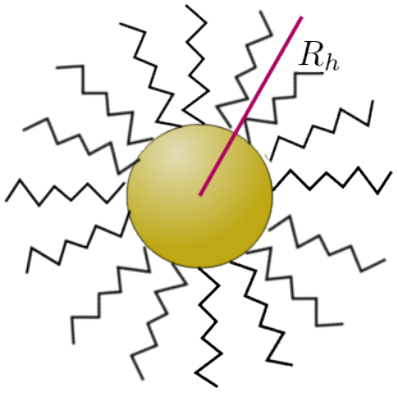


Figure 4: A gold nanoparticle with PEG coating to indicate the hydrodynamic radius R_h

$$\left\langle R^2(t) \right\rangle = 6Dt \tag{2}$$

with D the diffusion constant

$$D = \frac{k_b T}{6\pi\eta R_h} \tag{3}$$

This random motion causes the total scattered intensity $I(t)$ to fluctuate over time and a normalized autocorrelation function is defined which compares the intensity at time t with the intensity a time interval τ later (see figure).

$$g_2(\tau) = \frac{\langle I(t)I(t+\tau)\rangle}{\langle I(t)\rangle^2} \tag{4}$$

For a monodisperse sample this function can be written in function of the diffusion constant

$$g_2(\tau) = 1 + \beta|g_1(q,\tau)|^2 \quad \text{with } g_1(q,\tau) = \exp(-q^2\left\langle R^2(\tau)\right\rangle) = \exp(-q^2 6D\tau) \tag{5}$$

where β is an instrumental factor and q the wavevector of the scattered light [5]

$$q = \frac{4\pi n}{\lambda_0}\sin(\frac{\theta}{2}) \tag{6}$$

with n the refractive index of the medium, λ_0 the wavelength of the incident radiation and θ the scattering angle. Equation 5 clearly shows that if the particles are smaller the autocorrelation function will be steeper (see figure 5b). This is in agreement with the more rapid variation of the scattered intensity for smaller particles. For a polydisperse sample the same equation holds only now $g_1(q, \tau)$ is the sum of all exponential decays measured in the autocorrelation function. The specific analysis is executed by the *Vasoc- particle size analyzer* Nano-Q software using the Pade-Laplace method [3].

3.2.4 Stability of the colloid

There are several ways to create a stable colloid, to prevent the particles from aggregating. One way is to create steric repulsion by coating the particles with big molecules (mostly polymers). As mentioned in the previous sections the GNP in this project will be coated with a methoxy-PEG$_n$ thiol layer for optimal targeting to the tumor. This PEG layer immediately aids the stability of the colloid. Another way to stabilize the colloid is by using electrostatic repulsion. If all particles have an equal and high surface potential the coulomb repulsion will stop them from aggregating. Measurements of the surface potential is a typical characterization of gold nanoparticles and will be performed in this project. When no functionalization has happened the citrate ions (see section 3.1.2) surround the particles and the surface potential should be negative. If the particles are coated with an methoxy-PEG$_n$ thiol layer no surface potential should be present.

As figure 6 shows the naked gold particles in solution have one tightly bound layer of negative ions (citrate ions) and a second less tightly bound layer of positive and negative ions. The surface potential of this second layer will determine the electrostatic properties of the particles, it is called the ζ-potential. In general it is said that if the ζ-potential is bigger than $30mV$ the colloid is stable. That is, there's enough electrostatic repulsion to prevent the particles from aggregating.
To measure the ζ-potential the Laser-Dopler electrophoresis technique is used. When an electric field is applied over a sample the charged particles start to accelerate. Since the particles are in solution they undergo a drag force and will eventually move at a constant velocity v.

$$E \cdot q = \alpha \cdot v \Rightarrow v = \mu_e \cdot E \quad \text{with } \mu_e = \frac{q}{\alpha} \tag{7}$$

Here μ_e is called the electrophoretic mobility and it's link with the ζ-potential in the Smoluchowki approximation is given by the following equation.

$$\zeta = \frac{2\eta\mu_e}{3\epsilon} = \frac{2\eta v}{3\epsilon E} \tag{8}$$

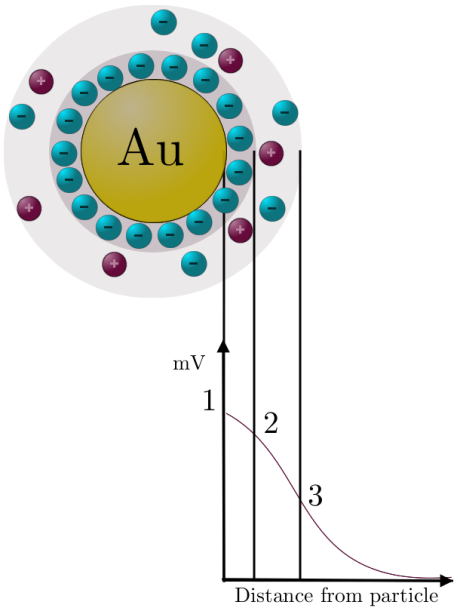


Figure 6: Schematic representation of the double layer configuration of particles in a colloid.

with η the viscosity and ϵ the dielectric constant of the medium. Measuring the constant velocity of the particles in electric field E is done by irradiating the moving particles with a laser of known wavelength and registrate the doppler shift in the scattered radiation.

4 Results and Discussion

4.1 Hydrodynamic Radius (DLS)

Table 1: DLS measurement results for three different particles, of expected size 15nm, 30nm and 45nm, with and without functionalisation using PEG. The length of the PEG chain varies from 20k to 1k .

Size (nm)	No PEG	1k PEG	5k PEG	10k PEG	20k PEG
15	28.37 ± 1.53	135.72 ± 38.01	40.26 ± 1.25	54.03 ± 0.56	66.00 ± 4.71
	9.70 ± 1.44			14.05 ± 4.13	
30	30.03 ± 1.25	99.61 ± 17.33	45.41 ± 1.26	53.28 ± 1.66	61.29 ± 1.50
	4.99 ± 1.55	25.37 ± 1.84	7.85 ± 1.05	5.90 ± 0.97	7.36 ± 1.73
45	50.75 ± 2.05	109.54 ± 0.69	70.04 ± 3.06	71.56 ± 1.87	76.14 ± 0.66
	3.28 ± 0.79		10.67 ± 1.44	10.74 ± 1.13	5.15 ± 0.74

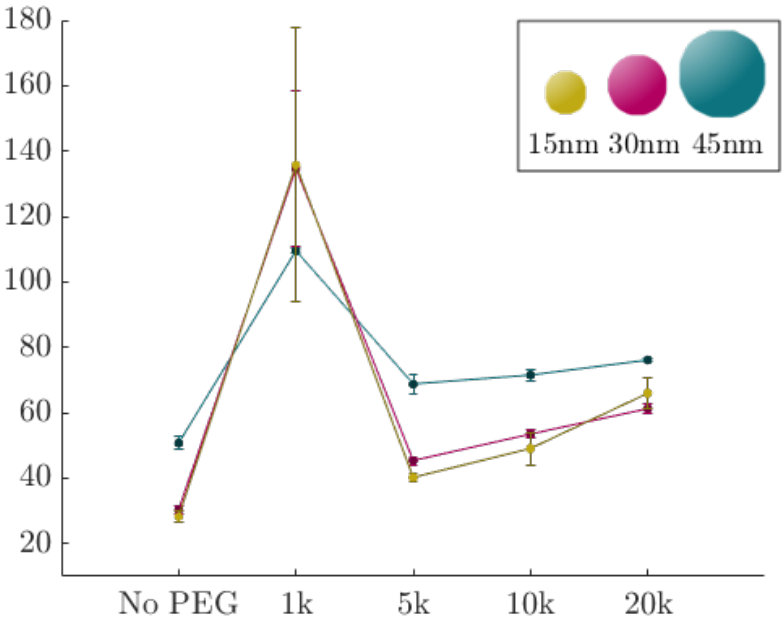


Figure 7: DLS measurement results for three different particles, of expected size 15nm, 30nm and 45nm, before (transparent) and after PEG functionalisation.

4.2 UV-Vis

4.3 Zeta potential

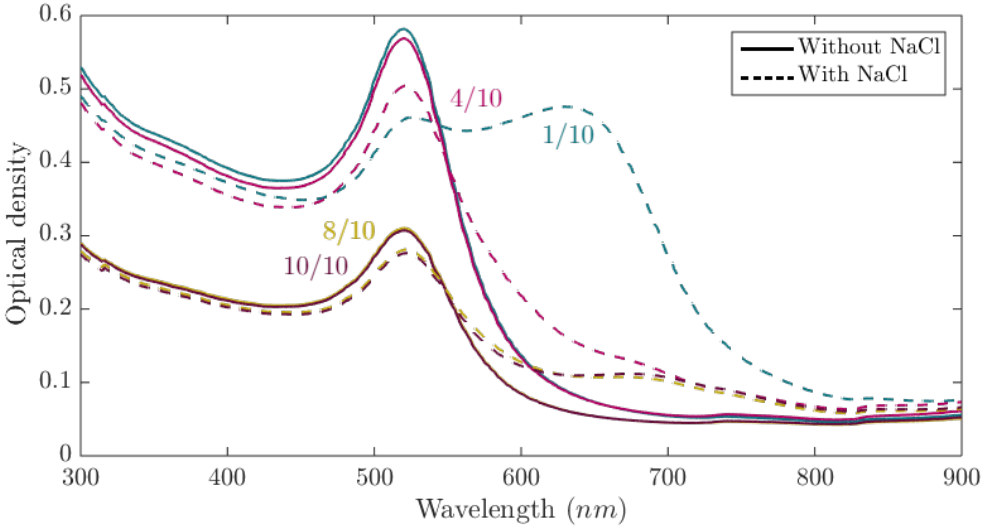


Figure 8: Optical density in function of wavelength for 15nm GNP with 20k PEG for different PEG/GNP proportions, with and without NaCl.

Table 2: Results of the mobility (μ) and zeta potential (ζ) for three different particles, with and without functionalisation using PEG.

Size (nm)	Data	No PEG	1k PEG	5k PEG	10k PEG	20k PEG
15	$\mu(\mu$m·cm/V·s)	-2.62 ± 0.15	-1.02 ± 0.13	-0.40 ± 0.11	-0.46 ± 0.13	-0.35 ± 0.10
	ζ(mV)	-33.73 ± 1.85	-13.08 ± 1.67	-5.20 ± 1.46	-5.87 ± 1.65	-4.45 ± 1.28
30	$\mu(\mu$m·cm/V·s)	-1.61 ± 0.15	0.35 ± 0.11	0.06 ± 0.10	0.08 ± 0.09	$\pm$
	ζ(mV)	-20.65 ± 1.89	4.48 ± 1.46	0.76 ± 1.20	1.06 ± 1.10	$\pm$
45	$\mu(\mu$m·cm/V·s)	-1.92 ± 0.28	-0.70 ± 0.10	0.14 ± 0.12	-0.27 ± 0.17	-0.61 ± 0.13
	ζ(mV)	-24.70 ± 3.57	-9.05 ± 1.32	1.77 ± 1.60	-3.51 ± 2.14	-7.90 ± 1.69

References

[1] Hannu Hakkinen. The gold-sulfur interface at the nanoscale. *nature chemistry*, 4:443–455, 2012.

[2] Peter Copper Stevenson John Turkevich and James Hillier. A study of the nucleation and growth processes in the synthesis of colloidal gold. *Discussion of the Faraday Society*, 11:55–75, 1951.

[3] Vasco Particle size analyser. Nanoq and vasco, instruction guide, 2013.

[4] Jenny Aveyard Wolfgang Haiss, Nguyen T. K. Thanh and David G. Fernig. Determination of size and concentration of gold nanoparticles form uv-vis spectra. *Analytical Chemistry*, 79 N. 11:4215–4221, 2007.

[5] Jenny Aveyard Wolfgang Haiss, Nguyen T. K. Thanh and David G. Fernig. Determination of size and concentration of gold nanoparticles form uv-vis spectra. *Analytical Chemistry*, 79 N. 11:4215–4221, 2007.

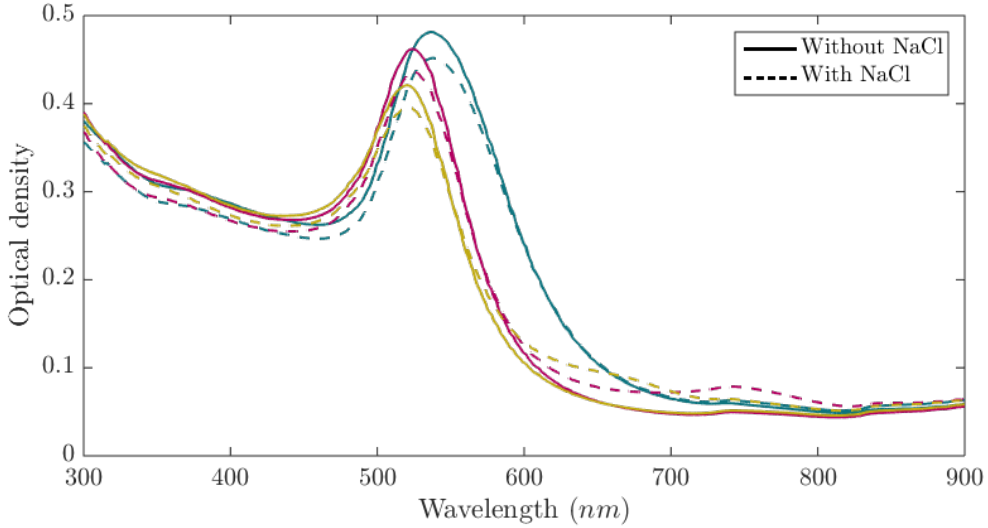


Figure 9: Optical density in function of wavelength for 15, 30 and 45nm GNP, with 20k PEG with PEG/GNP=8/10.

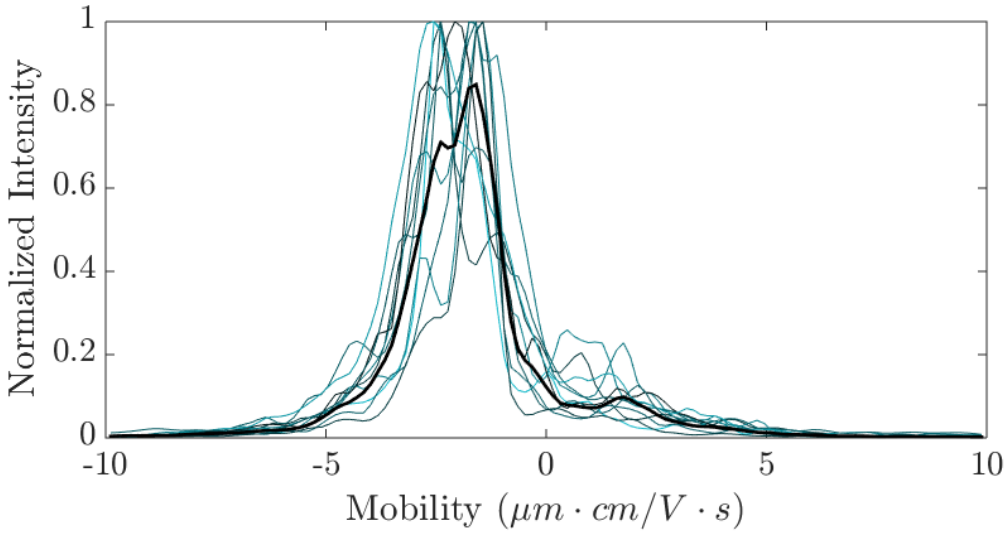


Figure 10: Mobility measurement results for particles of 45nm without functionalisation. Ten measurements were done and the average was calculated (black curve).

Appendix

Synthesis gold nanoparticles

To synthesis the gold nanoparticles following protocol is followed.

a. Perpare a solution of 100ml 0.01% $HAuCl_4$

b. Heat the solution till boiling temperature while stirred

c. Add 2.5ml, 1.24ml, 0.8ml of a 1% $Na_3C_6H_5O_7$ solution for particles of size respectively 15nm, 30nm and 45nm

d. Let the solution boil for 20 minutes while stirred

e. Let the solution cool down for at least 60min while protected from light

f. Store the solutoin at $4°C$ and protected from light

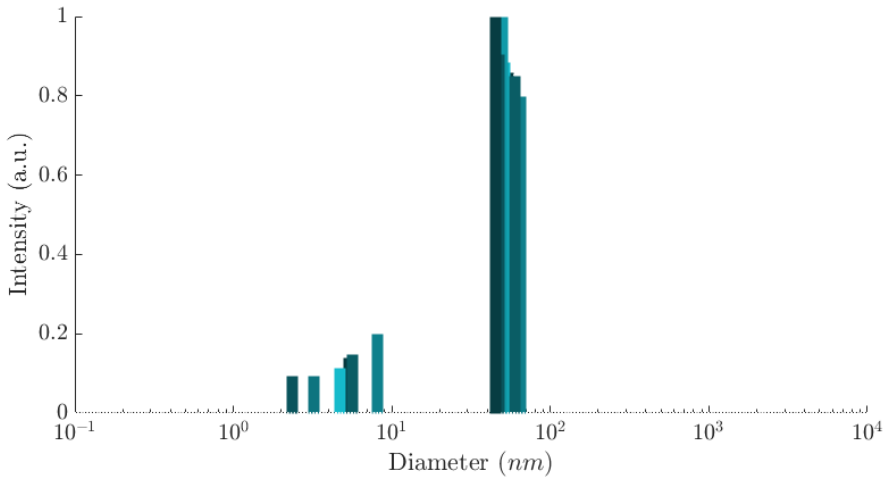


Figure 11: DLS measurement results for GNP with expected size 45nm, no PEG added

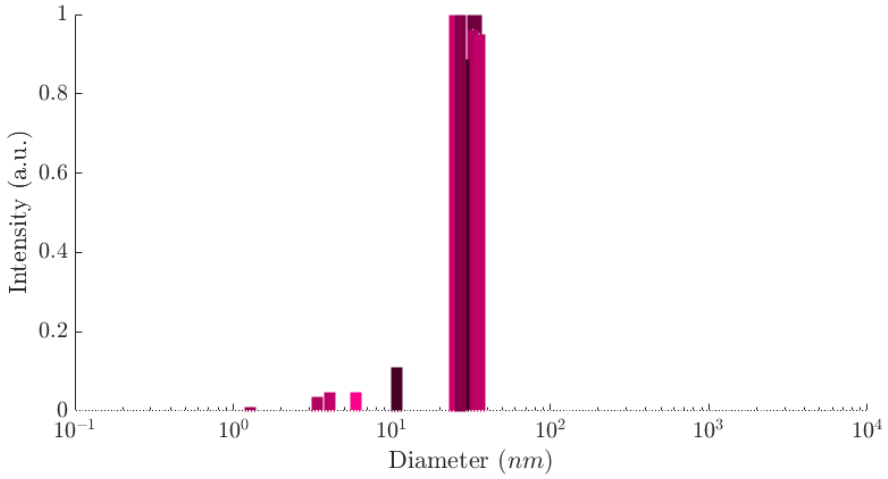


Figure 12: DLS measurement results for GNP with expected size 30nm, no PEG added

4.4 DLS measurements

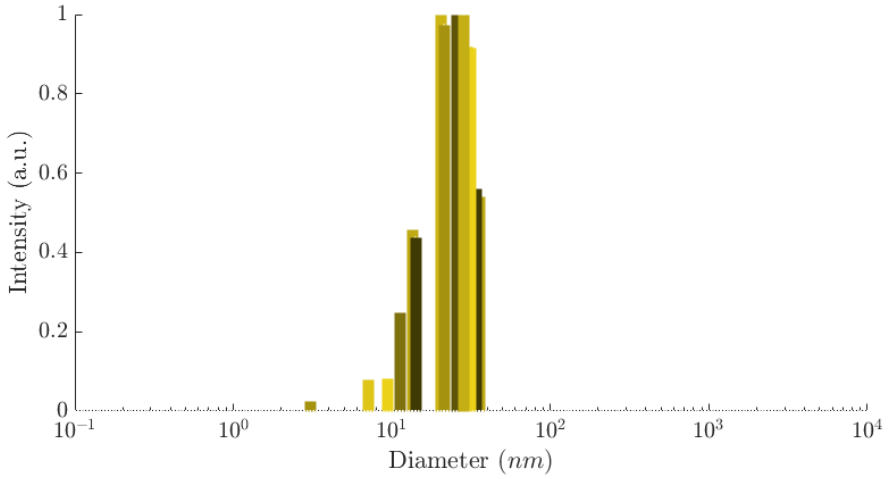


Figure 13: DLS measurement results for GNP with expected size 15nm, no PEG added