KU LEUVEN

Project work: Engineered Nanoparticles for Biomedical Applications

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1 Introduction

Cancer is one of the most occurring diseases in the world. It includes the group of diseases that are characterised by the uncontrolled growth and spread of cells. Each year millions of people are diagnosed with a certain type of cancer [1]. The development of treatments, which kill the malignant cancer cells, has thus become one of the main concerns of the medical world. Radiotherapy, which sends in a beam containing ionising particles, offers a possible cure for cancer. However, the main issue of this treatment is the nonspecific delivery of the irradiation energy. This will cause damage to the targeted cancer cells, but side effects will also be induced in the healthy cells in the vicinity of the beam track. Thus in order to improve this treatment, a focused radiation beam is required, which is capable of targeting only the malignant cancer cells. The use of gold nanoparticles (GNPs) can aid in localizing the effects of radiotherapy by acting as a radiosensitizer due to the emission of Auger electrons when they are targeted by an irradiation energy [2]. By this approach the beam energy can be significantly reduced.

The main goal of this project was to synthesize these GNPs with different sizes and characterise them via different analytical methods. Afterwards the naked gold nanoparticles were functionalised with polyethylene glycol (PEG) of various molecular weights in order to increase the in vitro stability of the GNPs. Consequently, the targeting control of the nanoparticle at the biomolecule interface is improved. Finally, particular sizes of the GNPs were chosen to perform irradiation experiments to illustrate the effect of GNPs on the denaturization of DNA.

2 Materials and methods

2.1 Synthesis of gold nanoparticles

Gold nanoparticles (GNPs) are produced by a reduction of a tetrachloroauric acid (HAuCl4) precursor solution. The formation of these GNPs is based on a thermodynamically driven precipitation process consisting of a nucleation step followed by particle growth. The nucleation phase is induced when the gold solution becomes supersaturated. This is induced by eventually increasing the temperature up to boiling temperature and adding a reducing agent. The decoupled gold ions Au(III+) will form the nanoparticles by ionic bonding. The growth phase, and thus the final particle size is controlled by the amount of reducing agent present. The effect of this last varies as illustrated in figure 1.

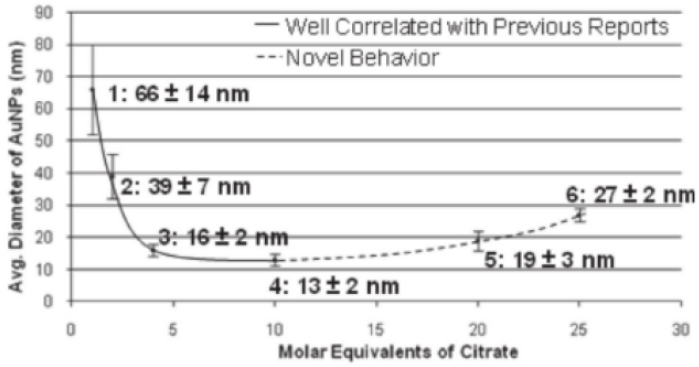

Figure 1: The diameter of the gold nanoparticle with respect to the amount of citrate [3].

The GNPs can be further be controlled in their synthesis by optimising the temperature, pH and used reagent. However, these nanoparticles will have a low thermodynamic stability due their small sizes. Thus, it is possible that these nanoparticles agglomerate due the attracting van der Waals interactions. In order to prevent this ag-

glomeration certain surface protecting reagents, such as polyethyleneglycol (PEG), can be added to the solution. These organic ligands provide a barrier, due to a steric hindrance effect, that is able to counteract the van der Waals interactions between nanoparticles [4].

Figure 2: Experimental set-up used for the synthesis of GNPs that required reflux.

There are many ways to make GNPs. In this project work GNPs ranging from 5 to 45 nm in diameter were synthesised with the procedure based on the reduction of tetrachloroauric acid ($HAuCl_4$) with trisodium citrate ($Na_3C_6H_5O_7$) or sodium borohydride ($NaBH_4$). To achieve GNPs with an even larger diameter a protocol based on citric acid ($C_6H_8O_7$) or ascorbic acid ($C_6H_8O_6$) were used. For the protocol based on ascorbic acid smaller GNPs, based on the reduction with trisodium citrate ($Na_3C_6H_5O_7$), are used as seeds in order to obtain GNPs with diameters up to 100 nm. Below, the protocols that were used during this project work are discribed. Note that all glassware were thorougly cleaned with aqua regia, which is composed of a 3:1 ratio of nitric acid (HNO_3) and hydrochloric acid (HCl). Next the glassware is rinsed with water and oven-dried in order to avoid possible contaminations of the samples by previously used chemicals. After the synthesis of the GNPs was completed, all the samples were stored at 4°C and covered with an aluminum foil in order to avoid any possible interactions with light that might lead to the aggregation of the GNPs.

Procedure of Patra et al. Sodium borohydride was used to make naked GNPs with a diameter of approximately 5 nm based on the synthesis procedure of Patra et al. [5]. This protocol states that the GNPs were synthesized from tetrachloroauric acid ($HAuCl_4$) by wet chemical methods using sodium borohydride ($NaBH_4$) as a reducing agent. First a stock solution of $HAuCl_4$ with a concentration of 5 mg/mL is prepared. Then 4 mg or 0.8 mL of this $HAuCl_4$ stock solution was taken and put into 99.2 ml deionized water (H_2O). Next 4.3 mg of $NaBH_4$ is dissolved in 50 ml deionized distilled water. By adding the last two solutions together, while being mixed under vigorous stirring, the desired GNPs are obtained after 12 hours of stirring at room temperature. Note that during the synthesis the solution is hidden from excess light.

Method of Turkevich For the synthesis of 15, 30 and 45 nm diameter particles, trisodium citrate ($Na_3C_6H_5O_7$) was used as the reducing agent. This synthesis is based on the classical method introduced by Turkevich [6]. 100

mL 0.01% tetrachloroauric acid ($HAuCl_4.4H_2O$) solution was refluxed and 2.5, 1.5, and 0.8 mL, respectively, of 1% sodium citrate solution was added to the boiling solution in order to obtain GNPs of different sizes. The reduction of gold ions by the citrate ions was completed after 5 min, but the solution was further boiled for another 30 min such that the nucleation process can occur. After this time period the sample was cooled down at room temperature, hidden from light. Later these naked GNPs will be functionalised with PEG as described in section 2.2 to stabilise the particles.

Protocol of Oh et al - citric acid For the synthesis of GNPs with larger diameter from 15 nm up to 130 nm the protocol of Oh et al. is used [7]. Starting with 100 ml of deionized water at 100°C, 100 µl of a 0.19 g/mL citric acid solution ($C_6H_8O_7$) is dissolved and the solution is agitated with a magnetic stirrer. Subsequently 150 µl of a 0.013126 g/mL tetrachloroauric acid stock solution is added together with 120 µl of a 0.2216 mg/mL PEG-SH (5000 g/mol) stock solution. After several minutes the colour of the solution colour from violet to pink.
A second injection follows after 2 minutes. The more injections made, the larger the nanoparticles will be. Next the solution is cooled, hidden from light, and after 2 hours additional PEG is added as explained in section 2.2. In figure 3 an overview is given of the steps of this protocol.

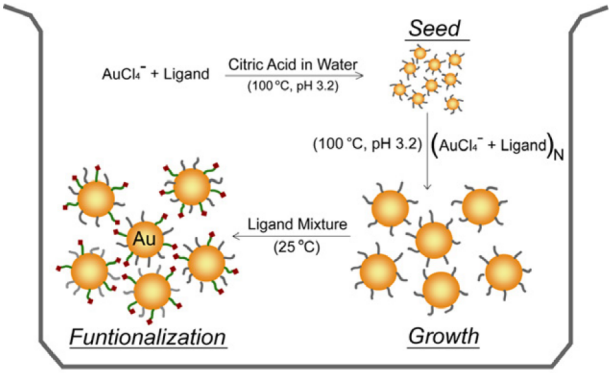

Figure 3: Overview of protocol to synthesise nanoparticles of diameter 15 nm upto 130 nm by Oh et al. [7].

Protocol based on ascorbic acid With this protocol, non-functionalised gold nanoparticle seeds of a varying diameter are used which were made with the previous Turkeyvich protocol based on reduction with trisodium citrate ($Na_3C_6H_5O_7$) or sodium borohydride ($NaBH_4$). By adding ascorbic acid ($C_6H_8O_6$) as the reducing agent, it is possible to further enlarge the seeds to even larger GNPs.. This is based on the following formula: $D_f = D_{seed}$ $\{([Au(0) + [Au(III)])/[Au(0)]\}^{1/3}$. The ascorbic acid also makes sure no new nucleation sites occur and that all the newly added Au(III) ions will be used to enlarge the existing particles. In this case ascorbic acid (10^{-2}mol/L) was added to a mixture of Au(III) (10^{-3}mol/L) and Au(0) (ranging from 1.5×10^{-4} mol/L to 7×10^{-5} mol/L) while stirring the solution at room temperature. This addition will then induce an immediate colour change indicating an increase in size of the nanoparticles [8].

2.2 Functionalisation of the gold nanoparticles

Due to the fact that small size GNPs are not really thermodynamically stable, their aggregation has to be stopped by adding certain surface coating agents after the synthesis reaction. The organic ligand that is used in this study is polyethylene glycol (PEG). These thiolated compounds provide favorable organic-inorganic interactions with the gold nanoparticles via thiol chemistry and are able to counteract the van der Waals attractions between nanoparticles due to an increased energetic barrier caused by sterical hindrance [4]. This prevents the nanoparticles to form clusters and will also contribute to an increase in hydrodynamic diameter of the particles, as will be explained in subsection 2.3.2.

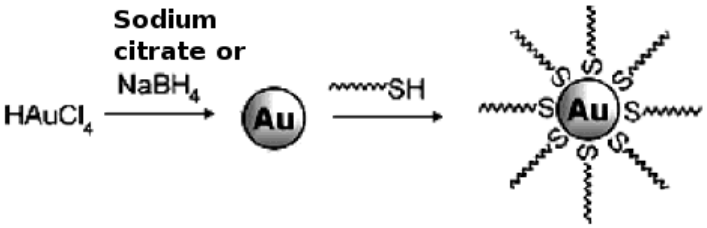

Figure 4: Functionalizing gold nanoparticles with organic compounds via thiol groups [9].

2.2.1 PEG coating

PEG is popular in biomedical applications due to their high in vivo tolerance and solubility features. The PEG functionalisation of the naked GNPs made with the protocol based on the reduction of tetrachloroauric acid ($HAuCl_4$) with trisodium citrate ($Na_3C_6H_5O_7$) or sodium borohydride ($NaBH_4$), was achieved by mixing 0.5 mg of PEG-SH of 1000 g/mol, 5000 g/mol, 10 000 g/mol or 20 000 g/mol respectively. Afterwards the particles were stirred for 1 hour to allow the modification of the surface of the GNPs with PEG by the formation of gold-sulfide bridges. For the particles made with ascorbic acid non functionalised seeds were used to be enlarged. After this enlargement PEG was also added in order to have a solution containing 0.5 mg PEG per 100 mL.

For the GNPs created by adding citric acid as the reducing agent, based on the protocol of Oh et al. [7], PEG was already added in the injection steps. However to make sure that in the end every gold nanoparticle is stabilised, extra PEG (amount depends on how much already was added) was added after two hours. Here also a overall concentration of 0.5 mg PEG per 100 mL solution is obtained.

2.3 Characterisation of the gold nanoparticles

Fabricating gold nanoparticles of different sizes theoretically is one thing, but in order to see what is actually formed is another. That's why the characterisation to determine the exact shape and size- of the synthesized GNPs is important. Several measurements like UV-Visible light spectroscopy (UV-Vis), dynamic light scattering (DLS) and transmission electron spectroscopy (TEM) are performed in order to do this. Also gel electrophoresis experiments have been used in order to understand the effect on the DNA stability. In order to avoid possible thermal influences the samples were stored in a fridge at 4°C and all experiments were performed under room temperature conditions.

2.3.1 UV-Vis

UV-Vis is a technique that analyses the absorption by a sample of incident photons in the UV (10-400 nm) and visible (400-760 nm) region. As illustrated in figure 5, the UV-Vis light will be radiated from the bottom of a 96-welled plastic microplate enabling an efficient way of analysing multiple samples sequentially in a short time, while keeping the reflection and scatter losses at a minimum level [10]. In this case collective oscillations will be induced by incident photons against the restoring force generated by the positively charged nucleus of the GNPs. The condition for surface plasmon resonance (SPR) will be fulfilled when the frequency of the incident light particles and oscillating surface electrons match. Metallic nanoparticles have unique optical properties allowing them to lie at the basis of the field of plasmonics for absorbance measurements. The nature of this surface plasmon band (SPB) can be described quantitatively by solving the Maxwell's equations for spherical particles by setting the correct boundary conditions [4, 9].

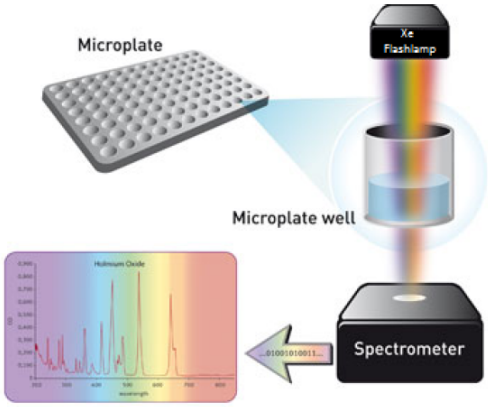

Figure 5: Different light intensities created due to the integration of an optical grating in the spectrometer will be measured by a linear array photomultiplier tube detector at the desired wavelength [4].

The optical properties of nanoparticles can differ based on their size, agglomeration state, shape, refractive index and concentration. Some of these phenomena will therefore influence the SPB (e.g. shape of the nanoparticles and refractive index of the medium). Next to that, UV-Vis spectrometry is also sensitive towards surface modifications (e.g. coatings), thus it is possible to examine the effect of adding functionalizing ligands in the aqueous medium or possible aggregation phenomena [9, 11]. The uniformity of size distributions of the GNPs are characterized by the steepness of the side slope. As illustrated in figure 6, when functionalizing ligands, like polyethylene glycol (PEG), are added on the surface of gold nanoparticles a redshift is observed. This shift is induced by the change of the dipolar oscillation of the free conduction electrons with respect to the heavier ion core, which is induced by the electric field an incoming light wave, when additional components are added to the nanoparticle [9].

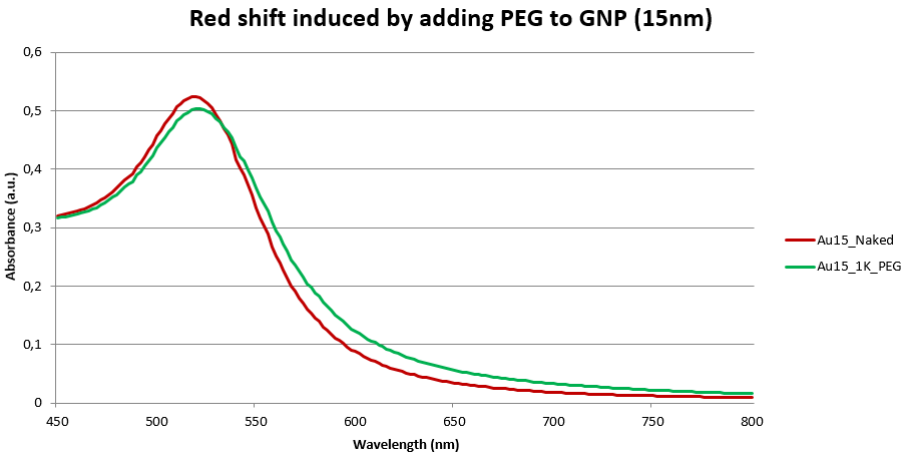

Figure 6: Red shift induced by adding a coating of PEG.

When nanoparticles aggregate scattering effects will dominate in solution. And due to the coupling of surface plasmons in aggregated colloids a red shift in the UV-vis spectrum can be observed[4, 11]. The intensity of the peaks of the SPB is a function of the amount of individual nanoparticles present in the solution. Also, when the functionalization density of the nanoparticle is higher an increase of the peak intensity can be observed [12].
This study will focus mostly on GNP diameters in the 5 to 45 nm range. Therefore the resulting peak in the absorbance measurements will be observed near 520-545 nm [4]. For these measurements the Tecan Infinite 200 Pro multimode spectrometer of the Solid State Physics and Magnetism department of the KU Leuven is used. However, in order to get a correct view of the spectrum of the sample, a correction is needed by subtracting the values of the blank, given by the wells that were filled with the dispersing medium (e.g. water). It was already possible to differentiate, with the naked eye, between different samples with nanoparticles of different size and those prepared via different preparation methods due to their colour. The prepared samples had an orange/red/purple colour dependent on the size of the GNPs that were produced.

2.3.2 Dynamic light scattering (DLS)

Another characterisation experiment for the synthesized GNPs is dynamic light scattering (DLS) or a photon correlation spectroscopy measurement. DLS measures the speed at which the particles are diffusing due to brownian motion by detecting fluctuations in the intensity of scattered light. The velocity of this brownian motion then can be related to the hydrodynamic diameter of the particles.

The brownian movement of particles originates from the fact that solvent molecules hit the particles of which you want to determine the size. This evokes the particles to perform a random movement in the solvent which is called brownian movement. The smaller the particles are, the more rapidly they will move. Hence the larger the particles, the slower this brownian movement will be.
The velocity of this Brownian motion typically is defined by a property known as the translational diffusion coefficient (usually given the symbol, D). This translational diffusion coefficient can be related to the size of the particle through the Stokes-Einstein relationship, where d(H) is the hydrodynamic diameter (size), η is the viscosity, k is Boltzmann's constant and T is the absolute temperature. So the size that is measured via DLS refers to the hydroynamic diameter, given by the following formula.

Algorithm 1 Hydrodynamic diameter

$$d(H) = \frac{k.T}{3\pi\eta D}$$

Furthermore anything that has an influence on the translational diffusion coefficient will hence influence the hydrodynamic diameter. That's why having a surface structure like for example the functionalised nanoparticles with PEG will lead to a larger hydrodynamic diameter than the uncoated particle. Also, note that this hydrodynamic diameter is always larger than the actual diameter of the nanoparticle itself due to the ion layer that surrounds the GNP. Also having a medium with for example a low conductivity layer can extend the thickness of the electric double layer of the particle (called the Debye length κ^{-1}) which will also lead to a larger hydrodynamic diameters of the particles. In order to measure the fluctuations scattered light, a laser beam is send trough the sample as depicted in figure 7. A speckle pattern is obtained by scattering. If you have small particles these fluctuations over time will be more rapidly than when having large particles.

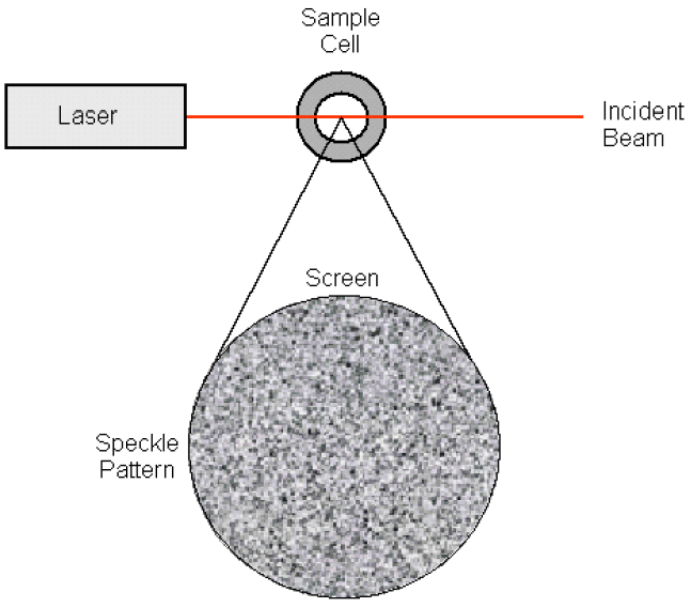

Figure 7: Emitting a laser beam through the sample results in a certain pattern caused by scattering effects.

A correlator is then used to see wether the intensities of two signals are well correlated or not. For large particles, this signal will change more slowly and a good correlation will maintain for a longer time. Whereas for smaller particles, which are moving much faster, this correlation will reduce more rapidly. The decay in correlation is an indication for the mean size of the sample. The steeper the line, the more monodisperse the sample is. Conversely, the more extended the decay becomes, the greater the sample polydispersity will be.
The mathematics behind this is that correlator will construct the correlation function of the scattered intensity. Via a cumulants analysis, a single exponential fit is applied to the correlation function to obtain the mean size of the particle (Z-average diameter) and the polydispersity index (PdI). If this PdI index is smaller than 0.1, the cumulants mean or intensity weighted mean hydrodynamic size of the particle collection can be used as Z-average. The value of this last will be referred to as the hydrodynamic radius of the GNPs. In case the PdI index is larger than 0.1, the Pade-Laplace inversion algorithm is used to determine the hydrodynamic radius of the GNPs. The Pade-Laplace inversion alogorithm is able to filter out peaks that correspond to aggregate peaks. For this work a VASCO particle size analyzer DL135 from Cordouan Technologies of the Solid State Physics and Magnetism department of the KU Leuven is used.

2.3.3 Transmission electron microscopy (TEM)

In order to get a clear image of the size, distribution and morphology of the synthesized GNPs a Philips CM200 FEG transmission electron microsope (TEM) is used. Instead of focusing a beam of light a TEM shoots out electrons, because they have a smaller wavelenght and thus they are able to signicifantly increase the resolution of the image. Variations in the amplitude and phase of the beam provide a contrast effect of the image. This is dependent on the thickness of the sample and the material which constitutes the sample, because the amount of electrons that are able to pass through a sample decays exponentially with its thickness and also heavier atoms decrease the electron mean free path due to an increasing occurence of scattering [13]. The sample is prepared by drying the GNP solutions on a copper grid, which is covered by a thin layer of carbon. Since the electron density of gold is higher than the amorphous carbon GNPs can be succesfully imaged [13]. When analysing a TEM image, the state of the nanoparticles, either individual or agglomerated particles, and their shape can be observed.

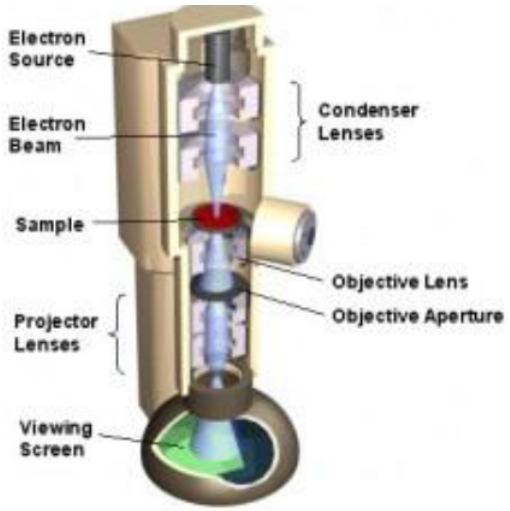

Figure 8: Schematic representation of a TEM [13].

2.4 Irradiation experiments

The most commonly used treatment for cancer is radiation therapy. The goal of this treatment is to induce the annihilation of the cancer cells by the delivery of a sequence of precise irradiation doses of 2 Gy with an energy up to 6 MeV to the tumor. In this work a Baltograph X-ray generator was used at a distance of 74.5 cm with an energy of 199 keV to irradiate samples of DNA with extra added GNPs. When these parameters are set, the radiation dosage can still be varied by adjusting the exposure time of the sample to the beam. The dosage of the irradiation was set at 20 Gy (4-5 min). For reference samples the irradiation dosage was set at 0 Gy.

2.5 Gel electrophoresis

In order to use GNPs for an irradiation sensitized cancer treatment, the damage that occurs to DNA needs to be qualified. For this purpose the gel electrophoresis experiment is performed after an irradiation experiment. In this case a 1% agarose gel plate was formed with the addition of a tris-acetate-ethylenediaminetetraacetic acid (TAE) buffer, which is required to maintain the stability and the charge of the negatively charged DNA [14]. These negatively charged DNA molecules can then move throughout the gel due to attraction by positive pole (anode) of the electrical cell. In this experiment supercoiled ΦX174 RF1 DNA was used, to which GNPs are added. After the irradiation experiments of the samples, these were loaded on the gel.

When supercoiled DNA (scDNA) is damaged, either by single-strand or double-strand breaks that are caused by the emitted Auger electrons, the scDNA will turn into circular or linear stranded DNA respectively [15]. These different types of DNA strands can be recognised with this technique due to their difference in mobility, which is dependent on their size. scDNA has a higher mobility than the relaxed (circular or linear) DNA, so this scDNA will be able to move faster through the gel towards the positive electrode [14].

In order to view this movement of the different DNA strands ethidium bromide was added. This addition of ethidium bromide, which is an intercalating agent, acts as a fluorescent tag that is visible under UV light. Also, in order to visualize the progress of the electrophoresis experiment blue juice is added to the GNP-DNA solution. This blue juice will run faster through the grid than the DNA and when it reaches the end of the grid, the electrophoresis experiment is terminated. When taking a UV picture the relative intensity of these lines, which are an indication of the amount of scDNA and circular DNA, is used as a measure for the damage done to the DNA molecules after irradiation. The size of the GNPs that were used for this gel electrophoresis experiment were chosen to be 15 nm and 30 nm respectively, because smaller nanoparticles interact less effectively with the DNA molecules. However using even larger nanoparticles for this experiment is unnecessary since they would have no potential use in further trials involving cell lines because they would never be taken up by the nucleus in a cell. Thus larger nanoparticles are unable to interact with the DNA in a cell. In addition, the damage of DNA after irradiation will be deteremined for different concentrations of GNPs that were added to the DNA before irradiation.

3 Results and discussion

3.1 Synthesis of GNPs

GNPs of different sizes were synthesized based on the different protocols as described in section 2.1. A UV-vis measurement of the GNPs which were fabricated based on the reduction of $HAuCl_4$ with either sodium borohydride ($NaBH_4$) or sodium citrate ($Na_3C_6H_5O_7$) is shown in figure 9 and table 1. This figure demonstrates that the red shift in the UV-vis spectrum is larger for larger GNPs. Note that larger GNPs are formed when less sodium citrate ($Na_3C_6H_5O_{7)}$ is added as reducing agent. In addition it is shown that via the method of Patra et al. it is possible to achieve GNPs with a diameter of approximatly 5 nm.
The redshift is due to the fact that when particles grow, conduction electrons near each particle surface become delocalized. This causes the surface plasmon resonance to shift to lower energies. The obtained results are in agreement with the particle sizes that have been given by previously performed experimental studies 2.1.

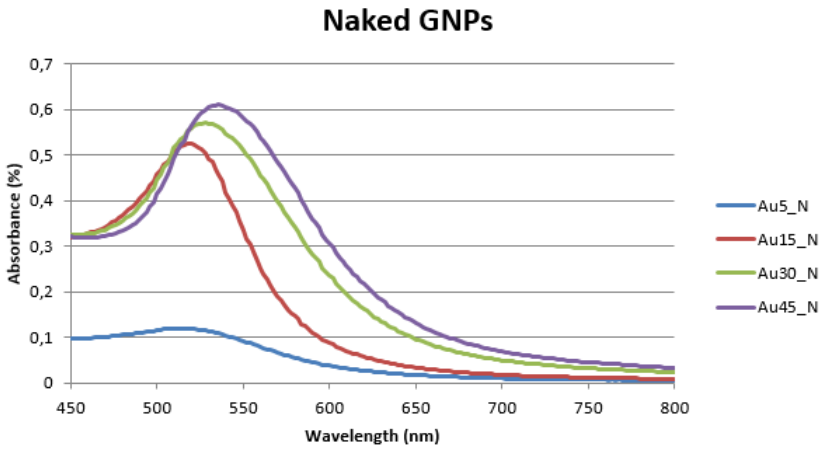

Figure 9: Different sizes of naked GNPs.

Table 1: Different sizes of naked GNPs

Sample	SPR (nm)
Au5_N	512
Au20_N	518
Au30_N	528
Au45_N	536

These naked particles were then further used as seeds to obtain even larger particles, as described in the protocol using ascorbic acid. When an additional 7×10^{-5} mol/L of Au(0) was added to a solution containing previously synthesized GNPs as seeds, it was possible to significantly increase their size. As expected these larger particles show even larger redshifts in the UV-vis spectra. As shown in figure 10 and table 2, when seeds of 45 nm GNPs were used to make 90nm sized GNPs, we see a redshift for the enlarged GNPs.

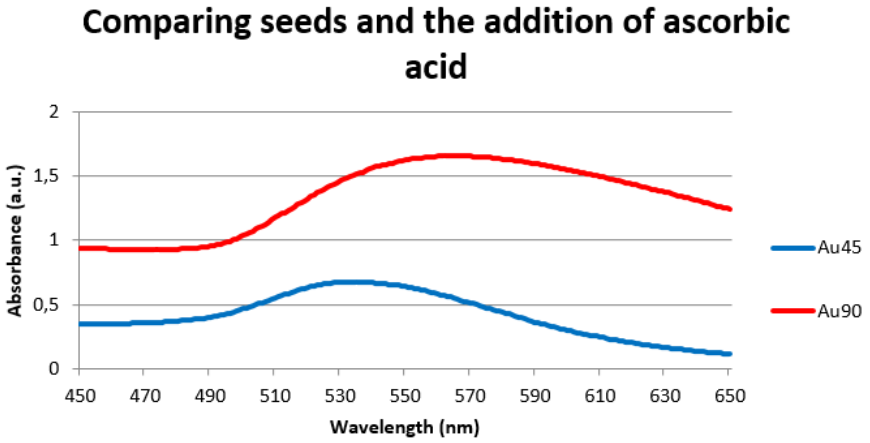

Figure 10: Red shift caused by increasing the GNPs size of particles via the ascorbic acid method. Seeds prepared with trisodium citrate were enlarged by adding ascorbic acid.

Table 2: Red shift caused by increasing the GNPs size via the ascorbic acid method.

Sample	SPR (nm)
Au45	535
Au90	548

When looking to the DLS measurements of these type of enlarged particles from figure 11 and table 3, it is shown that also an increase in hydrodynamic diameter occured, proving the fact that larger particles were formed. For this measurement, using the 15 nm GNPs as seed particles, the polydispersity index (PDI) is respectively 0.0175 and 0.2374. For PDI values smaller then 0.1 the cumulants curve is used to determine the hydrodynamic radius. Otherwise the Pade-Laplace inversion algorithm is used. Nevertheless one size for each measurement is clearly dominant.

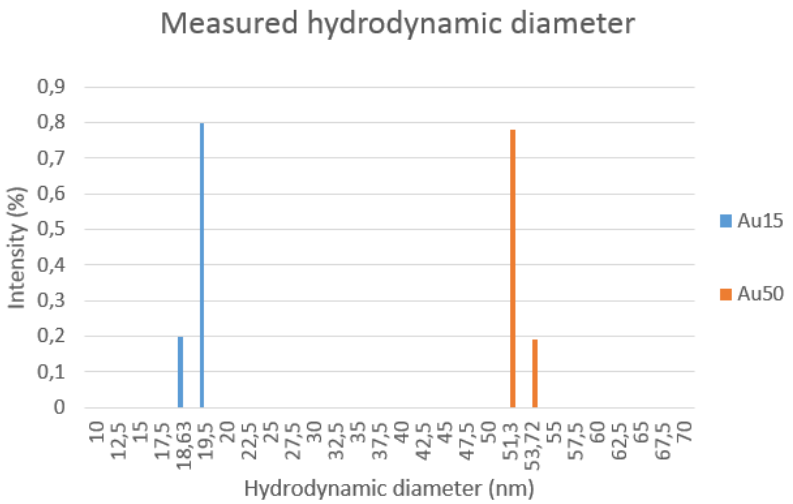

Figure 11: DLS measurements of 15 nm GNPs and after the addition of ascorbic acid which increased its size.

Table 3: DLS measurements of 15 nm GNPs and after the addition of ascorbic acid which increased its size

Sample	Hydrodynamic diameter (nm)	Intensity signal (%)
Au15	18.63	0.2
	19.5	0.8
Au50	51.3	0.78
	53.72	0.19

Hence all these results indicate that by using previously formed GNPs, via the method described in subsection 2.1, as seed particles for an additional reaction with ascorbic acid as reducing agent, even larger particle sizes can be achieved.

3.2 Functionalisation of GNPs

In order to see if the functionalisation of the GNPs with PEG results in a stable nanoparticle solution, stability measurements are performed. In this study, these are made by the addition of a certain salt (NaCl) concentration to the solutions that differ in amount of added PEG. The addition of this salt solution enforces the aggregation of uncovered or partially covered GNPs. For this stability measurement 140 mmol NaCl was added to the solutions. From figure 12, it is seen that fluctuations in the SPB peaks are noticeable. When these fluctuations are stabilised at the same level as the functionalised GNPs, without the addition of salt, it is possible to asses the amount of PEG that should be added in order to fully stabilise the nanoparticles. The results suggested that adding at least 2.8 µg/mL suffices. However, as a safety margin, a concentration of 5 µg/mL PEG was used to coat the GNPs. Extrapolating these results to batch volumes used in the protocols tells that 0.5 mg of a PEG solution should be added to the whole solution of 100 mL.

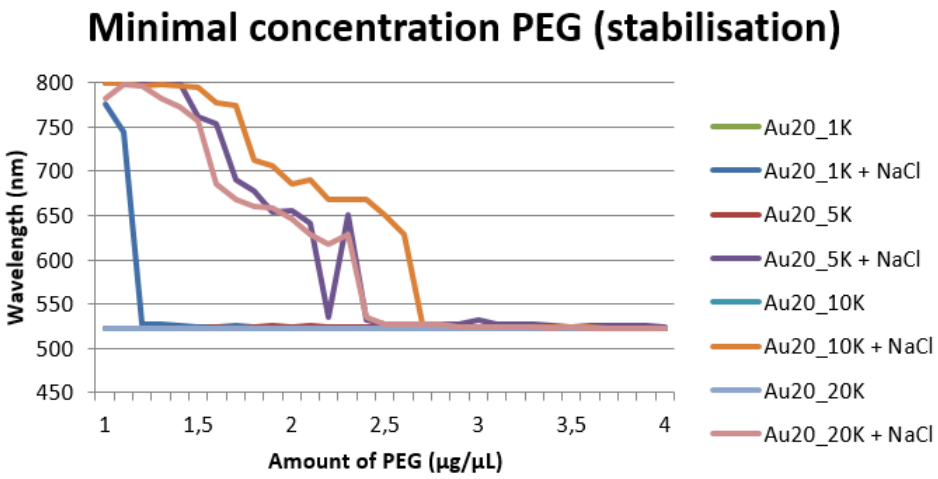

Figure 12: Stabilisation experiment with NaCl..

3.3 Characterisation of functionalised GNPs

As explained in the previous subsection, 500 µg of PEG must be added to a solution of 100 mL containing the GNPs in order to have the surface of the GNPs fully coated with PEG. This pegylation ensures that the GNPs are stable and prevents them to aggregate. Next several measurements like UV-vis, DLS, TEM are performed to qualify this PEG layer on the fabricated GNPs.

Next the effect of adding PEG to the naked particles is visualised. From the UV-vis measurements depicted in figure 13 and table 4, it is shown that by adding PEG to naked particles a redshift of 2 nm is visible. The same holds for the other GNP sizes.

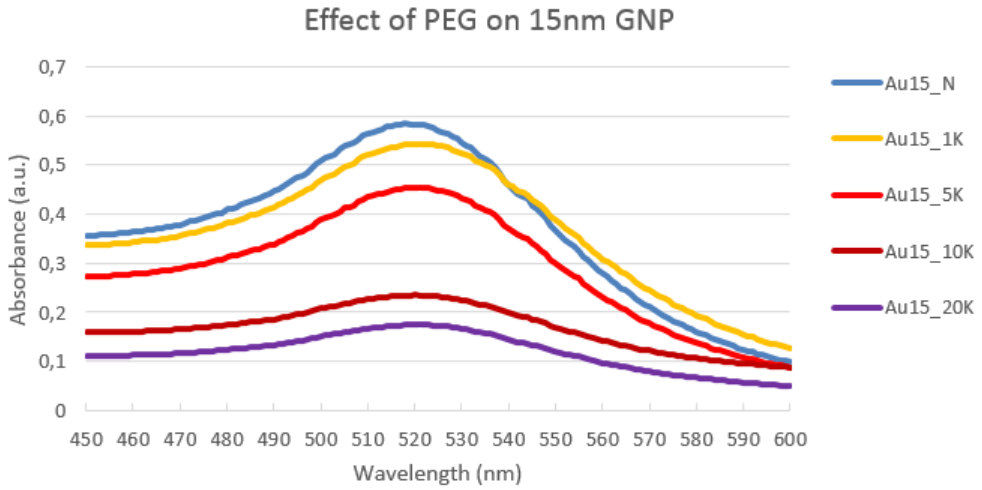

Figure 13: Effect of adding PEG with respect to shift of the SPR band.

Table 4: Effect of adding PEG with respect to shift of the SPR band

Sample	SPR (nm)
Au15_N	519
Au15_1K	521
Au15_5K	521
Au15_10K	521
Au15_20K	521

DLS can be used to verify the results from the UV-Vis. DLS measures the hydrodynamic diameter of a particle and thus more exact measurements of the coating and its thickness can be obtained. Thus, by comparing a sample with uncoated GNPs and coated GNPs it is possible to view an enlargement of the GNP when PEG is added as a coating agent. The GNPs that were made based on the citric acid protocol show a wide distribution in DLS, which indicate a non-uniform distribution of sizes. This is also confirmed by the PDI which is 0.2210 and 0.2230 respectively. However it is still possible to show the effect in DLS of adding PEG to these particles. Figure 14 shows that the hydrodynamic diameter of the particle increases when 1K PEG was added to the GNPs. Nevertheless these particles weren't used for further experiments because of the broad distribution of sizes.

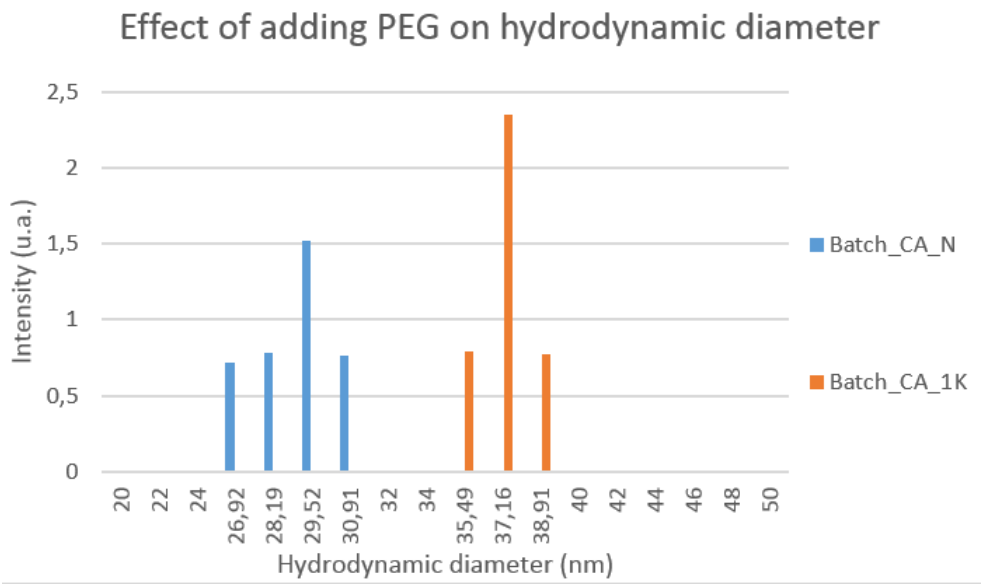

Figure 14: Comparing batches of GNPs with citric acid (CA) of naked particles and functionalised particles with 1K PEG.

3.4 TEM images

To investigate whether no clusters of GNPs were formed and the GNPs had the right size and shape, a TEM measurement was also performed. From these figures it is seen that particles of different sizes indeed can be fabricated, which are also not clustered. From figure 15 it is shown that GNPs are nicely distributed over the plate and no clusters were formed.

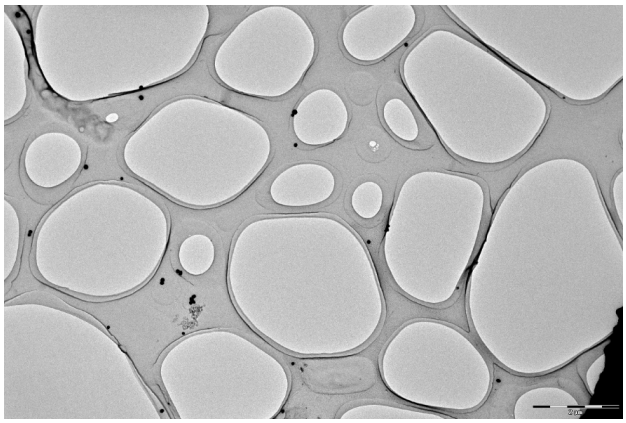

Figure 15: TEM image showing that GNPs are evenly distributed on the plate.

In figures 16, 17 and 18 the TEM images of particles generated via the method of Turkevich and from the protocol with ascorbic acid, respectively, are shown. By analysing these images with a special imaging programme (imageJ) the size of these nanoparticles can be accuratly measured. The particles generated with the Turkeyvich method were 15 nm and 34 nm respectively and the GNPs generated by using the 34 nm nanoparticles as seeds were 70 nm. Thus these results also prove the growth of the nanoparticles by using the method based on the addition of ascorbic acid as reducing agent.

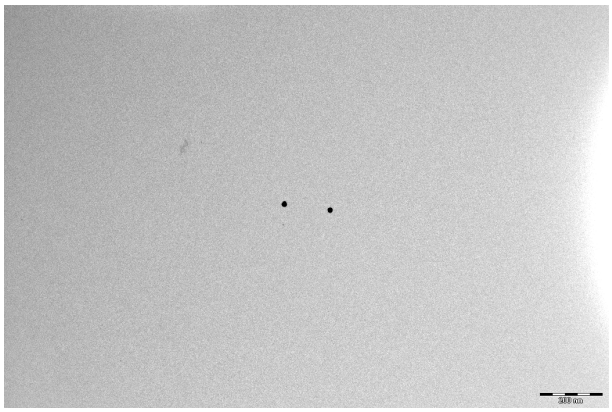

Figure 16: TEM image giving the exact size of the 15 nm GNP.

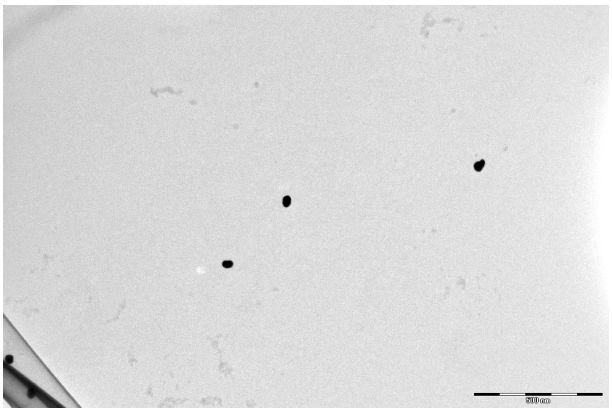

Figure 17: TEM image giving the exact size of the 30 nm GNP.

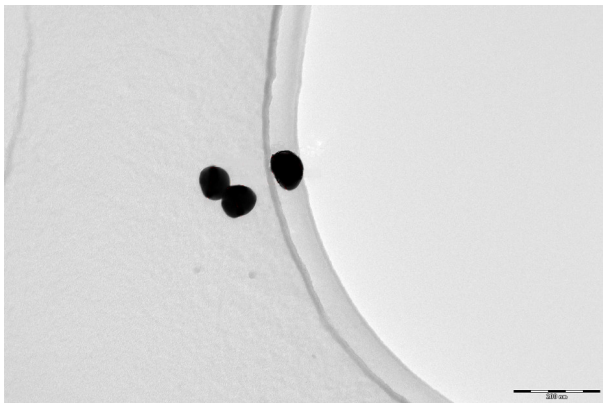

Figure 18: TEM image giving the exact size of the larger GNPs (70 nm) generated with ascorbic acid protocol using seeds of 30 nm.

3.5 Gel electrophoresis - DNA experiments

Finally, in order to determine the size of particles that induce the most beneficial effects when DNA is damaged by irradiation a gel electrophoresis experiment is performed after this irradiation. In order to achieve particles viable for in vivo experiments it is required that these solutions have been set at the physiological pH (7.365), because extreme pH values are capable of denaturing the DNA completely. This denaturation of DNA will provoke scDNA to be turned into relaxed circular DNA and hence the mobility of the DNA trough the gel will be slowed down, providing the possibility to qualify the damage done to scDNA. In this case the amounts of circular and supercoiled DNA resulting from the experiment are not relevant anymore.
Important to note is the fact that the prepared GNP solutions made by the protocol of Turkevich all showed a very acidic pH (3.64). Because this can cause denaturation of DNA, the physiological pH must be obtained by adding a phosphate buffered saline (PBS) to the acidic GNP samples. Also, as mentioned before, for this case only the particles that were 15 nm and 30 nm were chosen to perform the gel electrophoresis experiments since these sizes have a significant effect on the DNA in the nucleus. Larger particles are unable to reach the DNA in the nucleus and smaller particles target the DNA less effectively.

A smartladder is added as a molecular weight marker. Looking at the results shown in figure 19, it is shown that without irradiation (0 Gy lines) no visual damage to the DNA occurs so only the scDNA band is visible. For the lines of 15 nm and 30 nm sized GNPs added to DNA, which were then irradiated with 20 Gy, a second

band occurs proving that a part of the scDNA is converted into cDNA. This means that adding GNPs to DNA, followed by irradiation can induce more DNA damage, which is what we expected according to literature [15]. The intensity ratio of these two bands, corrected with the reference (20 Gy DNA), can give an idea about the damage due to irradiation. Qualitatively we can also conclude that the more GNPs that are added (1:1000), the more damage to the DNA is obtained. This can be visualised by comparing the relative intensities of the scDNA and cDNA lines for the Au30 (1:100), Au30 (1:500) and Au30 (1:1000).

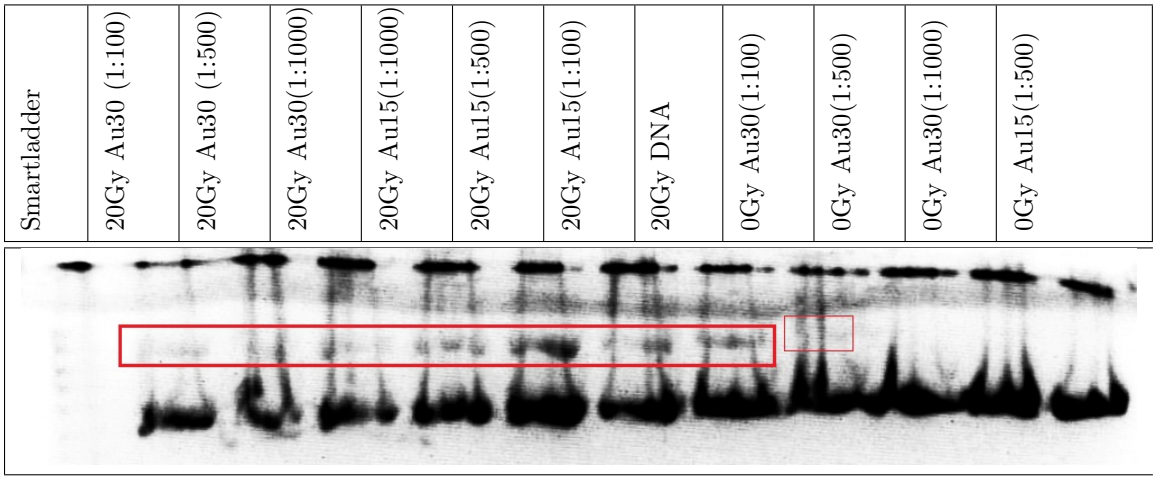

Figure 19: The beneficial effect of 1K pegylated GNPs (20 nm and 30 nm) in different molar ratios with the DNA on the damage of DNA.

It is also possible to see the effect of the different lengths of PEG molecules that were used to functionalise the GNPs. When analysing the gel electrophoresis, it is shown that 20K PEG induces less damage to DNA because the GNPs will be larger spaced from the DNA.

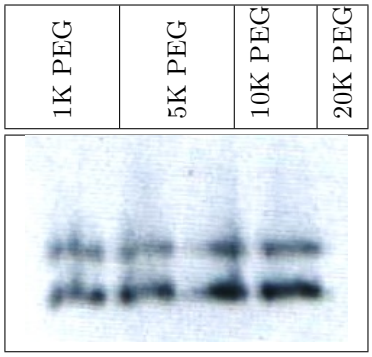

Figure 20: The effect of adding PEG of different molecular weights to GNPs (20 nm) on the damage of DNA when irradiated with 20 Gy.

4 Conclusion and future perspectives

It is proven with this project work that a broad range of GNPs can be fabricated in a controlled way. By using several characterisation methods such as UV-Vis spectrometry, DLS and TEM, the size and distribution of the synthesised particles can be visually determined. Particles in the range of 15 to 45 nm were produced by adding different amounts of trisodium citrate ($Na_3C_6H_5O_7$) as the reducing agent. Smaller GNPs (5nm) were produced using another reducing agent, namely sodium borohydride ($NaBH_4$). On the other hand, even larger nanoparticles (>50 nm) have been created by using previously formed GNPs as seeds and adding an additional reducing agent, ascorbic acid ($C_6H_8O_6$). In addition it is also proven that adding GNPs to DNA will enhance the damage to the DNA when irradiated. Thus incorporation of these GNPs with DNA molecules can be an important step towards a more effective cancer treatment based on radiation therapy.

Further research will also require that the gel electrophorese experiments are performed at least 3 times before any medical relevance can be stated. The next step of this research would be to implement the nanoparticles into cell cultures and investigate their effect.

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