Investigating the effect of gold nanoparticle size, shape and surface corona on cellular uptake and toxicity

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

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Declaration

I certify that except where due acknowledgement has been made, the work is that of the author alone; the work has not been submitted previously, in whole or in part, to qualify for any other academic award; the content of the thesis is the result of work which has been carried out since the official commencement date of the approved research program; any editorial work, paid or unpaid, carried out by a third party is acknowledged; and, ethics procedures and guidelines have been followed.

Catherine Carnovale

15th December 2015
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<th>Description</th>
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<tbody>
<tr>
<td>AAS</td>
<td>Atomic absorption spectroscopy</td>
</tr>
<tr>
<td>Au</td>
<td>Gold</td>
</tr>
<tr>
<td>AuNC</td>
<td>Gold nanocube</td>
</tr>
<tr>
<td>AuNP</td>
<td>Gold nanoparticle</td>
</tr>
<tr>
<td>AuNPr</td>
<td>Gold nanoprism</td>
</tr>
<tr>
<td>AuNR</td>
<td>Gold nanorod</td>
</tr>
<tr>
<td>AuNS</td>
<td>Gold nanosphere</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>CTAB</td>
<td>Cetyl trimethylammonium bromide</td>
</tr>
<tr>
<td>DLS</td>
<td>Dynamic light scattering</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>ENM</td>
<td>Engineered nanomaterial</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>fcc</td>
<td>Face centred cubic</td>
</tr>
<tr>
<td>FT-IR</td>
<td>Fourier transform infrared</td>
</tr>
<tr>
<td>HSA</td>
<td>Human serum albumin</td>
</tr>
<tr>
<td>ICP-MS</td>
<td>Inductively coupled plasma mass spectrometry</td>
</tr>
<tr>
<td>MTT</td>
<td>3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>NP</td>
<td>Nanoparticle</td>
</tr>
<tr>
<td>OD</td>
<td>Optical density</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PC3</td>
<td>Prostate cancer cells</td>
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<tr>
<td>ppb</td>
<td>Parts per billion</td>
</tr>
<tr>
<td>ppm</td>
<td>Parts per million</td>
</tr>
<tr>
<td>RPM</td>
<td>Revolutions per minute</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>SPR</td>
<td>Surface plasmon resonance</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>UV-vis</td>
<td>Ultraviolet visible</td>
</tr>
<tr>
<td>XPS</td>
<td>X-Ray photoelectron spectroscopy</td>
</tr>
<tr>
<td>XRD</td>
<td>X-Ray diffraction</td>
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Abstract

Colloidal gold is undoubtedly one of the most extensively studied nanomaterials, with thousands of different protocols currently available to synthesise gold nanoparticles (AuNPs). While methods for the synthesis of AuNPs have progressed rapidly in recent years, our understanding of their biological impact, and in particular to the effect of shape, size, surface characteristics and aggregation states, has struggled to keep pace. It is generally agreed that when AuNPs are exposed to biological systems, these parameters directly influence their pharmacokinetic and pharmacodynamic properties by influencing AuNP distribution, circulation time, metabolism and excretion in biological systems. However, the rules governing these properties, and the science behind them, are poorly understood. Therefore, a systematic understanding of the implications of these variables at the nano-bio interface has recently become a topic of major interest. This Thesis attempts to ignite a discussion around the influence of different physico-chemical parameters on the biological activity of AuNPs, while focussing on the critical aspects of cellular interaction, protein corona formation, cellular uptake and cytotoxicity. This Thesis also discusses emerging trends in AuNPs uptake and toxicity that may lead to technological advances through AuNPs-based therapy, diagnostics and imaging.

Chapter 1 presents an overview of the scientific literature related to the aims of this Thesis. A thorough discussion of the efforts and advances from various research groups around the world is presented, and as such, the scope for this study defined.

Chapter 2 presents an overview of the instruments employed for AuNP characterisation.

Chapter 3 then examines the synthesis methods in detail, and presents the characterisation outcomes for eight AuNPs which are examined in a biological context in subsequent chapters. The aim of this chapter is to ensure that a thorough understanding of the physico-chemical properties of each nanoparticle was obtained, to allow correlation of biological activity with these properties.

Broadly, two sets of AuNPs were synthesised for this study. The first set of AuNPs, comprises four gold nanospheres (citrate, tryptophan, tyrosine and CTAB/citrate), with the aim to create particles of comparable size and shape, despite having surface properties which differ significantly.
The second set of AuNPs, comprises four particles of differing shape (spherical, rod shaped, prismatic and cubic) which were created using a common chemical surfactant (CTAB) creating particles with analogous surface characteristics, despite different morphology.

Next, **Chapter 4** outlines the materials and methods used to perform cellular viability and uptake studies, the results of which are presented in **Chapter 5**. Both sets of AuNPs were tested on PC3 (prostate cancer) cells, to highlight biological trends related to AuNP surface composition, size, shape and protein corona formation. There currently exists no published study which details the synthesis of spherical, rod, prismatic and cubic AuNPs using a common chemical surfactant to subsequently test and compare their uptake and toxicity to mammalian cells. Further, the behaviour of AuNPs in environments of varied protein levels was probed, elucidating the role of the protein corona in mediating a cellular response.

**Chapter 6** presents a long term stability study of the AuNPs to determine their agglomeration behaviour over a period of six months. In addition to this, a fluorescent study of the interaction of various AuNPs with human serum albumin (HSA) is presented. It is commonly thought that size and shape play an important role in AuNP-protein interactions, however it is not entirely clear how to predict the changes which may arise due to these variables. While the study gives a clear indication that AuNP-HSA interaction is size dependant, the study also highlights the complexity of AuNP-protein interactions, suggesting the need for further work in this area.

Finally, **Chapter 7** summarises the findings presented in this Thesis and subsequently highlights several possibilities for future research in this field.
Chapter 1: Introduction
1.1 A History of Gold as a Nanomaterial

The use of gold to promote good health has been extensively documented in literature dating back to the 1st century [1-5], however it was not until 1856 that Faraday’s pioneering work set the foundation of modern colloidal chemistry, or rather, nanotechnology [6]. It is now well known that as a particle decreases in size towards the nanoscale – as shown in Figure 1.1- its inherent properties do not necessarily reflect the properties of the bulk material it is derived from, nor that of its individual atoms – this is indeed the case with gold nanoparticles (AuNPs) [7-12]. Generally speaking, as particle size decreases, the proportion of atoms which are localised on the particle surface grows, compared to those confined to the inside of the particle [13]. This effect gives rise to thermal, optical, electrical, magnetic, electronic and catalytic properties [13-31].

In the case of metal nanoparticles, particularly gold, silver and copper, when particle size becomes significantly small relative to the wavelength of light, the large number of surface electrons lead to interesting phenomena such as surface plasmon resonance (SPR) [32-35]. In the case of spherical AuNPs with sizes less than 60 nm, the SPR peak absorbance appears at around 520 nm, accounting for the ruby red colour commonly attributed to AuNPs [36]. Modifications to the size, shape and chemical environment of the particles alter the position of the plasmon band, and hence the apparent colour of the particles in solution [37-40]. It is this phenomenon which explains the use of AuNP suspensions throughout history to create the spectrum of colours possible in stained glass [9, 32, 36, 41, 42]. While size and chemical environment influence the position of AuNP plasmon bands, nanoparticle shape offers better opportunities to controllably fine-tune the optical properties of these materials [43, 44]. For instance, spherical AuNPs possess limited potential for SPR tuning as the intensity and position of the absorption bands are relatively fixed with only a small red shift and broadening seen with increasing particle size [37]. Conversely, altering the paths along which the oscillations are permitted to occur [45], which is achieved through altering the shape of AuNPs, gives rise to interesting optical properties that span the broader visible to near-infrared spectrum, making them more suitable for biological sensing, imaging and even therapy [46-54].
Considering the interesting optical properties of AuNPs and their perceived biocompatibility [55, 56], there has been significant interest in elucidating the interactions of AuNPs with biological systems, however much of this work pertains to spherical AuNPs. Further, it is commonly thought that changes in particle shape and size could influence the way that particles are recognised, processed and excreted by the body, however this conjecture remains largely untested [48, 49, 57-62]. Notably, when AuNPs are exposed to biological systems, their surface features, such as the presence of capping agents, unreduced metal ions and surface charge, can directly dictate the pharmacokinetic and pharmacodynamic properties of the nanoparticle [63-65], making it difficult to draw valid conclusions by simply comparing the biological activities of AuNPs originating from different laboratories. Therefore, a systematic understanding of the implications of these variables at the nano-bio interface has recently become a topic of major interest [65]. This review of the literature is an attempt to ignite a critical discussion around the influence of different physico-chemical parameters on the biological activity of AuNPs as depicted in Figure 1.2.

While concerns exist regarding the practicality of utilising AuNPs in vivo due to potential metal accumulation in the body, such concerns have not been thoroughly tested through long-term in vivo studies [66]. It is only recently that the importance of such studies has been recognised, and the research community has more seriously started to investigate the influence of in vivo factors, such as spontaneous protein corona formation on nanoparticle surfaces in response to exposure to biological fluids [67, 68]. New knowledge gained from nanoparticle-protein dynamic interactions will determine the way forward for tailoring AuNP-based systems for in vivo applications, and will also offer equally
valuable opportunities to take *in vitro* applications of AuNPs, such as diagnostics, to a commercialisation stage.

**Figure 1.2:** Different physico-chemical properties of AuNPs that may influence their interaction with biological systems.
1.2 Synthesis of gold nanoparticles

While Faraday first described the reduction of tetrachloroauric(III) acid (HAuCl$_4$) to form colloidal gold suspensions in 1857 [6], the most popular method used today was devised by Turkevich et al., in 1951 [69]. During the reaction, which yields ~ 20 nm gold spheres, HAuCl$_4$ is brought to a boil at which point a solution of trisodium citrate dihydrate is added. During the procedure described by Turkevich, as well as most other in situ methods of spherical AuNP synthesis, the reaction has two main stages, viz. nucleation and growth that seem to occur concurrently [70]. The first step in the Turkevich reaction involves citrate being oxidised to form dicarboxyacetone, while in parallel Au$^{3+}$ ions are reduced to Au$^{1+}$ ions and subsequently to Au$^0$ atoms. Following this step, gold atoms are generated from the disproportionation of aurous chloride molecules which can accommodate the adsorption of gold ions on their surface to form aggregates. During this process dicarboxy acetone acts as an “organiser” molecule to facilitate many of the steps in this reaction [71]. This synthesis has been subsequently adapted by many researchers to allow for size control [72], and varied chemical methods have been developed by later groups [73-76]. While all chemical methods must have a component which reduces gold, as well as a component to provide stabilisation of the newly formed particles, these chemicals can vary greatly leading to the creation of vastly different particles with chemically different surface species [70].

The use of stabilisers is of great importance in shape-controlled synthesis of AuNPs, and a range of stabilisers have been employed to achieve the many shapes researchers seek for various applications. Generally, shape controlled synthesis is performed through a seed-mediated growth process, wherein the nucleation and growth steps do not occur in parallel. Separating the two steps allows for tighter control over morphology and is generally favoured for larger spherical particles and AuNPs of different shapes [70]. The process begins by preparing small (<10 nm) spherical gold seed particles [77-79]. These seed particles are then added to a growth solution, typically containing a weak reducing agent, a stabilising agent and additional gold ions. While the seed particles added to the growth solution act as a catalyst for the reduction of the unreacted gold ions, the weak reducing
agent in the presence of an appropriate stabiliser allows Au\(^0\) nanocrystals to be further grown in specific morphologies [70].

While chemical methods of nanoparticle synthesis are commonly employed due to their relative convenience, non-chemical or pseudo-chemical means of creating nanomaterials also exist. Reducing gold ions without the use of a chemical reductant eliminates the presence of ‘contaminants’ or chemicals precursors which alter the nature of the particle surface. For instance, photochemical synthesis methods harness the energy of ultra-violet (UV) radiation to induce reduction of gold ions producing uniform spherical particles. This is possible as the rate of reduction can be controlled by the level of radiation [80].

Conversely, laser ablation is described as a “top-down” technique as the starting materials for synthesis are bulk materials, typically foils, pellets, or even liquids - as depicted in Figure 1.3. Top-down methods involve the removal of nanoscale material from a bulk surface, in this case, with the use of laser light. As with all synthesis routes, there are inherent disadvantages and limitations associated with laser ablation, including relatively low yield when compared with other methods as well as difficulties associated with shape and size control [81]. This is in part due to the absence of a stabilising agent, thus if the AuNP concentration exceeds a certain limit, the particles tend to agglomerate and collapse in the solution.

Additionally, a number of biological methods have been reported for the synthesis of nanoparticles of gold and other inorganic materials [53, 82-98]. These methodologies rely on the unique ability of certain microorganisms, plant extracts and biomolecules to produce nanoparticles. The biological approach to nanoparticle synthesis takes advantage of certain classes of biochemicals such as enzymes, oxidisable species, amino acids, etc. to allow these organisms to produce nanomaterials upon exposure to appropriate chemical precursors [99-101]. Since these biochemical species involved in nanoparticle synthesis are produced \textit{in situ} by the organisms, the biological synthesis of nanoparticles may be considered as a pseudo-chemical approach. Further understanding
about biological synthesis of nanoparticles can be obtained from some of the reviews recently published in this area [102-107].

Figure 1.3: Schematic depicting the two main approaches to nanoparticle synthesis.

1.2.1 Size controlled synthesis of gold nanoparticles

Many years after Turkevich published his method of AuNP synthesis, Frens identified the ratio of gold salt to trisodium citrate as the defining factor governing AuNP size. The method, which allowed synthesis of particles up to 150 nm in size, was achieved by simply modifying the amount of trisodium citrate in the reaction; reducing levels to create larger particles [72]. This is possible because while there is sufficient sodium citrate to reduce the gold ions slowly, there is less available oxidised sodium citrate to act as a stabiliser to cap the particles and prevent further growth. The disadvantage to this process is that as the particles get bigger, they also become more polydisperse due to the parallel formation of new Au nuclei while pre-existing Au nuclei grow in size. In contrast,
stronger reducing agents such as sodium borohydride provide a seemingly instant reduction of gold ions under ambient conditions, yielding smaller and less polydisperse particles compared to those achieved by sodium citrate [70]. While Frens pioneered the size controlled synthesis of AuNPs, the polydispersity of particles over 30 nm was significant and thus other methods for size control were developed. Another chemical method for increasing the size of spherical AuNPs involves seed mediated growth (originally called “germ-growth”), as described by Schmid et al. [108]. Using this approach, small AuNPs (such as those obtained using the Turkevich method) are used as nucleation sites for the growth of larger particles. The size of the final particles could be tuned by varying three factors; the diameter of the initial seed particles, the amount of seed added to the growth solution, and the amount of ionic gold present in the growth solution. However the size of the seed particles is of great importance, as this factor dictates the attainable size range of the final particles. While it is theoretically possible to employ this method to produce AuNPs ranging in size from 14-900 nm, the difficulty in synthesising seeds of low polydispersity in very small and very large size domains meant that the extreme ends of the size spectrum were still difficult to control.

An advancement to this type of seed mediated approach was developed by Sau et al., who combined the traditional chemical method with a photoirradiation technique first shown by Esumi et al. [109] and Itakura et al. [110] in 1995, and later by Zhou in 1999 [111]. Though the method was utilised by the initial research groups for shape control, it was hitherto not exploited as a means for controlling the size of spherical AuNPs. The new method eliminated the difficulty of synthesising monodisperse seed particles via chemical methods, replacing it with a method of seed production which proceeds via photoirradiation of chloroauric acid and Triton X-100 (poly(oxyethylene)iso-octylphenyl ether)) with UV light [112]. By varying the ratio of gold ions to Triton X-100 (the stabiliser/reductant) in the system the seed particle size could be tuned between 5-20 nm with low polydispersity. While only capable of attaining sizes between 5-110 nm, these seed particles could then be used in a manner similar to Schmid et al., gaining increased control over AuNPs at the smaller end of the size scale.
In 2001, the same year as the previously described Sau et al. method was published; Jana and co-workers published a method for synthesising AuNPs ranging in size from 5-40 nm [113]. In contrast to other methods, one seed particle, i.e., 3.5 nm citrate-capped, NaBH₄-reduced AuNPs were utilised initially, however the reaction was an iterative growth process with the particles obtained from one growth reaction being used to seed another and so on, creating larger particles with each growth step as shown in Figure 1.4. The range of sizes was also affected by varying the ratio of gold seed particles to metal salt in the growth solutions.

![Figure 1.4: TEM images of AuNPs prepared by using an iterative seed mediated approach. Particle size ranges from (a) 5.5 nm, (b) 8.0 nm, and (c) 17 nm to (d) 37 nm. Reprinted with permission from [113].](image)

While seed mediated processes (both iterative and non-iterative) have been used by many groups [114-117], there are additional methods for producing different sized spherical AuNPs which do not involve seed mediated growth.

Biological methods for producing various sized AuNPs have also been exploited, such as the use of plant extracts by Song et al. Using aqueous broths obtained by boiling the leaves of Magnolia (M. kobus) and Persimmon (D. kaki) plants in water as reducing agents, the group experimented with
temperature and leaf broth concentrations to study the effect of these variables on AuNP size. They noted that smaller particles were obtained (as well as a faster reaction time) with increasing temperature. For instance, 40 nm particles formed at 95 °C compared to 110 nm particles at room temperature, while leaf broth concentration had greater effect on the morphology of the particles rather than their size [118].

Organoamine-protected AuNP synthesis methods were introduced by Jana et al. in 2003 [5] and later refined by Hiramatsu et al. in the following year [119]. The simplified reaction involves refluxing a solution of HAuCl₄ and oleylamine (9-octadecenylamine) in toluene for 2 hours. These nanoparticles differ from those produced by chemical methods as they are isolated via precipitation to yield a stable powdered sample which can easily tolerate surface modification, allowing solubility in water or organic solvents. While the size range achievable is admittedly narrow, confined to 6-21 nm, the AuNPs were very stable, and the process by which they were synthesised is simple and can be readily scaled up. In this reaction the particle size is mainly dependant on the concentration of the gold salt with greater gold concentrations producing smaller particles. Conversely, the concentration of amine within the reaction primarily controls the polydispersity of the particles, with polydispersity decreasing with increased amine concentration.

In general, it may be noted that size control of spherical AuNPs is a balancing act, with the amount of available gold ions within the system being a key factor in influencing particle size, however as with all AuNP synthesis methods, factors such as pH, temperature, seed concentration and size, stabiliser strength and concentration, in addition to the choice of the solvent, contributing to the overall size and morphology of the resultant AuNPs [112].

Although size control of spherical AuNPs dominates the literature, size control of shapes other than spheres has been explored, though to a lesser extent. The size modification of rods or more specifically the tuning of their aspect ratio is reasonably well researched. When Yu et al. published the electrochemical method for synthesising nanorods (AuNRs) in 1997 [120], it not only uncovered the link between aspect ratio and the distinct dual band SPR peaks observed for AuNRs (a concept
explored in more detail in Section 1.2.2), but went further to demonstrate that the second peak (the longitudinal band) could be tuned by modifying the aspect ratio of the rods. The exact method by which the group accomplished this was not detailed, except to say that it was achieved by “carefully manipulating the experimental parameters”. However, in 2009, Wang and co-workers utilised the same method and provided details on how rods with differing aspect ratios could be obtained [121]. They noted that rod length was controlled by altering the ratio between the surfactant (cetyltrimethylammonium bromide - CTAB) and co-surfactant (TOAB or tetradecylammonium bromide). While they achieved some degree of size control, and were able to produce short rods with aspect ratios of 3-7, long rods which had aspect ratios of 20-35 occurred less frequently. The original work by Yu was also built upon by Mohamed et al. in 1998, who additionally noted that increasing the temperature of the reaction reduced the aspect ratio of the rods [122].

Much like spherical AuNPs, rod shaped AuNPs can also be produced using a seed mediated approach, with multiple publications on the subject by the Murphy group [123, 124]. The group found that the size of shorter rods with aspect ratios under 7 could be controlled by changing the ratio of gold seeds to gold salt in the growth solution, with increased aspect ratio rods forming with decreasing amount of gold seeds. There was a limit to this effect however, with the aspect ratio failing to increase above ~7-10. They did however note lengthening of the rods with the addition of a small amount of silver nitrate into the solution [124]. They expanded on this work in their next publication; producing higher aspect ratio rods in an alternate fashion, by varying the experimental conditions of the seed mediated process. They noted that altering the timing of the reaction could increase the aspect ratio from ~13 to ~18. The same seed and three identical growth solutions were utilised in both reactions, beginning with the addition of the seed to the first growth solution, from which an aliquot was sequentially added to the next growth solution in turn. For the ~13 aspect ratio rods the reactions were allowed to run to completion in each growth solution, with aliquots taken after 4-5 hours. In contrast, the ~18 aspect ratio rods were formed by transferring the growth solutions while the particles were still growing, after just 15-30 seconds in each solution [125]. Similar to spherical particles, the factors influencing the aspect ratio of AuNRs are varied and include: the length of surfactant chain
the size, surface charge and capping agent of the seed [127, 128]; and the amount of silver nitrate [79].

While there are relatively large bodies of work relating to the size tuning of rod and spherical AuNPs, other shapes such as prisms and cubes are less explored. There exists a small body of work on controlling the edge length of gold nanoprisms, however unlike silver nanoprisms which show a great ability to be tuned [129-131], the dimensions of gold nanoprisms are more difficult to control. Ha and co-workers demonstrated some ability to tune the edge length of gold nanoprisms by adjusting the pH within the narrow range of ~2.2-3.5. They observed increasing edge length with increased pH, however pH levels above 4 failed to induce prism formation entirely [132].

The majority of size control methods for gold nanoprisms use biological agents such as Aloe vera and lemongrass leaf extracts which were utilised by Chandran et al. [133] and Shankar et al. [82, 134], respectively in Sastry’s group. Both these studies observed that increasing the amount of leaf extract decreased the edge lengths in the prisms formed. Sastry group further expanded on this research by demonstrating that increased temperatures also led to the formation of prisms with shorter edge lengths [135].

Millstone and co-workers have conducted a large body of work on the synthesis of both gold and silver nanoprisms [7, 130, 136-138]. The group achieved shape control by using a seed mediated approach, effectively using freshly synthesised nanoprisms as seeds for further growth of larger prisms. By adding the prisms to a growth solution containing additional gold ions in the presence of excess ascorbic acid they produced nanoprisms with edge lengths varying from 100-300 nm [136].

While significant gains have been made in the area of size control of AuNPs, the resounding message in this field is that the process is influenced by many external factors. Due to the effect of size on the location and strength of corresponding SPR bands, the desire for shape control is sought by many researchers for applications of AuNPs which depend on their optical properties. Thus research in this area is expected to expand, with the goals of more precise tunability of common shapes and the possibility of controlling additional shapes.
1.2.2 Shape controlled synthesis of gold nanoparticles

In general terms, being able to control and finely adjust parameters for the synthesis of AuNPs can benefit many potential applications. While there is limited ability to tune the optical properties of AuNPs by altering their size, there is enormous scope for improvement to the range of possible SPR band positions by altering nanoparticle shape. Rod shaped AuNPs are arguably one of the most interesting in terms of their SPR potential as they exhibit two plasmon resonance bands, one of which can be easily tuned depending on the aspect ratio of the rod [120, 139, 140]. Similar to spherical AuNPs, rod shaped AuNPs display an absorption band at around 520 nm due to transverse oscillations or surface plasmons oscillating along the short edge of the rod; however, a strong longitudinal plasmon band, whose position is dependent on the length of the rod, is also present due to surface plasmons oscillating along the long edge of the rod. The position of the longitudinal band is generally seen at wavelengths at and above 750 nm in the near infrared region of the spectrum as shown in Figure 5, which coincides with the optical transparency window of many biological tissues [139, 141]. The optical window describes the wavelength range over which light is minimally scattered and absorbed by biological tissue. This means that light can penetrate deeper into the skin, without disturbing the tissue and hence allows for imaging of live subjects [142].

By including a capping agent in the nanoparticle synthesis process, greater control over size and shape can be attained. While the capping agents used to date include polymers, dendrimers, additive ionic species [143] and even microbial proteins and metabolites [70], the use of a surfactant is the most common way to achieve shape control for metal nanoparticles [144]. This is achieved by preferential binding of shape-directing chemical species to different facets of a growing gold crystal, based on the energy state of the facets, as well as steric interactions which relate to atomic density. [126]
Figure 1.5: UV-visible absorbance spectra (left) and TEM images of spherical (right, inset) and rod shaped AuNPs (right, main panel). Spherical AuNPs show a single absorbance peak at ~520 nm while rod-shaped AuNPs show a small transverse peak in this region and significant longitudinal band at ~900 nm.

While there are many surfactants which share similar traits, the most commonly used in AuNPs shape control is cetyltrimethylammonium bromide (CTAB) [145], which contains both a polar hydrophilic head group, and a non-polar hydrophobic tail as shown in Figure 1.6. This amphiphilic nature predisposes CTAB to form micellar arrangements in solution above a certain concentration (called the critical micelle concentration (CMC)), conferring soft-template like nature. A frequent constituent in topical antiseptics, its cationic head group is an effective antibacterial and antifungal agent which targets the outermost membrane of Gram negative bacteria [146]. This property, in combination with its surfactant nature justifies its inclusion in some cosmetics and personal care products [147, 148].

Figure 1.6: Structure of CTAB.
While Murphy and co-workers [123] are credited with publishing the wet chemical method for gold nanorod synthesis using CTAB, Yu *et al.* had previously suggested that the formation of nanorods via electrochemical synthesis was due to the cylindrical-shaped co-surfactant CTAB [120]. While its mechanism of action is still not definitively known, it was clear to early groups that CTAB acted as a soft template [78, 79, 149], which directed growth of nanorods by binding preferentially to certain facets of the growing gold crystal. CTAB is known to form a bilayer on AuNPs in solution as the surfactant molecules interdigitate, arranging their polar head groups both inward to the nanoparticle surface and outward to the interface with solution, assisting in stabilisation by preventing agglomeration [128, 150, 151]. The exploration of CTAB’s mechanism of action has led to several attempts to explain its behaviour during nanorod synthesis [77-79, 145, 152, 153]. In the years following Murphy’s publication in 2001, many groups stated that the yield of nanorods obtained by following this method was strikingly low (~4%) [154]. This led to several groups adapting the method or formulating new methods for synthesising gold nanorods in high yield [78, 79, 139, 155]. While groups including Murphy and co-workers [154, 155] published an improvised method in which they identified pH as a critical factor for rod formation within their system, methods also emerged using micromolar concentrations of silver nitrate (AgNO₃) to induce gold nanorod formation [78, 156]. Despite the similarity between methods, the addition of AgNO₃ causes the reaction to proceed via a different proposed mechanism, and results in a slightly different shaped rod (shown in Figure 1.7) as compared with a silver free system [78, 149, 157]. The advantage of using silver is a markedly higher yield (~90-100% yield of nanorods is possible in a silver containing system compared with ~20-40% in a silver free system [128]), however the aspect ratio of rods produced with silver is limited to ~6 (compared with up to ~25 for a silver free system) [128, 149].

The initial method for rod synthesis devised by Murphy *et al.*, utilises citrate capped 3.5 nm gold seed particles, which act in dual capacities as both the initial site of growth and as a catalyst to induce reduction [123]. The citrate seed particles develop facets as they grow, resulting in the formation of penta-twinwed AuNPs [149]. The penta-twinned seed particles are structured such that five {111} crystallographic faces are cyclically arranged on each end, separated by five side faces.
which can either be \{100\} or \{110\} [77]. Sterically, CTAB is accommodated with greater ease on the \{100\} or \{110\} faces, when compared with the closely packed \{111\} face, and hence, a bilayer of CTAB forms along these faces, blocking growth, while leaving the end face exposed for further growth [149]. CTAB helps to control the rate of the reaction by limiting the interaction of gold ions, and adding the weak reducing agent ascorbic acid to the rods allows growth to occur more quickly at the tips of the rods, which results in elongation [156].

**Figure 1.7:** Images modelling the crystallographic differences that arise if gold nanorods are synthesised using (a) citrate-capped seeds without silver or (b) CTAB-capped seeds in the presence of silver. Reprinted with permission from [156].

In contrast, CTAB capped seeds were also employed to synthesise gold nanorods in the presence of silver [78]. These 1.5 nm seed particles are single crystalline and appear to have four \{110\} side faces, and one \{100\} face on each end. The exact mechanism of action of silver is not known, however it is thought to significantly slow the reaction process, and also act as a template via silver underpotential deposition (UPD) [78, 152, 158]. Silver UPD occurs when silver becomes reduced at a metal surface which holds a surface potential that is lower than the expected reduction potential of silver. This is energetically favoured on the \{110\} surface [159], which leads to faster reduction of Ag\(^+\) to Ag\(^0\) compared to the \{100\} face. It is thought that the presence of silver allows
CTAB to bind more effectively to the \{110\} surface, and this synergistic effect promotes lengthening of the rods [156].

While CTAB is most commonly associated with gold nanorod formation, it can also be used to encourage cubic AuNP formation [157, 160-162]. Gold seed particles which are intended for cubic shaped particle growth such as those used by Kim et al., are typically stable, with \{111\} faces accessible [163]. When in the presence of CTAB, which preferentially binds to the \{100\} face over the \{111\} face, the \{100\} faces are effectively “blocked”, and growth is encouraged on the \{111\} face, forming cubes [161]. For this to occur, the synthesis requires elevated levels of a weak reducing agent such as ascorbic acid to be included in the system. Although incapable of facilitating a complete reduction of Au\(^{3+}\) ions, it ensures partial reduction to Au\(^{+}\) ions. The final step, a careful addition of sodium hydroxide into the reaction vessel, is done with no mixing, as immediately mixing the solution causes an influx in fully reduced Au\(^0\) atoms, hastening uncontrolled growth in various directions leading to octahedral growth. Delaying mixing for more than 6 hours allows a very slow generation of Au\(^0\) atoms, allowing CTAB to prevent undirected growth, forming cubes [157].

While there are many published methods for producing gold nanorods using CTAB, it has emerged more recently that a number of research groups found this work difficult to reproduce. The most common issue encountered was that despite closely following the published synthesis protocols, the result yielded only spheres, with no or negligible rods formed within the growth solution. The first report of this was in 2007 when Durr and co-workers found that the use of high-purity (>99%) CTAB did not result in rod formation, however a less pure (96%) CTAB produced rods in high yield [47]. Though the group’s focus was to utilise gold nanorods for medical imaging, they pursued this oddity further, testing five CTAB products from different suppliers, with purities ranging from 96-100%. When the products of lower purity returned favourable results, the group concluded that an unknown impurity was most probably responsible for this effect [47]. Members of the same group, Smith and Korgel pursued this further the following year, testing ten CTAB products under identical conditions for rod formation [153]. Of these samples, three failed to yield rods, sustaining their earlier reports that the impurity in the CTAB was critical for nanorod formation [47, 153]. While it was not
definitive whether the impurity induced or prevented nanorod formation, it was hypothesised that the
impurity disrupted nanorod formation during the growth phase. That same year, Millstone and co-
workers detected levels of iodide (I) on the surface of gold nanoprisms via inductively coupled
plasma mass spectroscopy (ICP-MS), which was surprising as it was not intentionally added during
the synthesis and had no obvious origin [7]. By performing ICP-MS on the starting constituents of the
synthesis, it was determined that iodide was present in the CTAB which possibly accounted for the
published inability to synthesis rods. This agreed with later reports by Smith’s group and Rayavarapu
and co-workers, and furthermore most of the researchers active in AuNP synthesis agreed that it was
possible to have batch to batch variation of iodide content despite using products from the same
supplier with a common product number [153, 164]. The mechanism responsible for the iodide
interference was attributed to its strong affinity for the {111} crystal surface [165]. In ideal conditions
for rod formation, the {111} surface must be left unblocked (or at the very least partially unblocked)
to allow for lengthening of the rods to occur. However when a growth system includes sufficient
levels of both iodide and CTAB, binding to the {111} and {100} or {110} surfaces respectively, rods
formation becomes improbable, with spheres being the most likely outcome. Some examples of
different nanoparticle shapes synthesised using CTAB are shown in Figure 1.8.

While this property makes iodide ions highly undesirable for inclusion in gold nanorod
synthesis, it is the key to forming triangular gold nanoprisms [135, 138, 166]. As with all crystals,
each plane possesses a distinct energy level which is dictated by the reactivity of its surface electrons.
For gold, a face centered cubic (fcc) metal, the order of its crystallographic planar energy is \( \sigma(111) < \sigma(110) < \sigma(100) \) [165]. While it is known that iodide preferentially adsorbs to the lowest energy facet
of a growing nanoparticle crystal, if concentrated sufficiently, it binds to all surfaces of the crystal [7].
When this occurs, growth is thought to favour the higher energy facets, (110) and (100), which results
in lengthening of the prism edges, rather than increasing the prism thickness [130]. It is postulated
that this growth effect may also have stoichiometric justification, as it requires fewer gold atoms to
lengthen an edge, as compared to increasing the overall thickness of the particle [130].


**Figure 1.8:** TEM images (clockwise from top left) of rod shaped, cubic, quasi-spherical and prismatic AuNPs synthesised using CTAB as a shape directing surfactant.

### 1.2.3 Effect of reducing agents and stabilisers on the synthesis of gold nanoparticles

With the Turkevich method of spherical AuNP synthesis so widely used, the effect of citrate present on the resulting AuNP surface has been noted by many groups. The negative charge of the citrate coating influences the interactions of the particles with other species and can limit researchers in terms of further surface functionalisation. Notably, the negative charge inherent to DNA hampers its electrostatic binding to citrate stabilised particles, although does not preclude it fully [167]. Similarly, bovine serum albumin (BSA) binding to citrate stabilised AuNPs is approximately two times less than that of bare gold [168], thus emphasising the idea that individual components chosen during nanoparticle synthesis can influence the surface chemistry of the final particles. For this reason surface modifications and the development of new methods of AuNP synthesis became necessary for biological applications, to create particles with controllable surface chemistry, surface charge and stability. There are many comprehensive reviews that cover the synthesis of AuNPs in great detail [70, 103, 105, 128, 169-171]. However, some of the key chemical agents used in nanoparticle synthesis which relate to the uptake and toxicity discussions are covered in Sections 1.3 and 1.4 of this Chapter.
Various chemicals can be used as reducing agents during AuNP synthesis, the most common being sodium borohydride, sodium citrate, ascorbic acid, and amino acids [14, 124, 149, 172]. The strength of the reducing agent (or the ease with which it is oxidised) plays an important role in controlling the rate of the reaction, thereby influencing the morphology of obtained AuNPs [135]. For instance, sodium citrate is a relatively weak reducing agent, and thus the reduction reaction does not occur instantaneously, but over approximately 10 minutes [70]. It is of no surprise that when citrate salts are employed as reducing agents, the AuNP synthesis is performed under boiling conditions to enhance the reaction rate, thus the modified Turkevich method devised by Frens reduces the amount of sodium citrate in order to achieve larger particles [72], a concept discussed in further detail in Section 1.2.1. Further, while reducing agents tend to stabilize AuNPs during synthesis, sometimes stabilisers are also used to achieve additional control over AuNPs, such as, halting further growth and preventing aggregation of the particles over time. As with reducing agents, a number of stabilisers can be used during AuNP synthesis such as sodium citrate, polyethylene glycol (PEG), tetraoctylammonium bromide (TOAB) and cetyltrimethylammonium bromide (CTAB) [70], as well as greener alternatives such as leaf [97, 173], seed [95, 96] and fruit extracts [174]. Typically, strong reducing agents such as TOAB and sodium borohydride only bind weakly to the surface of AuNPs during synthesis, such that over time, stored AuNP solutions stabilised with these agents tend to agglomerate. In such cases, the use of additional stabilisers provide an opportunity to prepare monodisperse yet stable AuNPs [175]. Stabilisers can also play an important role in controlling the morphology of AuNPs (explored in greater detail in Section 1.2.2) through binding preferentially to specific planes of a growing Au nanocrystal, and thereby blocking the further growth of that specific plane [176, 177].

In summary, methods for synthesizing AuNPs have grown exponentially since their discovery by Faraday over 150 years ago. We are now capable of synthesising different shaped AuNPs to suit a plethora of applications which differ from imaging [11, 47, 55, 139-184], to therapeutics [12, 53, 98, 142, 179, 184-193] and sensing [194-197]. These AuNPs can be tuned with varying degrees of size control, despite researchers not necessarily understanding the full mechanisms which underpin
the process. It is only through continued work and documentation in this field that we will understand
the intricacies through which these synthesis reactions proceed.

1.3 Toxicity of nanoparticles

As a global multi-billion dollar industry, the area of engineered nanomaterials (ENMs) is
rapidly growing, with an expanding list of applications [198-205]. While these applications are
diverse in terms of their end use, they form the basis of growing environmental, health and safety
concerns. Despite many applications of nanotechnology not being intended for medical or biological
applications, there remains a necessity to investigate all new nanomaterials in terms of risks related to
the manufacturing and use of the materials as well as the environmental ramifications of their use and
disposal [57, 206]. The innate size of a nanoparticle means that cellular interactions are highly likely.
Nanoparticles comparable to the size of a virion or bacterium are small enough to penetrate the
cellular membrane and accumulate within the cell [207, 208].

In reality, humans have been exposed to nano- and micron-sized materials for thousands of
years. Early exposure did not originate from the laboratory, but from nature, dictated by such events
as dust storms, eruptions of volcanic ash, and the evaporation of oceans which generates
nanoparticulate salt aerosols [207]. Since the nineteenth century, human exposure to nanoparticles has
increased markedly, with the proliferation of combustion engines and an increase in cigarette sales
among other causes [207, 209, 210]. The likelihood of exposure to intentionally designed
nanomaterials is relatively small for most of the population in comparison to exposure from other
anthropogenic and naturally occurring sources; however due to their common size, the investigation
of their health effects unites under the umbrella of nanotoxicology [57, 211].

Although nanoparticles can theoretically be created from any element, the vast majority of
research is performed on nanomaterials derived from carbon, silicon, polymers, metals and metal
oxides [199]. Each of these materials in their bulk form possesses vastly different characteristics
which may or may not be inherited by their nanoparticle counterparts [13]. While we know that size is
an important factor for defining a nanomaterial, when examining the interaction between a biological
interface and an ENM, there are over 30 variable factors which are known to have an impact on the way that nanoparticles are recognised, up-taken and processed intracellularly [211]. These variables include, but are not limited to size, shape, surface charge, surface area, surface functionality, suspending media, tendency for aggregation, chemical nature and the nature of the protein corona in biological fluids [212]. Hence the merits and dangers of individual ENMs must be assessed on a case by case basis, until enough is understood about ENMs to predict the properties and behaviours of new samples [213].

1.3.1 How to assess the toxicity of nanoparticles?

While it may be possible to predict biocompatible or bio-adverse results based on surface chemistry in silico, measuring the toxicity of nanoparticles in cellular systems in vitro is a necessary prerequisite for medical or consumer use of ENMs [57]. There are many established assays for assessing the toxicity of materials in cellular systems which can be grouped into general categories depending on the endpoint measured [214]. The question posed by many research groups [206, 211, 215-218] is whether assays which have been optimised for general chemical compounds can be used to measure the effects of ENMs or whether the assays require modification to assess nanoscale materials.

Permeability assays, which assess viability based on the integrity of the cellular membrane most commonly include dye inclusion/exclusion assays (e.g., trypan blue which selectively stains dead cells but cannot permeate the membrane of living cells) and cytolysis detection assays which measure specific intracellular substances (e.g. lactate dehydrogenase (LDH) which is released from lysed cells when they die) [57]. A general disadvantage to the use of permeability assays is related to the endpoint itself [214]. By measuring toxicity only at the point where the cell membrane ruptures, it would be incorrect to assume that the cells deemed viable are in fact fully functioning, healthy cells. In reality, permeability assays cannot detect early or more subtle forms of cellular stress or damage. While this fact remains true for trypan blue, there are no noted specific disadvantages related to the use of ENMs in this assay [218]. It should be noted however that it can become time consuming and
cumbersome to perform the trypan blue assay on a large number of samples when compared with other techniques. On the other hand, interference was noted by Han and co-workers between LDH assays and copper, silver and titanium dioxide nanoparticles [215]. This interference is due to adsorption of the LDH molecule onto the nanoparticles, which causes a decrease of free LDH within the system, which manifests as falsely reduced toxicity measurements.

Functional assays are often utilised as they relate to a specific metabolic pathway or function performed by the cell to a quantifiable level of cell viability. Common examples of this form of assay include tetrazolium-based assays such as MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] and MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] [219], as well as alternatives such as the Presto Blue metabolic assay [181], and Neutral Red assays [220]. Tetrazolium salts are able to selectively identify metabolically active cells because only these cells possess the mitochondrial activity required to cleave a tetrazolium ring thus creating formazan. Formazan formation results in a visible purple colour, which appears only in viable cells, giving rise to the ability to detect the amount of viable cells colorimetrically [219] – depicted in Figure 1.9. While this type of assay is very commonly used, they may not always be suitable for assessing ENMs. Carbon nanotubes (CNTs) cannot be reliably tested using this assay because they adsorb formazan which falsely appears as decreased colour formation and can be interpreted as decreased cell viability [217, 219]. This may be avoided by the addition of a step to remove the nanomaterials from the cell containing plate before adding the tetrazolium compound; however this is not traditionally commonplace [206]. As a precautionary measure, particles can be tested in a cell free system to check if they have any formazan reduction activity prior to performing the full assay. Such a test was carried out by Sayes et al. on zinc oxide nanoparticles to establish that they had no effect on formazan reduction [221]. Considering that a number of ENMs which utilize AuNPs possess wide ranging catalytic [222] and nanzyme properties [194, 195], it is likely that many ENMs may not be suitable for testing in all cytotoxicity assays. Unfortunately, most published reports on cytotoxicity of AuNPs and other ENMs fail to undertake such precautionary
tests, resulting in a vast amount of literature citing outcomes which may not be fully trusted without revalidation.

Another common problem encountered with assays which are read spectrophotometrically such as MTS and MTT is that each nanoparticle absorbs light over a particular wavelength band, which may coincide with the wavelength that the assay is read at. This has been reported to be the case for sodium titanate nanoparticles [223] as well as citrate stabilised AuNPs [218]; however this problem may be potentially alleviated by adding a centrifugation step to the MTT protocol or by removing the nanoparticles prior to the addition of the tetrazolium compound [218]. Despite this fact, it is not common practice in such assays to remove the analyte before continuing the assay. Moreover, the innately “sticky” nature of ENMs, (adhering to the reaction vessel, cellular surface, etc.) makes it difficult for the EMS to be removed without disturbing the surrounding cells when performing assays. Alternate strategies, such as those involving control experiments, wherein pre-fixed (dead) cells are exposed to ENMs to assess non-specific cellular binding of ENMs, may address the issue or ENM’s stickiness to the cellular surfaces.

![Figure 1.9: A 96 well plate after development of purple formazan crystals, denoting cell viability.](image)

Genomic or proteomic assays such the enzyme-linked immunosorbent assay (ELISA) are not impervious to similar forms of interference [206]. ELISA can be used to determine if proinflammatory markers or cytokines, such as TNFα [56], interleukin 1-β [56], interleukin-6 or interleukin-8 are being released from cells following treatment with nanoparticles. For instance, CNTs
were found to adsorb interleukin-8 as reported by Montiero-Riviere and Inman [224], while Veranth and co-workers [225] reported other nanoparticles including SiO\textsubscript{2} and TiO\textsubscript{2} behaving similarly in the case of interleukin-6, leading to the detection of lower than expected levels of cytokines.

The resounding message emerging from the literature regarding the toxicity of nanomaterials highlights the importance of choosing an appropriate assay for each individual ENM with consideration for the endpoint being examined, and to perform appropriate control experiments before critically analysing the results obtained. It is obvious that each assay must be optimised for new nanomaterials, adding additional steps to minimise interference when necessary. Where possible it is also advised to cross check results by using more than one assay to expose false readings [217]. Some of the foremost considerations that must be followed in choosing the correct assays for toxicity assessment of ENMs include, (i) appropriate assessment of the potential for nanoparticle aggregation in cell culture medium, (ii) careful assessment of potential adsorption of the assayed component onto the nanoparticle surface, and (iii) consideration of the nanoparticle’s inherent chemical activity in producing the reaction product. For instance, it is now well-established that gold and many other metal nanoparticles show strong reducing capabilities. Since MTS/MTT assays rely on reduction of these tetrazolium compounds into formazan crystals, one must be very cautious when choosing such assays for assessing biocompatibility of metal nanoparticles. Similarly, recent reports have demonstrated that some of the nanoparticles such as gold and iron oxide behave as nanoenzymes by demonstrating biological enzyme-like activity (peroxidase, catalase, etc.) [194, 195]. These new findings may further influence the way ENMs should be assessed for in vitro cytotoxicity.

Of equal importance in choosing the correct assay to assess the toxicity of ENMs is identifying an appropriate line of cells on which to perform the assay. It is commonly thought that incidental exposure to nanoparticles is via respiration, ingestion or through the skin [211, 213, 216]. For this reason, nanoparticle toxicity assays are commonly performed on skin cells or cells from the respiratory or gastrointestinal systems [60, 62, 224-226]. However researchers interested in assessing the therapeutic potential of ENMs in applications such as biological imaging [54, 183, 227, 228] and drug delivery [227, 229-234] perform tests on common tumour cell lines of the breast [144, 178],
prostate [178, 185, 235] and cervix [59, 236]. While it appears appropriate to choose cell lines depending on the end application, to assess the overall cytotoxicity of a particular ENM, it may be critical to test a number of different cell lines in a single comprehensive study for comparison.

### 1.3.2 Uptake and toxicity of gold nanoparticles

While gold in its bulk form, and particles of micron size range or larger, are generally thought of as non-catalytic, stable, inert and biocompatible, the uptake and toxicity of AuNPs is a far more complex issue [65]. With key variables such as the synthesis, size and shape of the nanoparticle as well as the cell type and endpoint examined [237], researchers have begun to form a matrix of results based on these findings. While some studies draw valid comparisons based on their own results [59], other reviews collate and compare the toxicity of particles synthesised by different groups over many years, using varied synthesis methods [64, 65, 238].

Shukla et al., published one of the pioneering reports on biocompatibility assessment of AuNPs [56]. This study examined the cytotoxicity and intracellular uptake dynamics of AuNPs in macrophage cells. Atomic force microscopy (AFM) studies shed light on endocytosis processes involved in AuNP uptake, while revealing that AuNPs taken-up by the cells could not be expelled out of the cells for extended time periods. One of the notable findings of this study was that these AuNPs were not only found to be highly biocompatible, they showed antioxidant properties at higher dosages and longer treatment points. This was concluded based on their ability to reduce the level of reactive oxygen species (ROS) and reactive nitrogen species (RNS) produced by the cells. This study also demonstrated for the first time that spherical AuNPs didn’t induce the formation of proinflammatory cytokines such as TNFα and interleukin 1-β by macrophage cells.

This study was followed by a comprehensive study by Chithrani and co-workers with regards to nanoparticle shape, size and toxicity [59]. The study examined five citrate stabilised spheres of different diameters and two CTAB stabilised rod-shaped AuNPs of different aspect ratio. To be able to compare the particles in terms of toxicity and uptake, the positively charged CTAB present on the surface of the gold nanorods was exchanged with negatively charged citric acid ligands so that it was
common across all seven nanoparticles. The study concluded that nanoparticles synthesised in this way did not cause toxicity to HeLa (immortal cervical cancer) cells in the doses examined. This finding of negligible toxicity has been validated by many other groups who tested spherical [56, 236, 239-241] and rod shaped [242] AuNPs in a number of different cells.

In contrast to these reports, some other groups such as Patra and co-workers synthesised one particular type of nanoparticle, e.g. citrate-reduced 33 nm spheres and tested its toxicity in three different cell lines [243]. Their results showed that the same nanoparticle was toxic in human carcinoma lung cells (A549), while remaining nontoxic over the same dose range in both baby hamster kidney cells (BHK21) and human hepatocellular liver carcinoma cells (HepG2). When better understood, this cell-selective effect could be harnessed as a possible treatment for certain forms of cancer.

In 2010, Alkilany and Murphy compiled a comprehensive review of other group’s results regarding the toxicity and cellular uptake of AuNPs [64]. Over 20 studies were examined, most commonly, groups studied spherical and rod shaped particles, however a range of chemical stabilisers were employed including citrate (most common), CTAB, various amino acids, and PEG both in vitro and in vivo. The authors found that there was no clear conclusion to draw on the toxicity of AuNPs, with such conflicting results between groups. The analysis of existing research outcomes led them to hypothesize that particle uptake, distribution and toxicity depended greatly on the interaction between the particle surface and the surrounding biological media [64, 238].

1.3.2.1 Effect of gold nanoparticle size on uptake and toxicity

Using methods such as ICP-MS and related techniques, it is possible to quantify the uptake of nanoparticles by relating the gold concentration of a known population of AuNP treated cells to the number of particles per cell. These results can be confirmed visually using electron microscopy. Chithrani and co-workers performed such a study on 14, 30, 50, 74 and 100 nm gold spheres [59]. The study estimated the number of nanoparticles per cell (calculated from the number of gold atoms obtained using inductively coupled plasma atomic emission spectroscopy), and found that maximum
uptake (by number) occurred for the 50 nm particles; however despite the difference in uptake quantity, none of the spherical particles tested induced any significant cytotoxic event. These findings correlate well with other groups who found similar size dependent trends for uptake maxima [244-246] as well as uptake speed maxima [247] for ~50 nm AuNPs. The studies, which performed additional electron microscopy found that 50 nm AuNPs could not enter inside the nucleus, a possible reason for their lack of toxicity [59, 247].

In an early investigations, using a combination of imaging tools such as TEM, AFM and confocal microscopy, it was demonstrated that when ~10 nm AuNPs were exposed to RAW264.7 macrophage cells, the particles were sequestered in the endosomes, and these endosomes migrated from the cellular membrane towards the nucleus in a time-dependant fashion [56]. Even after longer exposure times, while endosomes carrying these AuNPs were found to beautifully arrange themselves just outside the endoplasmic reticulum network emerging from the nuclear pore, none of the AuNPs were observed to penetrate the nucleus as shown in Figure 1.10. One proposed mechanism for seemingly enhanced uptake of AuNPs is that their size is within the range of viruses and lipid-carrying proteins which would typically be engulfed or endocytosed by the cell [244]. However, the broader literature is less clear, with reports varying depending on surface modifications and the cell line chosen. There are many reports which indicate that AuNPs of varying sizes demonstrate negligible toxicity [56, 236, 239-241]; however there are also significant number of papers which dispute this [248-250].

One notable in vivo example of size dependent toxicity was obtained by Chen and co-workers who tested eight AuNPs of sizes ranging from 3 to 100 nm, injecting the doses intraperitoneally into mice [250]. While nanoparticles of sizes 3, 5, 50 and 100 nm did not induce any apparent cytotoxic effects, AuNPs ranging from 8-37 nm initially caused changes in appetite and fur colour, progressing to bruising and bleeding under the skin, the development of a crooked spine and eventual death. Physiological changes were detected in lung, liver and spleen tissue samples taken from the mice treated with 8-37 nm AuNPs that were not seen in mice dosed with smaller or larger nanoparticles. These changes include the presence of emphysema like structures within the lungs, depletion of
immunologically active lymphoid tissue within the spleen and an increase in Kupffer cells inside the liver, which would suggest that it was the site of a large scale immune response [250].

![Image of a RAW264.7 macrophage cell dosed with ~10 nm AuNPs. Triangular-tipped arrows show the reticuloendothelial network emerging from a nuclear pore. The square-tipped arrows show AuNPs present inside lysosomal/endosomal bodies. Reprinted with permission from [56].](image)

**Figure 1.10:** TEM image of a RAW264.7 macrophage cell dosed with ~10 nm AuNPs. Triangular-tipped arrows show the reticuloendothelial network emerging from a nuclear pore. The square-tipped arrows show AuNPs present inside lysosomal/endosomal bodies. Reprinted with permission from [56].

While typical cell culture experiments are most often based on the methodology of single exposure and subsequent reading, the long term effects of exposure to AuNPs hold equal importance. By exposing human dermal fibroblasts (CF-31) with citrate-capped 13 and 45 nm spherical AuNPs for up to six days, Mironava *et al.*, determined that despite an initial increase in doubling time observed as a function of concentration for both particle sizes, the effect on the cells was not permanent [251]. Using electron microscopy, the cells were observed to transfer internalised AuNPs to their daughter cells during division, effectively decreasing the number of internalised particles in each cell over time. As the number of internalised particles decreased over generation, the cell doubling time returned to that similar of the control cells over a 5 day recovery period. These findings are in agreement with Liaw and co-workers who tested citrate-capped 10 nm spherical AuNPs on human osteogenic sarcoma cells (MG63) [252]. By exposing cells to the nanoparticles for a period of 20 hours (at concentrations of 1 and 10 ppm) and monitoring them for a further 21 days they concluded that there was no difference in viability, doubling time or morphology compared with the
control group at the final time point. These observations highlight the significance of exposure levels, such that if the organism is able to tolerate the chosen dose initially, it may be able to overcome cytotoxicity through in vivo metabolic mechanisms.

1.3.2.2 Effect of gold nanoparticle shape on uptake and toxicity

While there are studies that discuss the synthesis of triangular/prismatic [7, 78, 135, 144, 149, 253, 254] and cubic [41, 144, 149, 157, 161, 162] AuNPs and their potential for biological use, such particles have been the focus of relatively few biological studies in comparison to spherical nanoparticles. Following spherical AuNPs, the shape that has captured the highest interest from researchers is the gold nanorod, with the relationship between toxicity and nanorod length, or more specifically their aspect ratios, explored by many groups [64, 142, 242, 255-258].

As previously discussed, gold nanorods are popularly synthesised using CTAB, a cationic surfactant used to influence shape; however pristine CTAB above 1 μM concentration has been shown to be toxic to certain mammalian cells [62]. For this reason, some groups choose to modify gold nanorods originally synthesised using CTAB, by chemical exchange or surface functionalisation to give the particles a new, less toxic coating. Cellular uptake of these rods, when compared to spheres, has been shown to be slower, in lower quantities, and to induce a higher cytotoxic response [59, 60, 256, 257]. Surface modifications often involve the use of polymers such as PEG to dispel the toxicity associated with CTAB; however it is reported that this high molecular weight non-fouling molecule discourages cellular uptake [212, 259], and drastically slows the process of endocytosis [60]. When comparing the effect of changing the aspect ratio of rods it was found that lower aspect ratio rods are taken up in higher quantities than those with higher aspect ratios [59]. This is somewhat expected as spherical AuNPs, which have the lowest possible aspect ratio (1:1) are generally uptaken by cells in much higher quantities.

Other groups elected to test their gold nanorods as-synthesised, without any modification of the surface. Wang and co-workers examined the uptake and toxicity of various sized gold nanospheres (synthesised without CTAB) and gold nanorods (synthesised with CTAB) [62]. While they found no
toxic effect from all tested nanospheres, they found a drastic increase in toxicity from all gold nanorods. The group related the increased toxicity to levels of free CTAB within the sample, i.e. CTAB left unreacted after synthesis which is not bound to the surface of the particles, and performed a more thorough series of wash steps using centrifugation. After washing the particles three times, they observed a 60% improvement in cell viability compared with the same particles unwashed. Highlighting the degree of improvement that can be gained from washing gold nanorods thoroughly, Connor and co-workers eliminated toxic effects completely from CTAB stabilised gold nanospheres by centrifugation and washing, contrasting with high levels of toxicity for unwashed particles [239].

While less explored, preliminary studies have also been performed on the effect of prismatic AuNPs on various cells – both human and animal. Singh et al. [260] synthesised gold nanoprisms using fresh lemon grass extract, an example of one of the many biogenic synthesis methods developed to provide shape control. The difficulties related to making high yield nanoprisms (or purifying solutions to retain a high yield of nanoprisms) are well documented in the literature [82, 261, 262], and as such, TEM images of the particles used by the group depict many persistent small spherical particles, as well as prisms with “edges cut” – effectively hexagons. Negating potential issues with sample purity, the nanotriangles were found to be well tolerated by cancerous and non-cancerous cell lines, with 80% cell viability at doses of up to 800 μM. Further imaging studies confirmed that the cells do take up the prismatic particles; however these particles did not enter the nucleus, but rather remained within the cytoplasmic space.

While the effect of shape can be quite drastic when comparing forms such as spheres and rods, Wani and Ahmad performed a shape and size dependant study on the antifungal activity of two AuNP samples, viz. gold nanodisks and mixed polyhedral shapes [49]. While they drew a conclusion that the enhanced antifungal activity observed from the gold nanodisks was due to their size, in reality, the two sets of shapes were not clearly defined, with mixed populations and high polydispersity apparent. The two shapes were synthesised using different methods, utilising different reducing agents, which also has the potential to create a difference in activity.
Overall, the findings suggest that while there may indeed be shape specific effects on cellular uptake and toxicity of AuNPs, the importance of eliminating surface and size effects to draw clear conclusions on the effect of gold shape on biological activity remains paramount.

1.3.2.3 Effect of gold nanoparticle surface characteristics on uptake and toxicity

Use of the term “gold nanoparticle” typically refers to a nanoparticle synthesised with a hard inorganic gold core and almost always with a soft organic corona. However, depending on the method of synthesis, and any modifications applied post-synthesis, the surface may vary greatly between different AuNPs [211]. It is widely thought that the chemical composition of a nanoparticle is one of many factors which contribute to the level of cytotoxicity it exhibits; however there are relatively few studies which explicitly establish this precedent.

Connor and co-workers demonstrated that starting materials such as gold salt (AuCl₄⁻) and CTAB were cytotoxic when tested alone, however when they were utilised along with other chemicals during synthesis, the resulting nanoparticle did not necessarily show cytotoxic effects [239]. The group tested spherical particles surface modified with cysteine, citrate, glucose, biotin and CTAB, finding negligible cytotoxicity in human leukemic cells [239].

In a similar system, Das et al. looked at three spherical AuNPs with different surface modifications – aspartic acid, trisodium citrate dihydrate and BSA finding negligible cytotoxicity from all particles through in vitro testing [187]. The group also tested the nanoparticles on mice to monitor toxicity after ingestion. In this experiment, the particles stabilised with BSA were found to be the most biocompatible, while the particles stabilised with aspartic acid and trisodium citrate dehydrate exhibited damage to the liver and kidneys. The group identified stability of nanoparticles in biological media and surface charge as key factors in influencing biocompatibility of nanoparticles [187].

The effect of surface charge has been investigated by many groups [242, 263, 264] to determine its role in cytotoxicity. Rotello and co-workers found that identically synthesised cationic and anionic spherical AuNPs exhibited different cytotoxicity levels, with cationic particles observed
as moderately toxic while anionic particles were deemed nontoxic [263]. This disagrees with the findings of Schaeublin and co-workers who tested three spherical AuNPs; positively charged, negatively charged and neutral [264]. In this study, both the positively and negatively charged particles were found toxic, with the negatively charged particles eliciting a stronger cytotoxic effect. The authors concede that this may be related to the outer chemistry of the cationic particles not being similar, due to differing methods for charging the particles surface.

More recently, to understand how nanoparticle surface charge influences their uptake and toxicity, Daima et al. utilised tyrosine amino acid-reduced/capped AuNPs as a model system [172]. These negatively charged AuNPs showed no toxicity to Gram negative Escherichia coli bacteria and A549 human lung carcinoma. When the negative charge on the AuNPs surface was reduced through coating with a cationic amino acid lysine, it was noted that although nanoparticle uptake increased for both the bacteria and mammalian cells, it did not influence their toxicity. These observations were further validated by functionalising tyrosine-capped AuNPs independently either, with a cytotoxic polyoxometalate molecule or with polyoxometalate in combination with lysine. This investigation showed that the cytotoxicity of these polyoxometalate-functionalised AuNPs is dependent on the overall surface charge of the AuNP carrier system such that with an overall reduction in negative charge, the uptake and therefore cytotoxicity is enhanced. In contrast, when Daima et al. utilised polyoxometalate-functionalised tyrosine-capped silver nanoparticles for a similar study, they showed toxicity against both Gram negative bacteria E. coli and Gram positive bacteria Staphylococcus albus, while showing no cytotoxicity against PC3 human epithelial cells [265]. It remains unclear at this stage whether the difference in Ag and Au toxicity profiles of nanoparticles with similar surface characteristics was due to differences in nanomaterial composition or to the difference in the mammalian cell line [266].

Biological media are buffered solutions containing a mixture of electrolytes and proteins which can interact with the nanoparticle surface [242]. While nanoparticles may be stable in situ for extended periods of time, this alone does not confer stability in cell culture media [267, 268]. Alkilany and co-workers found that gold nanorods change surface charge when exposed to a cell culture
medium due to proteins adsorbing to the surface. The group tested positively charged rods synthesised using CTAB, against rods which were identical before being coated with polyacrylic acid (PAA) to switch the surface charge [242]. The initial charge of the rod was shown to be irrelevant as all rods had high levels of BSA adsorbed on their surface, which at physiological pH has a negative charge [269]. With surface charge constant, the toxic effect observed from the positively charged gold nanorods was attributed to free CTAB remaining in the nanoparticle samples. To make this point, and demonstrate the relatively low importance of initial charge, the group coated the negatively charged, non-toxic PAA-coated gold nanorods with positively charged polyallylamine hydrochloride (PAH). Unlike the positively charged CTAB rods, the PAH rods displayed negligible toxicity [242]. The group stressed the importance of studying the behaviour of nanoparticles after exposure to biological media as the nanoparticle-protein interaction is an important factor in uptake and toxicity.

This suggests that the surface characteristics of AuNPs play a rather complex role in determining their mode of biological action. It remains almost certain that the surface characteristics of as-synthesised AuNPs are instantaneously modified on their contact with biological fluids or cell growth media. It is these modified surface characteristics that are responsible for AuNPs final mode of action. However, it should be possible to fine-tune the surface features of as-synthesised AuNPs such that their interaction with the biological fluids (before interacting with cells) can be finely controlled. It is believed that this aspect has significant scope for future developments in the field, and therefore has elaborated on in Section 1.4.

### 1.4 Influence of biological protein corona formation on nanoparticle uptake and toxicity

With intravenous injection the optimal route of administration for many AuNP based therapies, it is fair to assume that the blood will be the initial biological environment encountered by many of these nanoparticles. In the same way that other materials including medical implants become coated with proteins almost immediately after introduction to the blood [270], the competitive
dynamic process of forming a protein corona commences at the nanoparticle surface almost instantaneously as depicted in Figure 1.11. All inorganic nanoparticles, including gold, invoke the formation of a protein corona when introduced to protein containing solutions such as biological media or blood [68, 238, 268, 271]. The time dependant process begins in as little as seconds to minutes with proteins of high mobility localising on the surface of the particle forming what is termed a soft corona due to its transient existence. Over the following hours, proteins which are less mobile but carry higher affinity for the surface of the particle may replace the initial proteins to form a more permanent hard corona in what is known as Vroman’s effect [272].

![Figure 1.11](image)

*Figure 1.11: The evolution of a protein corona begins almost immediately after introduction of a nanoparticle to protein rich conditions (I). Initially, the nanoparticle is covered with proteins which are abundant and highly mobile (II), the proteins species are exchanged over time resulting in hard corona of strongly bound proteins (III). Reprinted with permission from [273].*

To understand this effect, model proteins such as BSA or human serum albumin (HSA) are often used, as they represent albumin, the most abundant protein found in human blood. Despite the model used, the composition of the protein corona is unique, and influenced by many external variables relating not only to the nanoparticle itself, but also the nature of the biological system being probed. The composition of the protein corona is generally thought to consist of 10-50 proteins [238] and while highly abundant proteins in the blood such as albumin, immunoglobulin G (IgG), fibrinogen and apolipoproteins are the most common constituents of the protein coronas studied to date, their relative abundance on the nanoparticle surface does not necessarily correlate with their natural abundance in the blood [274].
While many mechanisms exist to explain the process of protein binding to the nanoparticle surface, the most widely accepted is the entropy-driven binding model [274, 275]. This exchange occurs because the release of water molecules from the area close to the nanoparticle surface is energetically favoured (due to high entropy increase) compared to the relatively small decrease in entropy caused by the binding of a protein in its place.

It is thought that cellular-nanoparticle interactions are greatly dependant on the composition of the protein corona; and that the nanoparticle core, hydrophobicity, size, shape, charge and surface functionalisation elicit some effect on the types of proteins that are attracted to form the corona [273, 274]. It is widely accepted that the protein corona confers the nanoparticle its biological identity, and thus it can be suggested that the uptake of AuNPs by cells is dictated by the species of proteins, their orientation and arrangement on the surface of the nanoparticle [242, 276]. Understanding the relationship between causal factors and the final composition of the protein corona may therefore prove insightful to the unpredictable and often contradictory results obtained by researchers probing the toxicity of AuNPs.

1.4.1 Effect of nanoparticle size on protein corona formation

The general paradigm linking nanoparticle size and protein corona formation is typically thought to hinge on the degree of curvature on the nanoparticle surface [68, 256, 270, 277, 278]. This is most commonly probed by examining spherical particles of different diameters, and comparing to binding observed on macroscopic flat surfaces of similar surface chemistry [271, 279-282]. Geometrically, it can be understood that differences in the degree of curvature may favour or inhibit proteins depending on their three dimensional structure, allowing for certain proteins to make contact with a greater area of the particle offering greater stability, or sterically inhibiting or possibly disrupting others upon making contact [277, 280, 283]. The knowledge that curvature is influential on protein-nanoparticle interactions is seemingly universal for all nanoparticles; however with respect to the protein perspective the results are less predictable. Having a large degree of variation in size, structure and composition between various proteins, the interactions must be probed or modelled for
each protein and nanoparticle in question, taking into account such variables as the preferred structure of each protein as well as the position and number of binding sites available on the nanoparticle surface. It must be noted that even with this knowledge, assessing the binding of each protein alone does not necessarily provide transferable information related to binding affinity of the proteins in a highly dynamic environment as would be expected within the blood.

Size dependant effects have been seen in many nanoparticles despite their elemental composition. For instance, Shang and co-workers examined the behaviour of cytochrome c proteins on the surface of different sized silica nanoparticles. Their finding showed that the cytochrome c structure became increasingly distorted upon binding to larger silica nanoparticles, while also becoming increasingly unstable in its new conformation [284]. Conversely, Roach and co-workers found that the structure of albumin becomes increasingly disordered after binding to larger silica nanoparticles while fibrinogen becomes increasingly compromised upon binding to smaller particles with higher surface curvature [280] as represented in Figure 1.12. Such size dependant effects were also recorded by Dobrovolskaia and co-workers in the case of 30 and 50 nm AuNPs with 30 nm particles binding to a greater range of protein species when compared with the 50 nm particles of similar surface chemistry [285].

**Figure 1.12:** Schematic showing how the size of a spherical particle (and thus the degree of its surface curvature) can affect the structure of bound proteins. While the structure of albumin becomes increasingly disordered after binding to larger silica nanoparticles, fibrinogen becomes increasingly compromised upon binding to smaller particles which possess higher surface curvature. Reprinted with permission from [280].
While it is clear that the composition of the corona is influenced by nanoparticle size, the question remains how to maximally manipulate this effect for therapeutical gain. For therapies that rely on long circulation times, avoiding nonspecific uptake of nanoparticles by the immune system, more specifically, the reticuloendothelial system (RES) and the mononuclear phagocyte system (MPS), is critical [259, 286, 287]. It is known that certain proteins within the blood act as flags for the RES and MPS systems; such proteins are termed opsonins (and their binding to the nanoparticle surface, opsonisation) and they provide the link between a nanoparticle and its capture by the MPS system [287-290]. Known opsonins include certain immunoglobulins (IgG and IgM), specific complement proteins (most notably C3), von Willebrand factor, thrombospondin, fibronectin, and mannose-binding protein. Conversely, other classes of proteins coexist that are deemed dysopsonic due to their opposing effect, suppressing the recognition and elimination of opsonised entities by the RES and MPS. PEGylation or the process of coating an object with linear chains of covalently attached PEG macromolecules is commonly employed for in vivo application of AuNPs to minimise such unwanted effects. As well as improving biocompatibility, PEGylation also decreases the extent of protein adsorption thus decreasing the risk of opsonisation [182, 287, 291].

An extensive study in this field performed by Casals and co-workers utilised citrate stabilised AuNPs in sizes ranging from 4-40 nm to probe the size effect on protein corona formation, with particular emphasis on the density and persistence of both hard and soft coronal states. The group found that 4 nm AuNPs were not able to form a stable protein corona despite prolonged incubation times, consistent with the hypothesis that particles of this size do not significantly activate RES or MPS [68, 278]. The 10 nm AuNPs behaved as expected for particles of this size, forming an initial transient soft corona followed by a persistent hard corona at longer incubation times. The larger 40 nm particles examined initially behaved like 10 nm particles, forming a soft corona transitioning into a hard corona over time; however the hard corona which formed on the 40 nm particles was both less dense and less strongly bound compared to the 10 nm particles. The group concluded that a particle of 10 nm is ideally sized for optimal protein coverage, being of a comparable size to the most abundant
serum proteins (albumins) while the 40 nm particles exceed the ideal size for serum protein binding, and were more prone to opsonisation [68].

In contrast to these trends, Lacerda and co-workers probed the kinetics of specific serum proteins such as albumin, fibrinogen, γ-globulin, histone and insulin, monitoring their binding on citrate stabilised AuNPs of sizes from 5-100 nm. Their findings highlighted a trend of increasing nanoparticle-protein binding strength with increasing nanoparticle size, until the size reached 60 nm, after which the trend dissipated. The cooperativity of the binding, i.e. the affinity of a particular protein for a nanoparticle after binding by successive proteins, decreased with nanoparticle size in all cases except insulin. These discrepancies draw attention to difficulties encountered by researchers in this area, whereby information gained by examining proteins individually does not necessarily correlate to results obtained in a competitive binding environment [292].

Walkey and co-workers examined citrate stabilised AuNPs ranging in size from 15-90 nm after surface modification with PEG. The study revealed two relationships, viz. a correlation between decreasing AuNP size and increased adsorption of serum proteins, as well as decreased adsorption of serum protein with increasing PEG grafting density. They reasoned that a decrease in nanoparticle size is synonymous with an increase in curvature on the surface of the nanoparticle, allowing better accommodation of PEG molecules with increased space, as opposed to the larger particles that forced a more crowded layer of PEG molecules. The fanned out arrangement of PEG molecules was claimed to lower the thermodynamic barrier for protein adsorption, leading to increased protein binding [281].

1.4.2 Effect of nanoparticle shape on protein corona formation

In a similar way that the size of a nanoparticle affects protein corona formation, shape plays an important, albeit less understood role. Much of the shape related effects on protein corona formation are indirectly gained though uptake studies, monitoring cell-nanoparticle interactions and inferring the state of the protein corona from these results [59, 185, 256]. Despite the small number of studies in this area, it is generally agreed that shape affects the manner in which a protein can bind to the surface of a nanoparticle, with the introduction of such features as curvature, flat planes, sharp
edges, corners, and pores on various shapes. Such features may favour or hinder binding of individual proteins depending on their conformation, or cause them to undergo structural changes [238].

Ramezani and co-workers performed a molecular dynamic simulation to compare the binding of HSA protein to cubic and spherical AuNPs with similar surface areas. The data showed that binding to cubic AuNPs causes the albumin to unfold more significantly than it does with spherical particles. Interestingly, the simulation showed that the distance between HSA molecules on the cubic AuNPs was larger than on the spherical AuNPs, highlighting that the unfolding effect is much stronger for cubic AuNPs than spherical AuNPs. The group also noted changes in the secondary structure of HSA upon binding to cubic particles, an effect not observed with spherical AuNPs. The authors reasoned that the curvature of spherical particles allows proteins to preserve their original structure by permitting greater gyration of the protein after binding, whereas a flat surface (consistent with the facets of a cubic particle) would not allow such movements [293].

Gagner and co-workers synthesised both spherical and rod shaped AuNPs using two enzymes, lysozyme and α-chymotrypsin, to monitor the structure and function of proteins after adsorption. They showed that higher density adsorption occurs on the surface of gold nanorods, as compared with spherical AuNPs, which they attributed to the long cylindrical surface of gold nanorods. When examining lysozyme, the group noted a higher degree of secondary structure disruption on rods, as compared with spheres (15% and 10%, respectively). Conversely, α-chymotrypsin was capable of retaining its secondary structure at low levels of coverage on both shapes, however as coverage became denser; a 40% loss of secondary structure was detected. Of particular interest is the fact that the change in α-chymotrypsin’s secondary structure led to an 86% reduction in activity [294].

Focussed on the link between nanoparticle shape and the stability of a bound protein, Asuri and co-workers compared the binding of enzyme soybean peroxidise with the surface of C-60 fullerenes and with flat graphite flakes. The group noted that upon binding to the highly curved surface of the fullerene, the enzyme possessed much greater stability with an enhanced (2.5 times) half-life, leading to enhanced enzymatic activity as compared to the flat supports. The authors
hypothesised that the effect of enhanced stability on highly curved surfaces may not be limited to carbon and could apply to other materials including gold [282].

Investigating rod shaped particles, Gagner performed ligand exchange to remove CTAB from the nanoparticle surface, and reasoned that the increased binding they observed on gold nanorods was due to their long cylindrical surface [294]. Conversely, Caswell [295] and Chang [296] probed protein binding on as-synthesised rods obtained using CTAB as shown in Figure 1.13. Both groups found that protein adsorbed preferentially to the ends of the rod shaped particles, and suggested that this may be due to the CTAB being tightly packed on the less highly curved long dimension, which may inhibit protein binding. With this knowledge, Chithrani and Chan examined rods coated with transferrin, a blood plasma protein vital in iron transportation [256]. They found that rods with lower aspect ratios displayed a higher degree of binding compared to rods with high aspect ratios. They reasoned that this finding, in agreement with Casswell and Chang, was due to increased curvature on the ends of the lower aspect ratio rods, and could also lead to more strongly bound proteins in these areas [256].

The effects reported for other materials are consistent with the findings for gold, suggesting that they are true shape effects, and not necessarily related to the core material. Deng and co-workers examined TiO$_2$ nanorods, nanotubes and nanospheres [297]. Consistant with many gold related reports, their results showed that nanospheres bound to higher amounts of protein compared to both rods and tubes. The most interesting result was the comparison of protein species found on the TiO$_2$ rods and tubes, with IgM and IgG the major constituents of the rod corona, while fibrinogen was the major constituent of the nanotube corona. With immunoglobulins IgM and IgG being known opsonins, this study highlights shape to be an important consideration for potentially avoiding nanoparticle detection by the immune system [238, 279].

It is possible however that shape may not have such noticeable effects in all settings. One study which did not see any significant shape related effects was conducted by Boulos and co-workers who examined gold nanorods of aspect ratios 3.5 and 18, along with 20 nm spherical AuNPs. They studied the particles after they were coated with PEG, as well as after a polyelectrolyte coating that
was used to recreate the original cationic state seen in CTAB synthesised particles. They found that PEG did not prevent binding of BSA protein to any particle, and all PEGylated particles showed cooperative binding regardless of nanoparticle shape. BSA was shown to have a higher affinity for polyelectrolyte coated particles which possessed a strongly positive surface charge, and this remained true of both shapes studied [298].

![Figure 1.13: TEM images of gold nanorods assembled end-to-end after exposure to mouse antibody/antigen biomolecules. By varying the concentration of biomolecules, chains of increasing length are assembled. Reproduced with permission from [296].](image)

1.4.3 Effect of nanoparticle surface characteristics on protein corona formation

The effect of a nanoparticle's surface characteristics on the formation a protein corona may be examined with varying levels of subtlety. The most fundamental aspect may involve comparison of the protein corona formed around nanoparticles of different inorganic compositions. Deng and co-workers analysed the composition of the protein coronas which formed on two commonly used metal oxide particles of comparable size and surface charge, viz. ZnO (31 nm diameter, -24 mV zetapotential) and TiO$_2$ (30 nm diameter, -26 mV zetapotential). The group found that the elemental core of the nanoparticle influenced both the species and concentration of bound proteins with dramatically different coronas elucidated from each nanoparticle [297].
The next layer of subtlety is surface coating or functionalisation, a practice often employed to fine-tune parameters such as biodistribution, circulation, accumulation and clearance of nanoparticles in drug delivery systems [274, 289]. In addition to their study on different sized AuNPs, Casals and co-workers studied the effect that surface functionalisation of AuNPs had on the formation of a protein corona. The group examined the protein coronas that formed on citrate stabilised AuNPs of sizes 4-40 nm, comparing them with those formed after modification of the nanoparticles with a self-assembled monolayer carrying a net positive (aminoundecanethiol) or negative (mercaptoundecanionic acid) charge. The group found that the negatively charged nanoparticles were unable to form a hard corona, with the loosely bound soft corona washing off easily despite an extended incubation period in cell culture medium. Conversely, the positively charge particles formed a soft transient corona more rapidly, driven by electrostatic forces between the positively charged surface and negatively charged proteins in the media. In this case, the soft corona possessed higher stability than the hard corona (formed on the citrate stabilised AuNPs), and was persistent after purification [68].

These findings are consistent with many other groups that have investigated the protein coronas which form on AuNPs after PEGylation. The process is commonly employed for in vivo application of gold nanorods, to avoid the potential toxicity derived from CTAB persistent on the surface after synthesis. While PEGylation of gold nanorods is performed as a way of ensuring biocompatibility, its presence is known to cause a reduction in uptake for the reasons described above. The complications surrounding this depend on the intended fate of the nanoparticle with a potential trade-off between longevity within the bloodstream and the likely degree of uptake. The ‘hydrophilic stealth coating’ that PEG molecules form around the particle [281, 291, 299], may protect it from elimination via immune intervention, but may also reduce uptake by cells via the same mechanism [59, 185].
Cui and co-workers compared the protein coronas which formed on nanoparticles with various surface modifications, such as citrate, thioglycolic acid, cysteine and PEG with varying molecular weights of 2 and 5 kDa. The 5 kDa PEGylated particles were unable to form a protein corona in any of the protein environments studied, while the 2 kDa PEGylated particles showed protein corona formation in BSA, but not with transferrin or fibrinogen [300]. This finding is consistent with the work of Cruje and Chithrani [301] as well as Dobrovolskaia and co-workers [285] who found that the amount of protein bound in the corona may be influenced by the molecular weight (i.e. chain length) of the PEG molecules within the AuNP coating, with longer PEG chain lengths consistent with lower levels of protein interaction with the nanoparticle surface, as presented results shown in Figure 1.14.

![Figure 1.14](image)

**Figure 1.14:** Graph showing the extent of bound proteins to 30 nm AuNPs uncoated or coated with PEG molecules of different molecular weights over time. Coating the AuNPs with longer PEG chain lengths leads to lower levels of bound protein. Reprinted with permission from reference [285].

Walkey and co-workers compared the protein corona composition between citrate-capped AuNPs and PEGylated citrate-capped AuNPs. Complement protein C3 was detected at levels of 30% w/w on the unmodified AuNPs, reducing to a level of 5% w/w after PEGylation, showing that modification was sufficient to significantly reduce, but not totally eliminate, opsonisation [281].
1.5 Technological advances in the use of gold nanoparticles

The use of gold to promote good health has been documented in literature dating back to at least the 1st century, when Pliny detailed its multiple functions in what is now regarded as the one of the earliest encyclopaedias ever compiled - *Naturalis Historia* [1]. Listed for both its medical and magico-religious properties, gold is described as being both a cure for fistulas, haemorrhoids and warts as well as an amulet to be worn for protection against harmful charms [1, 302].

Predating this publication, the use of gold as a purifying tonic or elixir by the Egyptians [2] and more tangibly the appliance of flat gold bands as dental prosthetics by the Etruscans [3] is believed to date back to the thirtieth century B.C. and the seventh century B.C., respectively. The Vedic age of ancient India (ca.1750–500 B.C.) saw gold utilised as a therapy for memory loss, poor eyesight and infertility and since the 8th century the use of *Swarnabhasma* (*swarna* meaning gold, and *bhasma* meaning ash) or gold ash (a nanoparticulate form of gold) began, a practice which is still continued by Ayurvedic followers to treat asthma, autoimmune and nervous disorders [303, 304]. During the Renaissance, physician and alchemist Paracelsus revived the idea of using gold as a medicine when he created *Aurum Potabile*, a colloidal gold suspension which he prescribed for sufferers of epilepsy [4].

Today, the use of AuNPs for biotechnological and medical advancement is progressing rapidly with biosensors, bioimaging techniques and therapeutic agent's dependant on the unique properties of AuNPs beginning to pass clinical phase trials with countless more in development.

1.5.1 Gold nanoparticles as diagnostic agents

Comprising both *in vitro* and *in vivo* applications, the use of AuNPs as biosensors is widespread, owing to the interesting SPR behaviour of AuNPs in varied environments [305]. Some examples of the use of AuNPs include sensors for bacteria [306-308], early cancer markers [309], and specific biomolecular interactions [310].
It is not widely known, but AuNPs have formed the basis of home pregnancy tests such as Carter Wallace’s “First Response” since the early 1990’s. Utilising the aggregation tendency of AuNPs, the tests combined micrometre sized latex particles and AuNPs functionalised with different epitopes of human chorionic gonadotropin antibodies. When the gonadotropin hormone present in the urine of a woman during pregnancy, contacts AuNPs within the sensor, the particles agglomerate forming visible pink aggregates [311]. Working via a similar principle, ImmunoCAP (Phadia, Inc) is a commercially available allergy test which uses whole blood samples to create an immunoglobulin E (IgE) profile. The test is used to differentiate allergy like symptoms (such as bronchospasm, rhinitis, conjunctivitis, eczema, angioedema, and nausea) and helps to provide a clinical diagnosis to a specific allergen. Preloaded with 10 common allergens including pet hair, pollen and dust mites, once blood is added, if IgG antibodies are present in the blood, they bind to the specific sections of the test strip containing the relevant allergen. The developer solution containing dried gold-anti-IgE conjugate is then released indicating an allergy to a particular trigger by forming pink/red lines due to the formation of complexes between the AuNPs and bound IgE antibodies. Similar principle are utilised by many other tests for detection of polynucleotides [312], proteins [313], antiproteins [314] and heavy metals [315].

Working via a different principal, SoPRano (ParmaDiagnostics, Belgium) uses localised surface plasmon resonance (LSPR) to detect and quantify binding effects which occur on the surface of AuNPs. Using negatively charged AuNRs, the system can be used to probe biomolecular interaction kinetics, antibody detection and blood brain barrier permeability. By first coating the rods with a biomolecule of interest and exposing them to different concentrations of analyte, the interaction causes a measurable difference in the absorbance value or an LSPR shift, which can be read using an absorbance plate reader.

As an alternative to methods which require a ‘lock and key’ approach to sensing (where specific recognition between analyte and receptor must takes place), the ‘chemical nose’ approach uses an array of AuNPs as receptors for protein analysis [316], and clinical diagnostics [317-319]). The technique uses the interaction between a fluorescent polymer and an array of AuNPs with various
surface characteristics (such as surface charge and surface functional groups). While the polymer’s fluorescence is quenched when attached to the AuNPs, once dissociated through competitive binding of proteins to certain AuNPs, a fluorescence pattern is generated, depicted in Figure 1.15. This pattern can be analysed to detect individual proteins and differentiate between protein species, giving both quantitative and qualitative data. The Rotello research group has made significant progress in this area, detecting and differentiating between bacterial strains [320], quantifying proteins [316, 321, 322], and identifying cancerous cells [323][324].

Another notable breakthrough in this area is the potential for diagnosis of lung cancer using the exhaled breath of subjects. While most published methods are performed with liquid samples (generally blood), the non-invasive method developed by Peng et al. requires the collection of exhaled air from patients to detect specific volatile organic compounds (VOCs) which have been linked to the presence of lung cancer at certain concentrations [318]. The group has since identified VOCs which are markers for breast, colorectal, prostate, head and neck cancers, and further developed exhaled breath analysis sensors for these VOCs with AuNP arrays [317, 319].

**Figure 1.15:** Schematic depicting a nanoparticle-conjugated polymer sensor array, wherein (a) anionic conjugated polymers bound on to cationic AuNPs are displaced by negatively charged bacteria surfaces, and (b) a fluorescent pattern is generated during the polymer displacement process by the bacteria. Reprinted with permission from [320].
1.5.2 Gold nanoparticles as therapeutic agents

The area of AuNPs as therapeutic agents is both diverse and rapidly growing. Researchers are attempting to treat HIV [325], bacterial infections [326], inflammation [327] and coronary diseases [328] with AuNP based therapies; however anticancer activity [12, 186, 190, 229, 329-339] forms the bulk of the research. While both auro (Au\(^{I}\)) and auric (Au\(^{III}\)) gold complexes are commonplace in pharmaceuticals [340, 341] [249, 342], the prevalence of AuNP (Au\(^{0}\)) based therapies is far less common. Presented in this section are the AuNP based therapies that are approaching or have progressed to clinical trial phase.

Possibly the first AuNP based therapy to reach (and pass) Phase I clinical trials, CYT-6091 is a 27 nm citrate stabilised AuNP surface functionalised with both tumour necrosis factor-\(\alpha\) (TNF-\(\alpha\)) and thiolated PEG [337]. The antitumor effects of TNF-\(\alpha\) (more specifically recombinant human TNF – rhTNF) have been known for decades; however its severe toxicity in humans has previously limited its dosage [339]. TNF-\(\alpha\) acts in multiple capacities acts as a proinflammatory cytokine, to disrupt tumour vasculature and when used in combination with chemotherapy, allows subsequent treatment to penetrate tumours more effectively eliciting greater results. The therapy first developed in 2004 by researchers as Aurimune by CytImmune Sciences Inc. (Rockville, MD, USA) [191] achieves circumvention of RES uptake, leading to increased circulation time in part due to its surface PEGylation, which was also shown to reduce the expected toxicity of rhTNF, and increased accumulation inside the tumours rather than in the surrounding tissues. This study used thiolated AuNPs as the vehicle for the therapy due to the strong binding affinity for rhTNF, noting that the AuNP bound rhTNF had a 5 fold longer half-life in plasma than native rhTNF. It was also noted that AuNPs restricted the biodistribution of the treatment, showing greater specificity for tumour sites, and were notably absent in healthy tissue. This allowed higher doses to be administered with reduced incidence of adverse side effects and systemic toxicity. After progressing through Phase I clinical trials, CYT-6091 is proposed to be next used to treat non-small lung cancer in combination with chemotherapy as part of Phase II trials [337].
Harnessing the SPR effects of AuNPs, researchers are employing plasmonic phototherapy to treat cardiovascular disease as an alternative to statin based therapy. Notably while statin therapy is successful in lowering cholesterol, its effectiveness in inducing atheroma (plaque) regression is less clear [343]. The research group led by Khalamov developed a plasmonic nanophotothermic treatment consisting of allogenic stem cells containing gold-coated silica nanoparticles [344]. The human study, which was preceded by an animal study using miniature swine [328], recruited 180 patients, of which 60 were given the silica-AuNP treatment to allow comparison to more conventional techniques. The nanoparticles were administered through a patch implanted into the affected artery and 7 days post-surgery, irradiation of the particles with near-infrared (NIR) irradiation took place (821 nm, 35–44 W/cm², 7 minutes). Due to the maximum light absorption by these particles in the NIR region, the absorbed photo-energy is converted into thermal energy, which in turn burns the surrounding tissues; in this case, the atheroma. This technique, which is referred to as nano-burning, saw success in Phase I trials with significant atheroma regression observed, though the second planned trial has been discontinued [345].

Cofounded by Naomi Halas, part of the team responsible for the invention of gold nanoshells for NIR therapy [346], Nanospectra Biosciences Inc. (Houston, TX) has focused their efforts over the past decade on the development of AuroLase therapy. The photothermal therapy which utilises AuroShell particles comprising of a 120 nm silica core surrounded by a 15 nm outer gold shell is used in combination with NIR irradiation to treat head and neck tumours. The nanoparticle treatment is delivered intravenously and shows accumulation in solid tumours due to the enhanced permeability and retention (EPR) effect of solid tumours because of their abnormally formed vasculature and poor drainage [347]. After sufficient accumulation of AuroShells (generally occurring 12-24 hours post-infusion), the area is illuminated by an 808 nm wavelength laser causing the nanoparticles to heat up, effectively burning the tumour. The FDA-approved pilot study was completed showing promising results with Phase II trials planned to target metastatic lung cancer [348].

Besides phototherapy, radioisotopes of gold have also been exploited for imaging and therapy of cancers. The low (Au¹⁹⁹: β_max - 0.46 MeV; half-life - 2.7 days), and moderate (Au¹⁹⁸: β_max - 0.96
keV; half-life 3.2 days) energy beta particles emitting isotopes, due to their desirable half-life characteristics offer ideal opportunities for radioimaging (Au\textsuperscript{199}) and therapy (Au\textsuperscript{198}). To circumvent the limitations associated with traditional metal based radiopharmaceuticals, nanoparticles of gold have been employed as radioactive probes for imaging and radiotherapy of solid tumours [349]. Several groups have demonstrated the utility of radioactive AuNP-based nanodevices in targeted radiopharmaceutical dose delivery to the tumours [53, 98, 350]. In a pioneering study, Shukla \textit{et al} recently reported a AuNP-based targeted radiopharmaceutical approach towards radiotherapy of prostate cancer [53]. This method demonstrated rapid, room temperature synthesis of \textsuperscript{198}AuNPs using epigallocatechin gallate (EGCG), a clinically-approved phytochemical derived from green tea. These EGCG-reduced radioactive AuNPs were amenable to facile manipulation of radioactive dosage within the nanoparticle matrix, offering a simple tool with potential for clinical translation. Since EGCG can target a 67 KDa receptor protein (Lam 67R) overexpressed on cancer cells, these nanoparticles exhibited selective uptake in prostate cancer cells. Furthermore, the nanoparticles showed remarkable stability in blood plasma and a desirable biodistribution profile in prostate tumour bearing mice, leading to an ~80% reduction in tumour volume. The levels of white blood cells, red blood cells, platelets and lymphocytes were found to be comparable to the control groups, thus supporting high therapeutic efficacy of these radioactive \textsuperscript{198}AuNPs.

1.5.3 Gold nanoparticles as bioimaging agents

The use of gold as an immunostaining agent was first documented in 1971 when Faulk and Taylor used antibody coated AuNPs to visualise specific antigens using TEM [351]. Relying on the ‘lock and key’ mechanisms of antibody-antigen interactions in combination with the high electron density properties of gold, immunolabelling methods have expanded with multiple labelling possibilities (using different sized AuNPs) [352], and adaptations have been made for visualisation using SEM [353, 354] and dark field microscopy [334].

The use of AuNPs as an alternative to iodine based contrast agents was discovered serendipitously by Wilhelm Röntgen in 1895 when taking an X-ray of a subject wearing a gold ring
Gold, both in its nano and macro forms, exhibits high X-ray attenuation due to its high electron density [356, 357]; however AuNPs offer additional enhanced permeability and retention effects inside tumour regions, unique SPR effects and are able to be surface functionalised to target specific tissues or organs, making it possible to combine imaging with therapeutic treatment. X-ray computed tomography (CT) imaging creates detailed structural scans of the body by exploiting the natural variation in X-ray absorption between different tissues [358]. The use of traditional contrast agents for CT imaging (typically iodine based) has allowed contrast to be created artificially in areas where it does not naturally exist. However, the downfalls with iodine-based CT contrast agents include low retention time, nephrotoxicity, thyroid gland abnormalities and anaphylaxis [359-361]. Further, the use of AuNPs as contrast agents – as shown in Figure 1.16, is not limited to CT scanning and can extend to optical coherence tomography [362] and photoacoustic tomography [363].

![Figure 1.16: CT images comparing Iopromide (commercial iodinated imaging agent) and AuNPs. Contrast agents are compared over a range of tube potentials (energy and intensity of the X-ray beam) with AuNPs showing superior contrast in all conditions tested. Reprinted with permission from [364].](image)

With sufficient work on the efficacy of AuNPs as bioimaging agents in vitro, many groups are testing AuNP based imaging agents on animal models in vivo [66, 141, 184, 258, 360]. CT imaging of the liver is an important tool for the diagnosis, treatment and monitoring of cancer, as it is a common site for secondary cancer presentation. However resolution of the liver’s microstructure is difficult, even with the use of conventional contrast agents [365]. Specifically, iodinated contrast agents exhibit low levels of liver uptake, demonstrating the demand for target (tissue or organ)
specific contrast agents. Sun and co-workers created AuNPs surface modified with heparin, a naturally occurring biomolecule with anticoagulant properties, to compare their performance with a commercial iodinated contrast agent. The heparin modified AuNPs displayed liver specificity [365, 366], allowing for differentiation of liver tissues and clear visualisation of vessels less than 1 mm in diameter. Comparatively, the iodine based agent provided markedly (3.2 fold) less contrast. The mice received a dosage of 200 mL of a 250 mg Au/kg solution in this study, after the AuNPs preparation were found to be biocompatible to human hepatocellular liver carcinoma (HepG2) cells up to levels of 100 mg/mL [365]. The authors acknowledge that while promising, extensive cytotoxicity testing is necessary for the progress of this technology.

Alternatively, researchers have used AuNPs to improve the short imaging window associated with iodinated CT contrast agents due to their low circulation times [360, 361]. By PEG-coating AuNPs to elude RES uptake, when injected into rats the PEGylated AuNPs demonstrated an X-ray absorption coefficient 5.7 times higher than a commercial iodinated CT contrast agent, as well as a blood circulation time 4 hours longer. While this study noted accumulation of AuNPs in the spleen and liver, there was no appreciable toxicity seen for up to one month, nor was toxicity observed after conducting an MTT test on human hepatocellular liver carcinoma (HepG2) cells using concentrations of the particles higher than the expected therapeutic dose levels [360].

To demonstrate this point further, Au et al. tested the circulation time of PEGylated AuNPs in vivo using mice. After injecting the mice with either PEGylated AuNPs or an iodinated contrast agent, the mice were CT imaged immediately and after 6 and 24 hours. While PEGylated rods showed clear contrast which continued to the last time point of the experiment at 24 hours, the iodinated contrast provided sufficient contrast immediately, failing at the subsequent 6 and 24 hour time points [367]. This study highlights the use of AuNPs for prolonged and delayed imaging of subjects which could be useful for real time imaging during procedures.

The ability to combine cancer diagnostics and therapeutics into a single process is a goal currently pursued by many research groups [331, 334, 336, 338, 368]. By harnessing the SPR
capabilities of AuNPs, the idea of imaging and delivering photothermal treatment using one diagnostic tool may become a reality in the near future. The research group led by El-Sayad employs gold nanorods of a chosen aspect ratio which strongly absorb and scatter light in the NIR region after surface modifying them with anti-epidermal growth factor receptor (anti-EGFR) monoclonal antibodies. Due to the high expression of EGFR on the surface of malignant cells, the rods were observed to have >2-fold higher uptake by malignant cells (oral epithelial cell lines HOC 313 clone 8 and HSC 3) versus non-malignant cells (human epithelial cells (HaCaT). Because of the increased uptake of AuNPs and thus strong light scattering capability, the malignant cells were clearly visible using dark field microscopy. Furthermore photothermal treatment using an 800 nm laser required half the dose to kill the malignant cells as compared with non-malignant cells. Similar results were obtained using folic acid functionalised AuNPs to target epithelial cancer cells by Bhattacharya et al. [229] and bombesin functionalised AuNPs to target breast and prostate cancer by Chanda et al. [178].

Inspired by such promising results, Niidome and co-workers performed an in vivo study of PEGylated and CTAB stabilised AuNRs to determine their cytotoxicity and biodistribution after intravenous delivery into mice. Using human cervical cells (HeLa) the cytotoxicity of PEGylated AuNRs was found to be very low, with ~90% cell viability at doses of 0.5 mM in contrast to the high cytotoxicity observed with CTAB stabilised AuNRs (which were only washed to remove free CTAB once). The group then injected the tail veins of mice with either PEGylated or CTAB stabilised AuNRs. After specific time points the animals were sacrificed, with their blood and organs collected and tested for gold content to determine the biodistribution of AuNRs. While CTAB stabilised rods were found primarily in the liver after 30 minutes (~30% of injected dose), <10% of the injected dose was present in the blood. PEGylated AuNRs in contrast had prolonged circulation time with 54% of the injected dose found in the blood after 30 minutes. This level decreased over time, until being completely removed from circulation at 72 hours. At this time 35% of the injected dose was found inside the liver while only a small amount was detected in the other organs tested (lung, spleen and kidney) [258]. This demonstrates the importance of surface functionalisation in terms of achieving controlled biodistribution of AuNPs, while supporting the efficacy of PEG for evading RES uptake of
AuNPs. In combination with PEGylation, targeted surface decoration of AuNPs could create tailored imaging and photothermal systems to treat various cancers.

1.6 Summary and outlook

Overall, this introduction has provided a critical analysis of different AuNP synthesis methods, while assessing relevant biological studies to highlight emerging trends in AuNP uptake and toxicity. Among different nanomaterials, AuNPs undoubtedly show some of the most interesting physico-chemical properties that make them useful for a number of biological applications including medical imaging [139, 141, 183, 184, 338], therapy [12, 53, 142, 179, 329, 338, 350], diagnostics [55, 180, 190, 194, 195, 369], biosensing [70, 370, 371] as well as gene [188, 192, 372-374] and drug delivery [142, 186, 189, 191]. Some of the major driving forces for their consistent interest in biomedical applications are that AuNPs appear to be well tolerated in biological systems causing minimal cytotoxicity, while providing opportunities for facile surface manipulations as well as exhibiting interesting optical properties. A major focus of this chapter has been to ignite a critical discussion around the influence of different physico-chemical properties of AuNPs on their biological activity. The ability to finely tune these properties of AuNPs by controlling the synthesis process puts great power in the hands of researchers by allowing them to tailor particles specifically for their intended purpose. While there seems to be some concerns about practical in vivo applicability of AuNPs due to potential metal accumulation in the body, such concerns have not been thoroughly validated through long-term in vivo studies. It is only recently that the importance of such studies has been recognised and the research community has more seriously started to investigate the influence of various in vivo factors such as spontaneous protein corona formation on nanoparticles in response to in vivo exposure. Among various aspects of research involving the use of AuNPs for nanomedicine, an in-depth understanding of dynamic biological corona formation on AuNP surface upon exposure to biological fluid, remains the cornerstone of achieving clinically translatable therapies. New knowledge gained from nanoparticle-protein corona dynamic interactions will not only lead the way forward for tailor designed AuNP-based in vivo therapies; they will also offer equally valuable
opportunities to take *in vitro* applications of AuNPs such as diagnostics to a commercialisation stage. It is foreseen that the impact of such studies on *in vitro* applications is likely to be more significant, as bioaccumulation and long-term cytotoxicity issues of gold outside the body are of least concern. Further, while the versatility of AuNP usage for biological applications remains undeniable, the scientific literature regularly shows conflicting outcomes in regards to AuNP-biological interactions. To overcome this inconsistency, a set of guiding principles need to be developed so that only appropriate assays are used to assess the toxicity of AuNPs; careful assessment of surface corona is performed before correlating biological action to nanoparticle surface chemistry; and the change in surface chemistry of AuNPs is carefully considered on exposure to biological fluids, whether under *in vitro* or under *in vivo* conditions. Such guiding principles will allow researchers to be able to predict the toxicity, uptake and action profile of AuNPs of different sizes, shapes and surface chemistry without necessarily warranting extensive testing. Overall, this Chapter has provided a critical analysis of different AuNPs synthesis methods, while assessing relevant biological studies to highlight emerging trends in AuNPs uptake and toxicity. It is hoped that this will ignite a critical discussion around new ways of assessing nanomaterial toxicity, while paying close attention to the influence of the biological corona formed on the nanomaterials surface after their exposure to the biological world.
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Chapter 2: Characterisation Techniques
2.1 Introduction

The research presented in this thesis can be divided into two main sections. The first of these sections details the synthesis methods used to create a library of varied gold nanoparticles. However the main aim of this thesis is to understand the behaviour of these gold nanoparticles at the biological interface. In pursuance of deeper knowledge of the effect that size, chemical nature and shape have on cellular uptake and toxicity, the particles must first undergo thorough characterisation in order to rationalise the effects observed. The characterisation techniques used include UV-Visible Absorption Spectroscopy (UV-vis spectroscopy), Dynamic Light Scattering (DLS), Fourier Transform Infrared Spectroscopy (FTIR), X-Ray Photoemission Spectroscopy (XPS), X-ray Diffraction (XRD), Transmission Electron Microscopy (TEM), Microwave Plasma Atomic Emission Spectroscopy (MPAES) and Zeta Potential analysis. The intention of this chapter is to explain the relevant principles on which each of these techniques is based.

2.2 UV-Visible Absorption Spectroscopy (UV-vis spectroscopy)

The electromagnetic spectrum represents the full range of light radiation; of which a small section is made up of ultra violet (UV) and visible radiation. UV-visible absorption spectroscopy is based around the interaction between matter and this part of the electromagnetic spectrum [1, 2].

Light interacts with molecules within a sample, promoting electrons from their ground state, to an excited energy state. These electronic transitions occur because valence electrons absorb discrete bundles of energy, i.e. photons, and move from a bonding or non bonding orbital to an anti-bonding orbital. The change in energy, \( \Delta E \), required for the electronic transition of a molecule can be described as:

\[
\Delta E = h\nu = \frac{hc}{\lambda}
\]

Equation 2.1
Where \( h \) is Planck’s constant \((h = 6.626 \times 10^{-34} \text{ J.s.})\), \( c \) is the speed of light \((c = 3 \times 10^8 \text{ ms}^{-1})\) and \( \lambda \) and \( \nu \) are the wavelength and frequency respectively, of the incident photon.

The wavelength and strength of energy absorbance is unique to each molecule and is influenced by its size, shape, chemical nature, surrounding environment and concentration [3]. To perform a measurement, the incident light is scanned through a range of wavelengths, with the light transmitted by the sample being detected. The difference between the intensity of the incident light, \( I_0 \), and exiting light, \( I \), is used to calculate the absorbance value, \( A \), at each wavelength. Using the Beer-Lambert law, this can be related to the concentration of molecules, \( c \), within the sample due to its proportional relationship with the light absorbed:

\[
\log_{10} \frac{I_0}{I} = \varepsilon cl = A
\]

Equation 2.2

Where \( \varepsilon \) is the molar absorption coefficient and \( l \) is the pathlength of the absorbing solution (cm).

UV-visible absorption spectroscopy has great pertinence to the analysis of nanomaterials due to the effects of surface plasmon resonance (SPR) [4]. The position and strength of absorption bands give both qualitative and quantitative information about nanoparticle samples. When nanoparticles interact with incident light, excitation of conduction band electrons occurs causing them to collectively oscillate and generate a sharp absorbance of light [5]. In the case of spherical gold nanoparticles (AuNPs) of sizes less than 60 nm, a single peak occurs at approximately 520 nm – as shown in Figure 2.1 [6], this is the result of oscillations along an axis of constant length which is expected for a shape of such symmetry.
In the case of anisotropic nanoparticles, such as rods which contain multiple axes, the existence of two surface plasmon resonance peaks is observed. The absorption profile for rods includes a peak at approximately 520 nm due to transverse oscillations or surface plasmons oscillating along the short edge of the rod, as well as a strong longitudinal plasmon band which is the product of surface plasmons oscillating along the long edge of the rod. The position and height of the longitudinal band is indicative of the length of the rods, with increased red-shift occurring with increasing aspect ratio and peak height increasing with yield. Similar effects are expected with all anisotropic metal nanoparticles, and this allows features such as shape and size to be examined in situ.

![Absorbance vs Wavelength](image)

**Figure 2.1: UV Visible spectra of different shaped gold nanoparticles (left) with corresponding TEM images (clockwise from top left: spherical, rod, cubic and prismatic AuNPs)**

For the work performed in this thesis, UV-visible absorption spectroscopy data was collected on a Cary 50 Bio Spectrophotometer, operating at a resolution of 1 nm over a wavelength range of 200-1100 nm.
2.3 Dynamic Light Scattering (DLS)

Dynamic Light Scattering (also known as Photon Correlation Spectroscopy or Quasi-Elastic Light Scattering) uses laser light to gain information relating to the shape and size distribution of colloidal particles [7, 8]. The laser’s oscillating electric field causes dipole oscillations of the molecules within the sample, causing them to scatter light in all directions.

In dynamic light scattering experiments, this light is collected by a detector, at a certain scattering vector \( \vec{q} \), defined as

\[
\vec{q} = \frac{4\pi n}{\lambda} \sin \left( \frac{\theta}{2} \right)
\]

\[ \text{Equation 2.3} \]

Where \( \theta \) is the scattering angle, \( n \) is a unit vector and \( \lambda \) the laser wavelength.

The scattering vector \( \vec{q} \) can be explained as the difference between the incoming light defined by propagation vector \( \vec{k}_i \) and the scattered light defined by propagating vector \( \vec{k}_f \) as demonstrated in Figure 2.2 [9].

![Figure 2.2: Schematic showing the geometry created after incoming light (\( \vec{k}_i \)) is scattered by the sample.](image)

As the illuminated volume can contain tens of thousands of particles, the scattered light rays from each particle will interact at the detector. As the particles are at different positions, the scattered light will have travelled different distances, and will therefore have different phases, leading to
constructive or destructive interference which is dependent on the position and orientation of the particles at a point in time. This interference will change as the particles move under Brownian motion, causing time dependant fluctuations in the intensity of the scattered light received by a detector. From these intensity fluctuations an intensity autocorrelation function can be formulated from which the diffusion coefficient D can be determined. If the liquid viscosity is known, the hydrodynamic radius of the particles can be determined using the Stokes-Einstein Diffusion equation:

$$D = \frac{K_B T}{6\pi \eta r}$$  \hspace{1cm} \text{Equation 2.4}$$

Where D is the diffusion coefficient, r is the particles radius, $K_B$ is the Boltzmann’s constant, T is the temperature in Kelvin, and $\eta$ is the viscosity of the solvent [8, 9].

It is important to note that in this context, radius refers to the hydrodynamic radius of the particles. In the case of particles which differ from the model of a hard sphere, the hydrodynamic radius is often larger than the radius value determined by direct imaging techniques such as electron microscopy. This is due to the interpretation of quasi-spherical particle movement within a dynamic system as well as interactions which may take place between molecules in the surrounding liquid and the particle surface.

After fitting the autocorrelation function, information relating to the average particle size, particle size distribution and polydispersity index (a measure of the broadness of the particle size distribution) can be extracted. For AuNPs prepared by chemical methods, some degree of polydispersity must be expected. Variables within the synthesis procedure (most notably the rate of reaction) have a large influence on the particle size distribution as shown in Figure 2.3.
Figure 2.3: Particle size distributions of two different AuNPs. Particle A is prepared in a rapid in situ synthesis resulting in a narrow particle size distribution in contrast to particle B, synthesised using a slower seed mediated approach which results in a broader particle size distribution.

An ALV– 5022F FAST correlator (ALV, Langen, Germany) was used to conduct all dynamic light scattering measurements. The system was equipped with a 22 mW, 632.8 nm He–Ne laser as well as an avalanche photodiode detector configured to collect scattered light at an angle of 90°. The sample was held at 22 °C by a temperature regulated sample holder (Digital Plus Refrigerated Bath – Thermo Scientific). To minimise vibration, static and temperature interference, the system was mounted on an optics bench fitted with a Newport RS-2000 laminar flow stabilizer unit.

2.4 Fourier Transform Infrared Spectroscopy (FTIR)

Vibrational spectroscopy techniques such as Fourier transform infrared spectroscopy (FTIR) examine the atomic vibration that occur in organic and inorganic materials after exposure to infrared light [10]. When a sample is exposed to electromagnetic radiation (at a wavelength or wavenumbers of 2.5 – 25 μm or 400-4000 cm⁻¹, respectively), the energy is transmitted or absorbed, with absorbance giving rise to enhanced vibrational movement at specific wavelengths. The wavelength at which this occurs is important, and indicative of the nature of the molecule being examined.
Atoms within a molecule are constantly in motion, displaying normal vibrations whereby the atoms exhibit simple harmonic movement around a mean position. Each molecule, depending on the number of atoms, N, that it contains, is capable of a set number of vibrational modes, $3N-5$ for linear molecules and $3N-6$ for non-linear molecules [11]. The frequency of an atom’s normal oscillations is dependent on two major factors: atomic weight - atomic bonds joining light atoms vibrate at a higher frequency as compared with bonds between heavier atoms; and bond length and force – stronger bonds resonate at higher frequencies - given by the following equation:

$$v = \frac{1}{2\pi} \sqrt{\frac{k}{\mu}}$$

Equation 2.5

Where $\mu$ is the reduced mass and $k$ is the force constant.

When a molecule encounters a photon with a frequency that is equal to its own unique normal oscillation frequency, it absorbs that photon. For this to occur however, the molecule must contain a dipole which oscillates during the vibration to confer the molecule’s infrared activity [12]. The absorbance is visualised as a function of wavenumber, a measure of the number of waves occurring in one centimetre, which is inversely proportional to wavelength.

The position of absorbance bands can be related to the presence of specific functional groups within the sample. All molecules have a fingerprint region of absorbance, thus seeing absorbance in a fingerprint region is a step in proving the existence of a particular bond within the sample.

While FTIR is used to gain information about the metal particle itself, it is a useful tool for nanoparticle characterisation to probe surface functionalisation properties. For surface functionalised or capped nanoparticles, information such as the extent of proteins binding [13], or the arrangement of surfactant molecules on the particles surface can be determined [14, 15].

While conventional IR spectroscopy techniques use monochromators to pass light through the sample one wavelength at a time, FTIR has the advantage that it uses an interferogram, so that all relevant frequencies of light are passed through the sample concurrently. This minimises the inherent signal to noise ratio which plagues other spectroscopic techniques by allowing many interferograms to
be performed over a short period of time. A Fourier transform (or more specifically a simplified fast Fourier transform (FFT) algorithm) is then used to transform the interferogram (which is a function of intensity versus time), to a function of intensity versus wavenumber as shown in Figure 2.4 [12].

**Figure 2.4:** *Example of the application of a fast Fourier transform (FFT) to translate an interferogram to a FTIR spectrum prior to analysis.*

The FTIR measurements contained in this thesis were collected on a Perkin Elmer Spectrum 100 instrument fitted with a Spotlight 400 attachment. Results were collected and averaged from 64 scans conducted over a range of 4000-750 cm\(^{-1}\) with a resolution of 4 cm\(^{-1}\).

## 2.5 X-ray Photoelectron Spectroscopy (XPS)

Often referred to as Photo Emission Spectroscopy (PES) or Electron Spectroscopy for Chemical Analysis (ESCA), X-ray photoelectron spectroscopy (XPS) is a surface spectroscopic technique that delivers elemental, compositional and chemical state information pertaining to the outermost layers of a surface.

The technique is based on the principles of the photoelectric event which describes the emission of electrons after a metal is irradiated with light [16, 17] - photon in/electron out - the discovery of which earned Albert Einstein the Nobel Prize in 1921 [18].
In XPS, a sample is irradiated with a monochromatic beam of soft x-rays with energy \((hv)\) of either Mg Kα, 1253.6 eV, or Al Kα, 1486.6 eV under ultra high vacuum. The incoming energy is absorbed by atoms within the sample, causing atoms to be ionised \((A)\), ejecting a photoelectron from a core or valence electron with some kinetic energy \((E_k)\). Photoionization proceeds via the following equation:

\[
A + hv = A^+ + e^- \tag{Equation 2.6}
\]

The energy of the emitted photoelectron is detected, and from this the binding energy \((E_b\) - the difference in energy between an atom’s charged and neutral states) is determined via the following equation:

\[
E_k = hv - E_b - \phi \tag{Equation 2.7}
\]

Where \(\phi\) is the work function of the spectrophotometer.

Because the electron configuration of each element is unique to atoms of that element, the elemental species can be determined based on the binding energy of the ejected electrons. However the binding energy of an electron is very sensitive to its chemical environment (in particular its proximity to strongly electronegative or electropositive species) and chemical shifts or subtle movement in the expected peak positions can occur. By noting these small shifts, inferences on the oxidation state and chemical environment of the sample can be drawn [11].

XPS measurements presented in this thesis were collected on a Thermo Scientific K-alpha X-ray Photoelectron Spectrometer at a pressure less than \(1 \times 10^{-9}\) Torr. An example spectrum is presented in Figure 2.5. Scans were collected using un-monochromatized Mg Kα radiation (photon energy of 1253.6 eV) at pass energy of 50 eV and electron take off angle of 90°. The overall resolution of all XPS experiments was 0.1 eV. Binding energy values collected were aligned with the C1s binding energy of 285 eV, the background data was corrected with the Shirley algorithm and chemically distinct species resolved using a nonlinear least squares fit.
2.6 X-ray Diffraction (XRD)

X-ray diffraction is an analytical technique capable of determining the crystalline structure or atomic arrangement of a material [19]. When x-rays interact with atoms, the electrons absorb the X-ray photons, and then reemit the X-rays in all directions. If the atoms are arranged randomly (such as in amorphous materials), the scattering X-rays will interfere to give an intensity vs. angle curve which is broad and relatively featureless. However if the X-rays interact with a crystalline material (which inherently is formed from periodically arranged atoms), constructive interference will occur at certain fixed angles – in other words, light is diffracted [20].

While all electromagnetic radiations are theoretically capable of inducing diffraction of regularly spaced atoms, using a wavelength comparable to the atomic spacing produces the strongest diffraction patterns accessible over a broad range of scattering angles. The position and intensity of the diffracted peaks contain information relating to the spacing of the parallel atomic planes and the arrangement of those atoms, respectively [20].

Because of the regular spacing of atoms within a crystal, diffracted beams contain a multitude of scattered rays which reinforce each other; as reflected x-rays originating at the surface of the crystal travel a shorter distance but are in phase with waves originating from an internal plane. The
difference in this path length, represented in Figure 2.6, is dependent on the spacing between the layers of atoms (d), and the angle of the incident x-rays (θ) described by the Bragg equation:

\[ n\lambda = 2d \sin \theta \]  

Equation 2.8

Where \( n \) is an integer number of wavelengths (\( \lambda \)).

Using Bragg’s equation, the atomic spacing (d), can be determined from the diffraction pattern. The diffraction pattern obtained is uniquely related to the arrangement of atoms within the material, hence, graphite and diamond produce different diffraction patterns, despite being both being pure carbon forms [21].

![Figure 2.6: Schematic showing the parameters of the Bragg equation](image)

Its use in nanoparticle characterisation can give an indication of purity or the presence of contaminants in the particle [22] as well as provide crystal structure information as shown in Figure 2.7 [23, 24].
Figure 2.7: Example of XRD pattern generated by gold nanoparticles.

XRD results presented in this thesis were gathered using a Bruker AXS D8 Discover with a General Area Detector Diffraction System (GADDS) micro diffraction instrument operating at 40 kV and 40 mA over an angular - 2θ range of 15-90°.

2.7 Transmission Electron Microscopy (TEM)

While techniques such as DLS and UV-vis spectroscopy are useful for the information they provide related to shape and size of nanoparticles, they are best used in conjunction with a direct imaging technique such as transmission electron microscopy (TEM) [25].

While light microscopes have resolutions limited by the wavelength of visible light, electron microscopy techniques use a focussed beam of electrons as an illumination source due to their much shorter wavelength. The wavelength (\(\lambda\)) of an electron is not fixed, but dependant on its accelerating voltage (\(V\)) given by this equation:

\[
\lambda = \frac{h}{\sqrt{2meV}}
\]

Equation 2.9
Where \( h \) is Planck’s constant \( (h = 6.626 \times 10^{-34} \text{ J.s.}) \), \( m \) is the electron mass \( (m = 9.109 \times 10^{-31} \text{ kg}) \), and \( e \) is the electronic charge \( (e = 1.6 \times 10^{-19} \text{ C}) \).

Hence using higher accelerating voltages results in shorter wavelengths making the resolution \( (d) \) achievable greater via Abbe’s equation:

\[
d = \frac{0.61\lambda}{n \sin a}
\]

Equation 2.10

Where \( n \) is the refractive index of the medium and \( a \) is the aperture angle.

TEM works by illuminating a thin sample with the electron beam under high vacuum conditions [26]. As the electrons pass through the column of the microscope, they are guided with electromagnetic lenses (as opposed to optical lenses used in light microscopy) to create a focussed beam. As the bean interacts with the sample, a portion of the electrons pass through the sample with their angle unchanged (referred to as the direct or transmitted beam), with the remaining electrons being scattered [27].

The unscattered electrons help to determine the thickness of the sample, with thicker areas transmitting fewer electrons and appearing as darker regions in the image. The scattering encompasses two different effects; elastic scattering – scattering with no effective loss of energy - and inelastic scattering – scattering which results in a loss of energy. Elastically scattered electrons give information relating to atomic arrangement, working on a similar principle to XRD. When the scattering results in a change of trajectory of the scattered electrons, but encounters areas with regular repeating arrays of atoms, the electrons are scattered by the same angle revealing diffraction patterns [27].

Inelastic scattering (often termed energy lost scattering) is valuable for probing the chemical properties of the specimen in more complex microanalytical modes, but this type of scattering also provides contrast information, with higher density materials exhibiting higher scattering probability than low density regions which appear lighter [27].
In TEM, an image is generated by collecting all of the electrons from the area below the sample, with shading and contrast possible from the scattering events described above. All TEM images presented in this thesis, including those shown in Figure 2.1 were obtained using a Jeol 1010 transmission electron microscope operating an accelerating voltage of 100 kV, fitted with a Gatan Orius charge coupled device camera.

2.8 Microwave Plasma Atomic Emission Spectroscopy (MPAES)

Microwave plasma atomic emission spectroscopy or flame analysis is a quantitative technique used to measure the concentration of a specific element within a sample. The sample is atomised via a peristaltic pump after which it is excited by a microwave induced nitrogen plasma source. Figure 2.8 shows how additional energy causes excitation of gold atoms to a higher energy state followed by a subsequent emission of radiation. The emission radiation can be quantified by measuring its intensity at a particular resonant wavelength (267.595 nm for gold) [28]. Such emissions, or more specifically high energy atomization emission lines, are discrete packets of energy and thus unique to a particular element.

![Figure 2.8: Schematic depicting the generation of MP AES signal.](image)

Following similar principles as inductively coupled plasma atomic emission spectroscopy (ICPAES), the technique differs by abandoning the conventional radio frequency inductively coupled plasma in favour of a magnetic plasma source operating at 2.455 GHz [29]. Using this technique,
elemental concentration can be accurately estimated based on the radiation emission intensity, which increases with an increasing number of atoms [30].

Each solution of AuNPs as well as five gold standard solutions were introduced to the instrument three times, and each reading recorded. The concentration of elemental gold was determined by using the mean value of the readings and interpolating from the standard curve established as shown in Figure 2.9.

![Figure 2.9: Calibration of gold standards to determine concentration of gold in AuNP samples.](image)

All atomic emission spectroscopy measurements were carried out using an Agilent 4200 MP-AES.

### 2.9 Zetasizer

When any particle is in solution, a net charge exists at the particle’s surface. The particles' net charge dictates the surrounding environment by drawing in ions of the opposing charge due to the electric double layer effect. These ions form a liquid layer around each particle which consists of two distinct regions. The inner layer – or the Stern region - is made up of strongly bound ions; while the outer layer – or diffuse region - consists of ions which are comparatively more weakly bound [31, 32].
The diffuse layer can be probed for information relating to the particle’s stability in its surrounding environment over time. Within the outer layer, the ions and particle form a stable unit, creating a barrier (called the surface of hydrodynamic shear or slipping plane) between the layer and the surrounding bulk dispersant. The potential at this barrier is the zeta potential and its magnitude (regardless of charge) is indicative of stability due to the large magnitude of repulsive forces. Zeta potentials are measured by applying an oscillating field, and then measuring the response of the particle’s scattered light using techniques similar to DLS to generate a plot such as the example shown in Figure 2.10.

![Figure 2.10: Example of a zeta potential plot generated by CTAB stabilised AuNPs.](image)

All atomic zeta potential measurements were carried out using folded capillary zeta cells (DTS1070) using a Malvern Zetasizer Nano ZS instrument at pH 7.
2.10 References


Chapter 3: Creating a Nanoparticle Library: Synthesis and Characterisation
3.1 Introduction

The quantity of published material detailing the synthesis of gold nanoparticles (AuNPs) [1-3] is constantly increasing. As such, the increased level to which researchers are able to manipulate material on the nanoscale has seen a significant expansion in the variety of nanoparticles synthesised. Unfortunately, the advance in AuNP synthesis capabilities has not been matched by our knowledge of their biological effects [4]. The variables that can be modified during the synthesis process extend far beyond the core element from which the nanomaterial is derived and includes shape, size, chemical nature, charge, porosity and crystal structure [1, 5-7]. While a comparison of nanoparticles with deliberately modified variables in itself is complex, it is worth noting that further changes arise depending on the surrounding environment of the nanoparticle. These variables include aggregation state, stability, protein corona composition, surface charge, SPR profile, shape and size [8]. While there are many studies which focus on single or small groups of gold nanoparticles [9-12], this practice has not allowed us to form a definitive picture relating to the toxicity of gold nanomaterials. We are currently unable to predict the toxicity of gold nanoparticles due to a lack of knowledge of what modifications to each variable mean in terms of toxicity and uptake in mammalian cells.

Researchers predict that shape, size and chemical composition are important parameters influencing gold nanoparticle toxicity; however the trends relating to each variable are unclear [8, 13]. To date, there does not appear to be a clear relationship between toxicity and nanoparticle size, but rather a fluid relationship which may involve complex cellular uptake mechanisms and interface dynamics.

To piece together the “big picture” of gold nanoparticle toxicity, it is necessary to systematically compare multiple nanoparticles which differ in only one variable at a time. While there exists much literature comparing groups of nanoparticles, often the nanoparticles differ in multiple variables, making it difficult to discern the mechanism responsible for the effects observed.
Notably, the vast majority of literature related to gold nanoparticle uptake and toxicity relates to spherical gold nanoparticles (AuNS) – which generally speaking are the most straightforward particles to make [14, 15]. For this reason they are often the model system used for nanoparticle studies irrespective of the element being observed. Next most common are rod shaped gold nanoparticles (AuNR) – of particular interest to researchers for their complex optical properties which could be harnessed as a useful biological imaging tool [10, 16].

Despite advances in the synthesis of different shaped gold nanoparticles, this degree of progress has not fully extended into the field of nanotoxicology. While there exists a large quantity of literature detailing the toxicity of various spherical gold nanoparticles, we are yet to fully explain the impact of different synthesis methods on these outcomes. Similarly, the synthesis of popcorn shaped, hexagonal, cubic, triangular, wire and branched gold nanoparticles have been described extensively in literature, yet the majority of these shapes are yet to be extensively tested in vitro. With the gap in knowledge evident, a systematic approach to this research combining controlled synthesis methods and extensive biological testing is the objective of this Thesis.

In this chapter the synthesis methods for all nanoparticles utilised in the subsequent chapters of this Thesis have been described. Each nanoparticle synthesis method was optimised and prepared for characterisation via the methods detailed below.

### 3.2 Synthesis and Characterisation of Chemically Distinct Small Spherical Gold Nanoparticles

#### 3.2.1 Rationale

It is hypothesised that changes in the chemical composition of elementally similar nanoparticles can create a difference in their toxicity profile [17, 18]. The exact mechanism behind this argument remains unclear; however chemical differences at the surface of the nanoparticle are known to give rise to variations to the particle's protein corona and biological identity [8].
To demonstrate the effect of different chemical coatings, four gold nanoparticles were synthesised using different chemical routes while keeping size and shape constant to allow comparison.

Of the four synthesis methods chosen, three are representative of commonly studied small spherical gold nanoparticles—tyrosine, tryptophan and citrate AuNPs. The fourth particle—a citrate stabilised, CTAB coated AuNP was included to provide a point of reference for the shape dependant study which follows.

### 3.2.2 Materials

Cetyl trimethylammonium bromide (CTAB) (Sigma H9151), tyrosine (TYR) (Sigma T8566), tryptophan (TRP) (Sigma T0254), trisodium citrate (Na₃C₆H₅O₇) (Aldrich 39,807-1), and tetrachloroauric acid (HAuCl₄) (Sigma G4022) were purchased from the supplier shown and used without further modification unless indicated.

All reactions were carried out using Milli-Q water, using glassware washed with aqua regia and rinsed thoroughly with deionised water.

### 3.2.3 Experimental

#### 3.2.3.1 Preparation of Citrate Stabilised Spherical Gold Nanoparticles

The method described by Li et al. was employed for synthesising concentrated citrate stabilised gold nanoparticles [19].

In a typical synthesis, 2.0 mL of 25 mM HAuCl₄ was mixed with 7.7 mL of 20 mM NaOH before bringing the total volume up to 20 mL with the addition of 10.3 mL of MilliQ water. The tube was placed in a thermomixer set at 85 °C and allowed to come to temperature. Following this, 600 μL of 0.0283 M trisodium citrate (Na₃Cit) was injected into the solution while vigorously stirring after which the temperature and stirring were maintained for a further 15 minutes. The solution was allowed to stabilise for a further 24 hours at room temperature. The method described produced
sufficiently concentrated AuNPs and as such, this sample did not undergo any further concentration prior to use.

3.2.3.2 Preparation of Tryptophan Stabilised Spherical Gold Nanoparticles

A modified version of the method described by Daima et al. was used to create both tryptophan and tyrosine reduced AuNPs [20]. In a typical synthesis for tryptophan reduced gold nanoparticles, 92 mL of Milli-Q water, 1 mL of KOH and 5 mL of 0.01 M tryptophan were added to a 250 mL conical flask with magnetic stirrer. The flask was heated while being rapidly stirred for approximately 4 minutes, before 2 mL of 0.01 M H\(\text{AuCl}_4\) was added. The solution was left to stir on the heat until it turned a red wine colour (approximately 15 minutes), after which the heat was turned off and the solution allowed to stir for a further 10 minutes. Following this, the flask was sealed with plastic paraffin film and allowed to stabilise for a further 24 hours.

To concentrate the sample, the solution was rotary evaporated under reduced pressure at 40 °C until the desired concentration was achieved.

3.2.3.3 Preparation of Tyrosine Stabilised Spherical Gold Nanoparticles

In a typical synthesis, 92 mL of Milli-Q water, 1 mL of 0.1 M KOH and 5 mL of 1 mM tyrosine were added to a 250 mL conical flask containing a magnetic stirrer. The flask was heated while being rapidly stirred for approximately 4 minutes, before 2 mL of 0.01 M H\(\text{AuCl}_4\) was added. The solution was left to stir on the heat until it turned a red wine colour (approximately 15 minutes), after which the heat was turned off and the solution allowed to stir for a further 10 minutes. Following this, the flask was sealed with plastic paraffin film and allowed to stabilise for a further 24 hours.

To concentrate the sample, the solution was rotary evaporated under reduced pressure at 40 °C until the desired concentration was achieved.
3.2.3.4 Preparation of CTAB/citrate Stabilised Spherical Gold Nanoparticles.

In a typical synthesis, citrate stabilised gold nanoparticles were synthesised by adding 100 mL of 0.25 mM HAuCl₄ solution into a 250 mL conical flask and bringing the solution to a boil while stirring. To the boiling solution, 0.7 mL of 0.17 M sodium citrate solution was added before being left to stir on the heat until the solution turned a purple hue, before progressing to red wine colour (approximately 10 minutes), after which the heat was turned off and the solution allowed to stir for a further 10 minutes. Following this, the flask was sealed with plastic paraffin film and allowed to stabilise for a further 24 hours.

Following this period, a 10 mL solution of 0.001 M CTAB solution was prepared and stirred while the entire volume of the previously prepared citrate AuNP solution was added drop wise over a period of approximately 10 minutes. The final solution was allowed to stabilise for a further 24 hours. To concentrate the sample, the solution was rotary evaporated under reduced pressure at 40 °C until the desired concentration was achieved.

3.2.4 Preparation of AuNPs for Characterisation

The concentrated nanoparticle samples were dialysed (citrate, tryptophan, tyrosine and CTAB/citrate AuNS) or centrifuged (all CTAB stabilized nanoparticles) to ensure removal of unreacted species prior to characterisation.

For dialysis, tubing (Sigma D9527) with a molecular cut off weight of 14000 was used to purify the AuNPs by allowing the removal of unreacted chemicals. Prior to use, the dialysis tubing was rinsed well in deionised (DI) water before being boiled in DI water for approximately 5 minutes. Following this, the tube was rinsed again before being filled with the nanoparticle solution and ends clamped.

The sealed dialysis bag was then placed in a 2 L beaker filled with Milli-Q water, with a magnetic stirrer and allowed to dialyse for 24 hours. During this time, 2 further water changes (at 3 hours, and again at 10 hours) were performed. In the case of CTAB/citrate gold nanospheres, a shorter
dialysis period of 6 hours was necessary due to agglomeration of the particles during prolonged
dialysis.

To prepare the samples for UV-Visible absorption spectroscopy, DLS and zeta potential
measurements, the AuNP solutions were diluted with Milli-Q water such that their absorption peak
was ≃ 1 optical density unit prior to reading.

To prepare the samples for XRD and XPS, the concentrated nanoparticle solutions were drop
cast onto a silicon wafer. The samples were layered by carefully dropping ~10 µL onto the same spot
on the wafer three times successively, allowing the nanoparticles to dry before each reapplication.

To prepare the samples for FTIR analysis, the concentrated nanoparticle solutions were drop
cast onto a silver substrate and allowed to dry prior to analysis.

To prepare the samples for TEM, ~ 3 µL of concentrated nanoparticle solution was drop cast
onto lacey carbon support grids and allowed to dry before imaging.

3.2.5 Results and Discussion

3.2.5.1 UV-Visible Spectroscopy of Chemically Distinct Spherical Gold Nanoparticles

Generally speaking, gold nanospheres (AuNS) of sizes less than 60 nm show a maximum absorbance
around 520 nm [21]. The sharp absorbance of light in this region is generated by the collective
oscillation of conduction electrons on the surface of the nanoparticle - commonly known as surface
plasmon resonance (SPR) [22]. For AuNS, a single peak is expected solely due to transverse
oscillations, however a decrease in the symmetry of the particles (i.e. quasi spherical particles) can
cause broadening or shifting of the peak [23]. In addition to this, the position of this peak is sensitive
to many other factors including the size, geometry, surface characteristics and aggregation state of the
particles, in addition to the refractive index of the surrounding medium [22, 24].

Figure 3.1 shows the UV-Visible spectra of the four chemically distinct AuNS. All spectra show peak
maxima between 520-530 nm which is in agreement with expected values from literature [19, 20, 25]
Citrate AuNS produced a sharp absorbance of light at 520 nm consistent with a sample of low polydispersity in this size range. Tryptophan AuNS generated an absorbance maximum of 530 nm, with a notably broader peak when compared with citrate AuNS. The slight redshift observed (or a shift toward higher wavelengths) is generally attributed to a small increase in size, while the broadening is generally associated with polydispersity (wider size distribution) or aggregation within the sample [26]. Both the tyrosine and CTAB/citrate stabilised AuNS show typical absorbance spectra with maximum absorption for both particles at 525 nm. Both samples show slightly broadened peaks as compared with citrate AuNS suggestive of higher size polydispersity [26].

SPR is very sensitive to changes in the external environment of the nanoparticle, with increases in the refractive index of the surrounding medium causing redshift of the absorbance maxima. Notably tryptophan has the highest refractive index at 1.485 [27] of all the stabilisers used (citrate 1.39 [28], tyrosine 1.47 [29], CTAB 1.43 [30]), which provides further justification for its absorption maximum, towards higher wavelengths.
3.2.5.2 Dynamic Light Scattering Measurements of Chemically Distinct Spherical Gold Nanoparticles

All AuNS were further characterised using dynamic light scattering (DLS) to obtain information relating to the size of the nanoparticles within solution. Unlike TEM which determines the physical size of the particles in a dried sample, by visualising the projected area diameter, DLS provides measurements related to the apparent size of the particles moving in solution, or the hydrodynamic radius [31]. The hydrodynamic size is defined as the size of a hypothetical hard sphere that diffuses at the same rate as the particles being characterized and is calculated using the Stokes – Einstein relation [32]. While TEM accounts only for the physical or core size of the particles, the size obtained using DLS includes any species (ligands, proteins, molecules, ions etc.) which are associated with the surface of the particle. For this reason in part, DLS measurements are typically larger than those obtained using TEM [31, 33]. Furthermore, sample polydispersity causes larger particle size determinations as larger particles will scatter more light as compared to smaller particles, dominating the DLS signal.

Figure 3.2 shows the particles size distributions of citrate, tryptophan, tyrosine and CTAB/citrate AuNS as determined by dynamic light scattering. The average hydrodynamic radii of the particles were found to be 10 nm (diameter equal to 20 nm), 10.5 nm (diameter equal to 21 nm), 10.5 nm (diameter equal to 21 nm) and 13.5 (diameter equal to 27 nm) respectively. As expected, these hydrodynamic values are larger than the physical values obtained using TEM, as detailed later in Section 3.2.5.3.
Figure 3.2: Dynamic light scattering size distributions of (clockwise from top left) citrate, tryptophan, tyrosine and CTAB/citrate stabilised AuNSs.

3.2.5.3 Transmission Electron Microscopy analysis of Chemically Distinct Spherical Gold Nanoparticles

TEM images of each AuNS are presented in Figure 3.3 providing fair representation of each sample. All samples appear not to be aggregated which is in agreement with UV-Visible and DLS profiles.

Figure 3.3: TEM images representative of (a) citrate AuNS, (b) tryptophan AuNS, (c) tyrosine AuNS and (d) CTAB/citrate AuNS.
There is some degree of polydispersity within all samples which is presented using particle size distribution histograms in Figure 3.4. At least 100 particles were analysed to determine average particle diameters as well as the standard deviation (SD) for each sample. These measurements are shown in Table 3.1 alongside the hydrodynamic diameter as determined by DLS for comparison.

Figure 3.4: Particle size distribution histograms of (a) citrate AuNS, (b) tryptophan AuNS, (c) tyrosine AuNS and (d) CTAB/citrate AuNS.

<table>
<thead>
<tr>
<th></th>
<th>TEM - Average diameter ± SD</th>
<th>DLS - Hydrodynamic diameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrate AuNS</td>
<td>12.5 ± 1.0 nm</td>
<td>20 nm</td>
</tr>
<tr>
<td>Tryptophan AuNS</td>
<td>8.4 ± 1.7 nm</td>
<td>21 nm</td>
</tr>
<tr>
<td>Tyrosine AuNS</td>
<td>9.9 ± 1.5 nm</td>
<td>21 nm</td>
</tr>
<tr>
<td>CTAB/citrate AuNS</td>
<td>8.9 ± 7.6 nm</td>
<td>27 nm</td>
</tr>
</tbody>
</table>

Table 3.1: Comparison of average particle diameters as determined by TEM and DLS.

3.2.5.4 X-ray Diffraction studies of Chemically Distinct Spherical Gold Nanoparticles

Generally speaking, gold crystallites -as with most metals- exhibit a face centred cubic (fcc) structure [2]. The fcc unit cell can be visualised as a cube with all sides having equal length and all faces perpendicular. The fcc structure is defined by having one atom located in each corner as well as one in the centre of each face of the unit cell as pictured in Figure 3.5.
The XRD results shown in Figure 3.6 confirm the presence of crystalline structures by showing well defined Bragg reflections corresponding to (111), (200), (220), (311), and (222) planes which are commonly associated with fcc bulk gold [34]. The sharp unassigned peak (prevalent in the tryptophan sample) is most likely attributed to the formation of polytryptophan during AuNP synthesis, as previously observed through electrochemical studies [35]. As expected for spherical particles, growth along the (111) facet is favoured for all samples as determined by peak area comparison [36].
3.2.5.5 Fourier Transform Infrared Spectroscopy Analysis of Chemically Distinct Spherical Gold Nanoparticles

Trisodium citrate (Na$_3$Ct) is utilised as both a reducing agent and stabiliser in the synthesis of spherical gold nanoparticles – structure shown in Figure 3.7. The reaction, which is dependent on variables including temperature, pH and the ratio of gold ions to citrate concentration [19], produces AuNPs which are stabilised by the attachment of citrate anions to the nanoparticle surface via terminal carboxylate group linkage [37].

![Trisodium Citrate](image)

**Figure 3.7: Structure of trisodium citrate.**

Figure 3.8 compares the functional groups present in (Na$_3$Ct) with those on the surface of citrate stabilised AuNPs (Citrate AuNS). The Na$_3$Ct spectrum shows characteristic asymmetric and symmetric stretching vibrations of the carboxylate group at 1573 and 1394 cm$^{-1}$ respectively. In the case of citrate AuNS the $\nu_{as}$(COO$^-$) and $\nu_s$(COO$^-$) peaks are shifted to 1582 cm$^{-1}$ and 1403 cm$^{-1}$ respectively, as denoted by blue stars in Figure 3.8. This demonstrates the adsorption of the terminal carboxyl group with the particle surface.

The $\nu_{as}$(COO$^-$) peak at 1582 cm$^{-1}$ obscures other peaks in this region where various hydrogen bonds including the bending vibration of water molecules adsorbed to the surface of metal nanoparticles are generally observed. This can be observed through the broadened shoulder of the $\nu_{as}$(COO$^-$) peak in the citrate AuNS as compared with (Na$_3$Ct). The broad and strong absorption band observed at 3370 cm$^{-1}$ for both (Na$_3$Ct) and Citrate AuNS is due to hydrogen bonded hydroxyl groups. The change in appearance (but not its position) may be due to a slight shift of the $\nu$(OH)$_{COOH}$ peak.
(which is expected around 3500 cm$^{-1}$) after coordination of the terminal carboxyl group with the AuNP surface.

![FTIR spectrum of trisodium citrate solution for comparison with citrate AuNS (a) and enlarged area from 1350 – 1650 cm$^{-1}$ to show shifted peaks (b).](image)

**Figure 3.8:** FTIR spectrum of trisodium citrate solution for comparison with citrate AuNS (a) and enlarged area from 1350 – 1650 cm$^{-1}$ to show shifted peaks (b).

Tryptophan (TRP), one of the essential amino acids, can be used to synthesise spherical gold nanoparticles that are often regarded as biocompatible [38]. Like all amino acids it comprises an amino and carboxyl groups, however in addition to this, tryptophan is an aromatised amino acid, and as such contains an indole ring on the side chain of the molecule as shown in Figure 3.9.

![Structure of tryptophan.](image)

**Figure 3.9:** Structure of tryptophan.

The FTIR spectra of tryptophan and tryptophan AuNS are presented in Figure 3.10. In the mechanistic study performed by Sastry *et al.* the indole group was reported to be responsible for the reduction of chloroauric acid during nanoparticle synthesis [25]. The group also determined that tryptophan molecules were likely attached to the surface of the nanoparticle via a coordination complex with the primary and secondary (pyrole) amine groups. This is validated via the following observations;
- C-N stretching vibrations in the indole ring for tryptophan at 1359 cm\(^{-1}\) is shifted to 1353 cm\(^{-1}\) in tryptophan AuNS, (yellow star),
- Stretching of the carbonyl group attributed to the peak at 1669 cm\(^{-1}\) in the tryptophan spectrum. This peak is shifted to 1663 cm\(^{-1}\) in tryptophan AuNS after the formation of carboxylate ions during synthesis (green star) [39],
- The almost weakened \(\nu_{as}(\text{NH}_3^{+})\) peak at 2068 cm\(^{-1}\) in the tryptophan AuNS spectrum, demonstrating deprotonation of the amine group during the reduction of gold ions (orange star),
- A weak, shifted peak which represents stretching of the primary amine observed in tryptophan at 3406 cm\(^{-1}\), shifted to 3400 cm\(^{-1}\) in tryptophan AuNS after adsorbing to the particle’s surface (purple star),
- The benzene ring structure generates bands at 1417 and 1459 cm\(^{-1}\) represented by \(\nu(\text{C-C})_{\text{aromatic}}\) stretching and are preserved along with \(\nu(\text{CH})_{\text{aromatic}}\) observed at 3031 cm\(^{-1}\) for both tryptophan and tryptophan AuNS [40].

![Figure 3.10: FTIR spectrum tryptophan solution (tryptophan) for comparison with tryptophan stabilised AuNPs (tryptophan AuNS).](image)

Tyrosine (TYR), another essential amino acid is made up of amino, aromatic and hydroxyl group as shown in Figure 3.11.
**Figure 3.11:** *Structure of tyrosine.*

The FTIR of pure tyrosine as well as tyrosine AuNS are presented in Figure 3.12. As with most amino acid spectra, a strong, broad band is observed around 3000 cm\(^{-1}\) [39] which is attributed to \(\nu(NH_3^+)\). This band is marked by the horizontal dotted line in Figure 3.12. In the case of tyrosine, this overlaps with the stretching band of \(\nu(OH)\) which is seen at slightly lower wavenumbers (\(\geq 2380\) cm\(^{-1}\)). The absence of the \(\nu(OH)\) peak is apparent in the case of tyrosine AuNS as the NH\(_3^+\) peak becomes sharper which supports the theory that tyrosine’s phenolic group becomes oxidised during the reduction of chloroauric acid [41]. Sastry *et al.* also suggests that during nanoparticle synthesis, after oxidation of the phenolic group occurs, a quinone like structure evolves which ultimately binds to the surface of the tyrosine AuNS [41].

**Figure 3.12:** *FTIR spectrum tyrosine solution (TYR) for comparison with tyrosine stabilised AuNPs (TYR AuNPs).*

In the case of pure tyrosine, \(v(NH_3^+)\) and \(v_{as}(NH_3^+)\) are observed at 1520 cm\(^{-1}\) and 2080 cm\(^{-1}\) respectively (orange stars), however in the case of tyrosine AuNS, they are absent and shifted 2127 cm\(^{-1}\).
cm⁻¹ respectively. This demonstrates that deprotonation of the amine group occurs during the reduction of gold ions similarly to tryptophan AuNS.

The synthesis of the CTAB/citrate AuNS differs from the other spherical particles in this section in its methodology. While citrate, tryptophan and tyrosine AuNS use the same reagent to reduce and stabilise (cap) the particles, the CTAB/citrate AuNS are initially reduced and stabilised with trisodium citrate, and then further capped with CTAB -structure shown in Figure 3.13.

![Structure of CTAB](image)

**Figure 3.13: Structure of CTAB.**

The full FTIR spectra for trisodium citrate, CTAB and CTAB/citrate AuNS are shown in Figure 3.14i. Due to the complexity of the spectra, three enlarged areas (A, B and C in Figures 3.14ii, iii, and iv respectively) are presented for analysis.

Area A, displays wavelengths from 750 – 1250 cm⁻¹ in Figure 3.14ii. In this section, ν(C-N) modes are shown at 937, 961 and 982 cm⁻¹ (shown by green stars) in the pure CTAB sample. The inclusion of these bands in the CTAB/citrate AuNS spectrum indicates preservation of the CTA⁺ head group after it undergoes dissociation [42]. In addition to this, the inclusion of bands ~1000-1250 cm⁻¹ indicates the formation of a CTAB bilayer on the NP surface via a CTA⁺-Au linkage (denoted by the black star).

Area B displays wavenumbers from 1250-1750 cm⁻¹ (Figure 3.14iii) for trisodium citrate, CTAB and CTAB/citrate AuNS. As described in the citrate AuNS section previously, the citrate spectrum shows asymmetric and symmetric stretching vibrations of the carboxylate group at 1573 and 1394 cm⁻¹, respectively (marked by blue stars). In the case of CTAB/citrate AuNS the νas(COO⁻) and νs(COO⁻) peaks are shifted to 1584 cm⁻¹ and 1396 cm⁻¹, respectively. This demonstrates the involvement of the carboxyl group with the particle surface via η¹-COO⁻. The νas(COO⁻) peak from 118
the persistent citrate layer on the AuNS has obscured the expected peak at ~1600 cm\(^{-1}\) which would be attributed to the bending of water around the CTAB head groups bound to the surface of the particles (denoted by aqua star).

![FTIR spectra](image)

**Figures 3.14**: FTIR spectra of for (i) trisodium citrate, CTAB and CTAB/citrate AuNSs; (ii) shows an enlargement of 750-1250 cm\(^{-1}\) (area A), (iii) 1250-1750 cm\(^{-1}\) (area B) and (iv) 2750-3100 cm\(^{-1}\) (area C).

While pure trisodium citrate does not contribute any peaks in this region, the area contains four CTAB peaks relating to the presence of a methylene chain structure. These peaks; \( \nu_s(CH_2) \) and \( \nu_a(CH_2) \) stretching bonds at 2850 and 2945 cm\(^{-1}\) (green and orange stars) as well as \( \nu_s(CH_3) \) and \( \nu_a(CH_3) \) stretching bonds at 2920 and 3017 cm\(^{-1}\) (yellow and purple stars), respectively are all observed unshifted in the CTAB/citrate AuNS. Venkataraman and Vasudevan report that this feature confirms
the presence of highly ordered methylene chains within the CTAB bilayer along the particle surface [43].

3.2.5.6 X-ray Photoelectron Spectroscopy Analysis of Chemically Distinct Spherical Gold Nanoparticles

To further support the conclusions made using FTIR, the highly sensitive surface sensitive technique XPS was employed. Figure 3.15 shows the C 1s and O 1s core level XPS spectra for Na₃Ct and Citrate AuNS, as well as the Au 4f spectra for Citrate AuNS.

In the case of Na₃Ct and Citrate AuNS, the C 1s spectra are resolved with 3 individual binding energy (BE) peaks. Both samples show an adventitious carbon component at 285 eV, a higher BE component corresponding to C-O bonds (at 286.1 and 286.7 eV for Na₃Ct and Citrate AuNS respectively) as well as a BE component corresponding to C=O bonds (at 287.5 and 288.6 eV for Na₃Ct and Citrate AuNS respectively).

![XPS spectra](image)

**Figure 3.15:** XPS spectra showing C 1s, and O 1s, (upper) and C 1s, O 1s, and Au 4f (lower) binding energies arising from (Na₃Ct) and citrate AuNS, respectively.
The BEs for the O 1s spectrum showed two peak maxima at 531.8 and 533.1 eV for Na₃Ct corresponding to carboxyl and hydroxyl group, respectively. In the case of citrate AuNS, 3 peaks were resolved at 531.6, 533.1 and 535.8 eV. The highest BE value can be attributed to the proximity of neighbouring chloride (arising from the gold chloride precursor) after coordination of the terminal carboxyl group with the AuNP surface.

In the case of tryptophan and tyrosine based systems, the high degree of structural similarity of the amino acids allows for combined discussion. Shown in Figure 3.16 in the case of tryptophan AuNS and tyrosine AuNS, the Au 4f spectrum shows 2 spin orbit pairs at 83.5 eV and 85.5 eV corresponding to reduced Au⁰ and unreduced AuCl₄⁻ species, respectively. The persistence of low levels of unreduced gold species (despite extensive dialysis of the sample) demonstrates the formation of a complex between AuNP bound amino acids and AuCl₄⁻ ions.

Decomposition of the C 1s spectra from both tryptophan and tyrosine resolves four individual peaks at 285.0, 286.4, 289.1 and 291.9 eV. These binding energies relate to;

- Alkyl and aromatic carbon (285.0 eV),
- C-O and C-N (286.4 eV),
- C(O)OH (289.1 eV) and
- Benzene group (291.9 eV).

In the case of tryptophan AuNS and tyrosine AuNS the C 1s spectra can be decomposed to 3 peaks at 285.0, 286.4 and 288.3 eV which related to;

- Adventitious carbon (285.0 eV)
- C-O and C-N (286.4 eV)
- Carboxylate carbon (–COO–) from surface bound tryptophan (288.3 eV)

The N 1s core level XPS spectra for tryptophan AuNS and tyrosine AuNS show a single peak in each case, due to surface bound primary amines.
Figure 3.16: XPS spectra showing Au 4f spectra for tryptophan AuNS (a), tyrosine AuNS (b), C 1s spectra for tryptophan (c), tyrosine (d), tryptophan AuNS (e), tyrosine AuNS (f) and N 1s spectra for tryptophan and tyrosine AuNS (g).

The C 1s, O 1s and N 1s core level spectra for CTAB and CTAB/citrate AuNS are presented in Figure 3.17. The C 1s spectra for CTAB can be deconvoluted into three peaks with maxima with adventitious carbon at 285.0, 286.2 (methylene chain) and 287.6 (methyl head groups) eV. The CTAB/citrate AuNPs present maxima at 286.6 (methylene chain), 289.7 (carboxyl group) and 291.3 (methyl head groups after binding with particle surface) eV. The presence of higher binding energy features indicate the presence of citrate as a stabiliser in addition to CTAB on the AuNP surface.
Figure 3.17: XPS spectra showing C 1s core level binding energy for Na$_3$Ct, CTAB and CTAB/citrate AuNS (left to right).

3.2.5.7 Zeta Potential Measurements of Chemically Distinct Spherical Gold Nanoparticles

It is generally agreed that the zeta (ζ) potential of AuNPs is chiefly determined by the stabilising agent or surface modifier employed during synthesis [44]. Table 3.2 contains the ζ-potential measurements for all chemically distinct spherical AuNPs. Quantifying the charge at the nanoparticles surface, these measurements provide additional confirmation for the presence of the stabilising or capping agent on the AuNP surface. In addition to this, the magnitude of the zeta potential is a mark of the particles stability or likelihood to aggregate in solution.

The measurements obtained are in agreements with literature (and expected) values. Citrate AuNS generated the strongest negative surface value, and notably (with a negative value > 30 mV) possess the highest degree of stability of all the AuNS [45].

Despite undergoing dialysis to remove unreacted ionic species, the eventual pH of the tryptophan and tyrosine NP solutions were found to be moderately alkaline due to the addition of KOH during synthesis. With the isoelectric points (pI) of tryptophan and tyrosine occurring at 5.9 and 5.7 [46] respectively, it was expected that both molecules would provide a negative charge on the particles surface [20].
Due to the addition of cationic surfactant CTAB to citrate stabilised particles, it was predicted the overall charge of the CTAB/citrate AuNS would be positive which was confirmed with a value of +21.9 mV [47].

<table>
<thead>
<tr>
<th>Nanoparticle</th>
<th>Zeta Potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrate AuNS</td>
<td>-38.4</td>
</tr>
<tr>
<td>Tryptophan AuNS</td>
<td>-27.6</td>
</tr>
<tr>
<td>Tyrosine AuNS</td>
<td>-22.6</td>
</tr>
<tr>
<td>CTAB/citrate AuNS</td>
<td>+21.9</td>
</tr>
</tbody>
</table>

Table 3.2: Zeta potential measurements for all chemically distinct spherical AuNPs.

3.3 Synthesis and Characterisation of Distinct Shaped Gold Nanoparticles

3.3.1 Rationale

Increasing interest is being paid to the difference in biological responses generated by gold nanoparticles of varying shapes. However it is unclear if any differences observed are primarily due to differences in geometry, or variations in other factors such as chemical nature, surface charge or a mixture of both [16].

Due to their geometry, interesting optical properties emerge from nanoparticle with altered shapes, providing potential myriad uses with diagnostic and therapeutic potential [21, 22]. While synthesis methods are increasing in number, biological response data is rarely available for these new materials, particularly for cubic (AuNC) and prismatic (AuNPr) gold nanoparticles. It is surprisingly rare that these materials have been tested in an environment which would allow comparison with other nanoparticle species.

To form each nanoparticle into its desired shape, a surfactant is employed to act as a template [48]. For the purpose of this study, a single surfactant was chosen which showed versatility in the range of shapes possible. Cetyl trimethylammonium bromide (CTAB) - a cationic surfactant commonly incorporated into antiseptic formulations - is often documented to be toxic to cells in its free form. Various research groups have shown however that with careful preparation, nanoparticles
which utilise CTAB for shape direction do not necessarily exhibit the same level of toxicity observed with free CTAB [49].

For this study, spherical, cubic, rod, and prismatic shaped particles have been synthesised using similar chemical routes and a common chemical surfactant - CTAB. A well known issue with the synthesis of rod shaped particles is the relationship between yield and silver ions. While it is possible to synthesise gold nanorods in a silver free environment, at best, the yield achievable is approximately 40% [50, 51]. With the introduction of silver ions in the form of silver nitrate, the yield of nanorods increases to 90-100% [52, 53]. For this reason, silver containing rods have been used for this study to obtain a high quality sample of rods in high yield.

3.3.2 Materials

Tetrachloroauric acid (HAuCl₄) (Sigma G4022), cetyl trimethylammonium bromide (CTAB) (Sigma H9151), L-ascorbic acid (C₆H₈O₆) (Sigma A0278), sodium borohydride (NaBH₄) (Labchem A2334), silver nitrate (AgNO₃) (Sigma 209139), hydrochloric acid (Sigma 30720), trisodium citrate (Na₃C₆H₅O₇) (Aldrich 39,807-1) and potassium iodide (KI) (Univar A409) were purchased from the supplier indicated and used without further modification unless indicated.

All reactions were carried out using Milli-Q water, using glassware washed with aqua regia and rinsed thoroughly with deionised water. All chemicals were used as purchased without any further purification unless noted.

3.3.3 Experimental

3.3.3.1 Preparation of CTAB Stabilised Gold Nanospheres (AuNS (CTAB))

Preparation of gold nanospheres was achieved by following the method described by Rayavarapu et al [54]. In a typical synthesis a gold seed was created by combining 9.75 mL of 0.1 M CTAB with 250 µL of 10 mM HAuCl₄ while stirring. To this solution, 600 µL of ice cold freshly prepared 10 mM NaBH₄ was injected while stirring vigorously. The solution was stirred for a further
2 minutes to allow excess NaBH$_4$ to breakdown before being used within 5 minutes as a seed in the following reaction.

To prepare the growth solution, 38 mL of 0.1 M CTAB solution was prepared and brought to a temperature of 25 °C after which 2.0 mL of 10 mM HAuCl$_4$ and 210 µL of 0.1 M L-ascorbic acid were added, stirring after each addition. Lastly, 48 µL of gold seed solution was injected into the growth solution and gently stirred before being left at 25 °C for 24 hours.

Following this period, the solutions were washed to remove excess CTAB and unreacted constituents. The solutions were first centrifuged at 2000 RPM for 6 minutes to settle excess CTAB, the supernatants collected and the process repeated while the pellet (made up of precipitated excess CTAB) was discarded. Following this, the supernatant was centrifuged at 6000 RPM, 12 minutes, 25 °C, repeated 3 times, each wash discarding the supernatants and redispersing the pellet in Milli-Q water.

3.3.3.2 Preparation of CTAB Stabilised Rod Shaped Gold Nanoparticles (AuNR (CTAB))

A modified version of the method described by Liu and Guyot-Sionnest [52] was employed to synthesise rod shaped gold nanoparticles. In a typical synthesis a gold seed was created using a method devised by Nikoobakht and El-Sayed [55]. 10 mL of 0.1 M CTAB was prepared and brought to 30 °C, to which 250 µL of 10 mM HAuCl$_4$ was added while stirring. To this solution, 600 µL of freshly prepared 10 mM NaBH$_4$ was injected while stirring vigorously. The solution was stirred for a further 5 minutes to allow excess NaBH$_4$ to breakdown, before being used as a seed in the following reaction.

During the decomposition of NaBH$_4$, 40 mL of 0.1 M CTAB solution was prepared and brought to a temperature of 30 °C after which 2.0 mL of 10 mM HAuCl$_4$, 0.4 mL of 10 mM AgNO$_3$, 800 µL of 0.1 M HCl and 320 µL of 0.1 M L-ascorbic acid was added, stirring after each addition. Lastly, 96 µL of gold seed solution was injected into the growth solution and gently stirred before being left for 2 hours at 30 °C.
The solution was allowed to stabilise at room temperature for 24 hours before being washed to remove excess CTAB and unreacted constituents. The solutions were first centrifuged at 2000 RPM for 6 minutes to settle excess CTAB, the supernatants collected, while the pellet (made up of precipitated excess CTAB) was discarded. Following this, the collected supernatant was centrifuged at 8000 RPM, 15 minutes, 12 °C, repeated 3 times, each wash discarding the supernatants and redispersing the pellet in Milli-Q water.

3.3.3.3 Preparation of CTAB Stabilised Prismatic Gold Nanoparticles (AuNPr (CTAB))

The method devised by Millstone et al. was employed to synthesise prismatic shaped gold nanoparticles [56]. In a typical synthesis, a 5 nm gold seed was prepared by sequentially adding 1 mL of 0.01 M sodium citrate dihydrate, 1 mL of 0.01 M HAuCl₄ and 1 mL freshly prepared 0.1 M NaBH₄ to 36 mL of Milli-Q water while stirring vigorously. The gold seeds were allowed to mix for a further 1 minute then left undisturbed for 2 hours before using used in the following reaction.

An iodide doped CTAB mixture was prepared for the growth solutions by adding 2.733 g of purified CTAB in 150 mL of Milli-Q water, followed by the addition of 75 μL of 0.1 M KI. The solution was sealed, gently heated until the CTAB dissolved and sonicated for 30 seconds before being set aside at room temperature for 2 hours.

Following this period, three growth solutions were prepared, such that growth solutions 1 and 2 were each made up of 9 mL of the prepared iodide doped CTAB solution, 250 μL of 0.01 M HAuCl₄, 50 μL of 0.1 M NaOH and 50 μL of 0.1 M L-ascorbic acid, while solution 3 contained 90 mL of prepared iodide doped CTAB solution, 2.5 mL of 0.01 M HAuCl₄, 500 μL of 0.1 M NaOH and 500 μL of 0.1 M L-ascorbic acid.

To commence the reaction, 1 mL of the 5 nm seed solution was added to growth solution 1 which was gently stirred before transferring 1 mL of growth solution 1 into growth solution 2, which was stirred before adding its total contents into growth solution 3. The reaction was allowed to proceed undisturbed for 30 minutes until completion.
The solution was left to stabilise for 24 hours before being washed to remove excess CTAB and unreacted constituents. The solutions were first centrifuged at 2000 RPM for 6 minutes to settle excess CTAB, the supernatant removed and kept while the pellet (made up of precipitated excess CTAB) was discarded. Following this, the supernatant was centrifuged at 8000 RPM for 3 minutes at room temperature. This process was repeated 3 times, discarding the supernatants and redispersing the pellet in Milli-Q water after each wash.

3.3.3.4 Preparation of CTAB stabilised cubic shaped gold nanoparticles (AuNC (CTAB))

A modified version of the method detailed by Kim et al. was employed to synthesise cubic shaped gold nanoparticles [57]. In a typical synthesis 40 mL of 10 mM CTAB was made in a 50 mL tube to which 500 μL of 10 mM HauCl₄ was added. The solution was mixed by gentle inversion before 200 μL of 100 mM L-ascorbic acid was added, followed by repeated inversion to mix.

The solution was allowed to stand for 30 minutes during which time; the ascorbic acid was allowed to begin the reduction of Au³⁺ to Au⁺. After this period, 60 μL of 100 mM NaOH was slowly added to the bottom of each tube using a pipette, which was removed carefully with minimal disturbance to the solution. The solution was allowed to stand at 25 °C for 6 hours before the tubes were inverted. By this time, the addition of NaOH had further reduced Au⁺ to Au⁰ which seeded the growth of cubic shaped gold nanoparticles.

The solution was allowed to stabilise for 24 hours before being washed to remove excess CTAB and unreacted constituents. The solutions were first centrifuged at 2000 RPM, 6 minutes, room temperature to settle excess CTAB, the supernatants kept, while the pellet (made up of precipitated excess CTAB) was discarded. Following this, the solutions were centrifuged at 9000 RPM, 3 minutes, repeated 3 times, discarding the supernatants and redispersing the pellet in MilliQ water after each wash.

Nanoparticle solutions were prepared for characterisation as described in Section 3.2.4.
3.3.4 Results and Discussion

3.3.4.1 UV-Visible Spectroscopy of Distinct Shaped Gold Nanoparticles

While spherical AuNPs characteristically give a single absorption peak in the UV-visible spectrum, various shapes with inherent anisotropy such as rods, prisms, stars and branches can give rise to two or more absorption peaks as their complex geometry creates varied paths along which the surface plasmons oscillate [16].

The UV-visible absorption spectrum for the AuNS (CTAB) sample is characteristic of spherical particles with a single absorption band at 540 nm shown in Figure 3.18. This suggests that AuNS (CTAB) are larger than the spheres presented in Section 3.2.4.1 as the redshift is indicative of a particle with a larger cross section generating increased scattering [26]. This finding is in agreement with literature values [58] as well as the DLS results obtained which returned an average size of 80.0 nm.

Rod shaped nanoparticles typically produce UV spectra with two plasmon resonance bands due to the oscillations of surface plasmons on the transverse (short) and longitudinal (long) edges of the rod [59-61]. The position of the transverse band is generally fixed at around 520 nm, however the longitudinal band is dependent on the length of the rod and typically present at wavelengths of 750 nm and above. The AuNR (CTAB) synthesised in this report returned a typical spectra for nanorod sample of high purity (low concentration of spheres) which is determined by the strength of the transverse peak at 510 nm in relation to the longitudinal peak - shown at 875 nm shown in Figure 3.18. From the spectra obtained, it can be estimated using literature values, that the AuNR would have an aspect ratio of approximately 4 [55, 59], which is comparable to the result obtained after TEM analysis (aspect ratio 3.8).
Figure 3.18: *UV-visible absorption spectra of (clockwise from top left) AuNS (CTAB), AuNR (CTAB), AuNPr (CTAB) and AuNC (CTAB).*

Similarly, prismatic or triangular shaped gold nanoparticles are often characterised by having multiple absorption peaks - with two or three peaks typically present [22]. The position of the longitudinal peak/s is determined by the edge lengths of the particles [62, 63], with increasing edge length generating greater redshift, while the position of the first (transverse) peak is generally observed between 500-600 nm. In the case of the AuNPr synthesised in this chapter the transverse peak is observed at 550 nm with the longitudinal peak seen as a shoulder at 630 nm in Figure 3.18. This is comparable with published results for several synthesis methods of AuNPr [62, 63].

The symmetry associated with cubic structures means that cubic AuNPs generally have a single absorption peak [64]. Like spherical particles, the position of the peak is dependent on the size of the particles synthesised [65]. Reported literature values for gold nanocubes place the single peak at 546 nm for cubes with an average side length of 33 nm [66]. Red shift is expected as the side length
increases which is consistent with the spectrum obtained from AuNC synthesised in this chapter which show a single peak at 554 nm (average side length obtained using TEM 47.5 ± 11.8 nm).

3.3.4.2 Dynamic Light Scattering Measurements of Distinct Shaped Gold Nanoparticles

While the size of spherical particles can be easily described with a single number – a diameter – size analysis of non spherical shapes is a more complex issue [67]. DLS inherently assumes a spherical shape for all particles analysed, and as such, caution must be taken when interpreting results of different shaped particles. More complex shapes such as prisms, cubes and rods require edge length, height, width or depth measurements for accurate particle size analysis, which are more easily obtained using electron microscopy techniques. When such particles are analysed with DLS, the measurement returned can be described as the equivalent spherical hydrodynamic radius. That is, the radius of a hard sphere that would diffuse in an identical way to the particles being analysed [32].

While useful data can be obtained from some non spherical shapes, shapes with high aspect ratios such as rods are difficult to analyse without employing more complex models [68]. For this reason, the DLS data for AuNR is presented in Figure 3.20 to portray size distribution only, but has been omitted from Table 3.3 due to its unreliability as a hydrodynamic measurement. Notably, the size distribution data shows the presence of much larger particles (not observed in TEM analysis). It can therefore be assumed that when in solution, some of the rod shaped particles agglomerate, or alternatively form chains linked end to end.

As expected, the DLS result of 80 nm obtained for AuNS (CTAB) is in the range of expected values based on an average diameter of 78 nm using TEM. Interestingly AuNPr (CTAB) returned the only DLS result less than the corresponding measurement obtained using TEM. To understand this value, we must think about the difference between a diffusing prism and a diffusing sphere. While a sphere diffuses in the same way regardless of orientation, differences in the orientation of the prism will cause different diffusion rates. Considering the prism depicted in Figure 3.19, if it is moving out of the page, the cross sectional area is larger than if it were moving vertically, which is slightly larger
than if it is moving horizontally. Thus the hydrodynamic diameter obtained is the orientational average of all of these faces including the larger flat face (which would be interpreted as a sphere of a roughly the size of the actual AuNPr) as well as the thin side faces and corners (which would be calculated as much smaller equivalent spheres).

**Figure 3.19: Various orientations of a diffusing prismatic particle.**

Analysis of the AUNCs returned a hydrodynamic diameter of 115 nm, much greater than the average side length of the cubes determined using TEM (47.5 nm). However much like the AuNPr described above, different orientations of the diffusing cubes must be accounted for.

The diagonal length of the cube is possibly a more relevant measurement of comparison for an equivalent sphere. The average diagonal measurement for AuNCs was determined using $\sqrt{3} \times$ average side length of the particles which is equal to 82.3 nm.

In addition to this, the impact of the larger cubes within the sample shown in Figure 3.21 would have a much greater effect on the light scattering signal, skewing the hydrodynamic diameter toward larger sizes. Accounting for both of these factors provides a clearer understanding for the difference in side length as determined using TEM and the hydrodynamic diameter determined by DLS.
Figure 3.20: Dynamic light scattering size distributions of (clockwise from top left) AuNS (CTAB), AuNR (CTAB), AuNPr (CTAB) and AuNC (CTAB).

3.3.4.3 Transmission Electron Microscopy analysis of Distinct Shaped Gold Nanoparticles

TEM images of each AuNP are presented in Figure 3.21 providing fair representation of each sample. All samples appear not to be aggregated once dried.

Figure 3.21: TEM images representative of (a) AuNS (CTAB), (b) AuNR (CTAB), (c) AuNPr (CTAB) and (d) AuNC (CTAB).
At least 100 particles were analysed to determine an average particle diameter in the case of AuNS or in the case of AuNR, AuNPr and AuNC, an average side length. There is some degree of polydispersity within all samples which is represented using particle size distribution histograms in Figure 3.22. These measurements, as well as the standard deviation (SD) for each value, are shown in Table 3.3 alongside the hydrodynamic diameters as determined using DLS for comparison.

![Figure 3.22: Particle size distribution histograms of (a) AuNS (CTAB), (b) AuNR (CTAB), (c) AuNPr (CTAB) and (d) AuNC (CTAB).](image)

TEM analysis confirms that the AUNS (CTAB) are pseudo spherical (sometimes referred to as quasi spherical) particles. This irregularity is commonly seen in large spherical AuNPs as the seed mediated growth process proceeds slowly. During this time, the concentration of available capping agent is reduced, causing a higher degree of irregular growth along different facets of the growing crystal [69].

The morphology of AuNR shown in Figure 3.21 is consistent with previously published methods also using Ag⁺. In contrast with the pentatwined structure of rods synthesised without Ag⁺, the rods presented here have square, untapered extremities. The aspect ratio of these rods (determined using the average side lengths determined with TEM) is 3.8.

TEM analysis of AuNPr (CTAB) returned an average side length of 94.7 nm, however it also uncovered the presence of spherical particles persistent within solution. This is a common issue encountered with the synthesis of AuNPr, which are notoriously difficult to produce in high yield [1].
In addition to this, “snipped” triangles can be observed in varying degrees ranging from corner rounding, to one or all corners appearing to be cut off (which can appear as irregular hexagonal plates). The anomaly occurs during synthesis due to disorder or lack of aggregation between gold particle segments and has impacted on the particle size analysis leading to a large size distribution within the sample. [70].

<table>
<thead>
<tr>
<th></th>
<th>TEM - Average measurement ± SD</th>
<th>DLS - Hydrodynamic diameter</th>
</tr>
</thead>
<tbody>
<tr>
<td>AuNS (CTAB)</td>
<td>77.9 ± 3.5 nm (diameter)</td>
<td>80 nm</td>
</tr>
<tr>
<td>AUNR (CTAB)</td>
<td>58.8 ± 10.6 nm (length)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>15.3 ± 5.1 nm (width)</td>
<td>-</td>
</tr>
<tr>
<td>AuNPr (CTAB)</td>
<td>94.7 ± 15.6 nm (side length)</td>
<td>90 nm</td>
</tr>
<tr>
<td>AuNC (CTAB)</td>
<td>47.5 ± 11.8 nm (side length)</td>
<td>115 nm</td>
</tr>
</tbody>
</table>

Table 3.3: Comparison of average particle measurements as determined by TEM and DLS.

AuNC (CTAB) also presented with a large standard error, with the average side length determined to be 47.5 nm ± 11.8 nm. This degree of size variation can be observed in the TEM image representing the sample in Figure 3.21. The majority of cubes within the sample have rounded edges; however their morphology is significantly different from AuNS (CTAB).

3.3.4.4 X-ray Diffraction Studies of Distinct Shaped Gold Nanoparticles

As discussed in Section 3.2.5.4, AuNPs generally exhibit a face centred cubic (fcc) structure as pictured in Figure 3.5 [2]. The results presented in Figure 3.23 confirmed the presence of highly crystalline structures, showing well defined Bragg reflections corresponding to (111), (200), (220), (311), and (222) planes which are commonly associated with fcc bulk gold [34]. The broad unassigned peaks (particularly prevalent in the AuNR (CTAB) sample are most likely attributed to amorphous impurities within the crystal which can occur during nanoparticle growth [71].
While the chemical composition was kept constant for this set of AuNPs, it is well known that the growth mechanisms for different shaped AuNPs favours growth along different crystallographic planes [72]. As expected for gold nanospheres, growth along the (111) facet is favoured due to its high stability, which is consistent with the spherical AuNPs synthesised in this report [73]. Interestingly, the results obtained for AuNPr show that they too have (111) faces, however in the case of thin plate like structures, it is indicative of an obstruction of growth along the (111) face. This is due to the addition of iodide during the synthesis of AuNPr which preferentially binds to the (111) facet, blocking further growth [74]. For this reason, the large faces of the prisms (111) generate a strong peak [34] which is apparent in Figure 3.23.

![Figure 3.23: XRD of different shaped, chemically similar AuNPs.](image)

Gold nanorods have been shown to have various combinations of \{110\}\{111\} and \{100\} facets dominant depending on the synthesis method employed and the length of the rod formed [75]. Short AuNRs grown with the assistance of Ag⁺ (such as those synthesised in this Thesis) have been shown to have \{110\} and \{100\} dominant facets [53, 76, 77]. As \{100\} and \{110\} diffractions do not satisfy the Bragg equation for fcc gold, diffraction of the parallel (200) and (220) planes, respectively was observed instead. This is consistent with the XRD results presented in Figure 3.23 which show AuNRs as the only shape presenting with this unique pattern.
The AuNC presented are synthesised using ascorbic acid as well as CTAB [57]. The relationship between these two species was determined by Sau and Murphy who noted that varying the relative concentrations of the two chemicals led to particles with different dominant facets [78]. They determined that a higher ascorbic acid concentration (or lower CTAB concentrations) was desirable for cubic particle growth. These cubes had increased {100} facets, which is seen in Figure 3.23 as increased (200) peaks.

3.3.4.5 Fourier Transform Infrared Spectroscopy Analysis of Distinct Shaped Gold Nanoparticles

Due to the employment of CTAB -structure shown in Figure 3.13 - for the synthesis of all AuNPs in this section, the FTIR spectrum for CTAB alone is presented in Figure 3.24a, (as well as the enlarged section shown in Figure 3.24b). At lower wavenumbers, \( \nu(CN) \) stretching modes are shown at 937, 961 and 982 cm\(^{-1}\) (shown by red star) in Figure 3.24a.

**Figure 3.24:** Full FTIR spectrum of CTAB (a) with indicated section enlarged (b).

For the range of wavenumbers between 1250-1750 cm\(^{-1}\), a complex array of peaks is generated by the pure CTAB sample. These peaks are mainly due to the different vibrational modes of the methylene groups from within the sample. The origin of each lettered peak in this spectrum is explained in Table 3.4.

Moving to higher wavenumbers, four distinct peaks arise due to the presence of the methylene chain structures found within CTAB. These peaks correspond to \( \nu_s(CH_2) \) and \( \nu_as(CH_2) \) at 2850 and 2850 cm\(^{-1}\).
2945 cm\(^{-1}\) (green and orange stars) as well as \(\nu_s(\text{CH}_3)\) and \(\nu_{as}(\text{CH}_3)\) stretching bonds at 2920 and 3017 cm\(^{-1}\) (yellow and purple stars), respectively. The peak seen at 3420 cm\(^{-1}\) are commonly seen in FTIR samples due to the high sensitivity of FTIR towards water [39].

<table>
<thead>
<tr>
<th>Letter of peak (from Figure 3.25b)</th>
<th>Wavenumber (cm(^{-1}))</th>
<th>Bond</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1285</td>
<td>(\omega(\text{CH}_2))</td>
</tr>
<tr>
<td>B</td>
<td>1303</td>
<td>(\nu(\text{CH}_2)) \textit{torsional}</td>
</tr>
<tr>
<td>C</td>
<td>1360</td>
<td>(\omega(\text{CH}_2))</td>
</tr>
<tr>
<td>D</td>
<td>1396</td>
<td>(\delta_1(\text{N-CH}_3))</td>
</tr>
<tr>
<td>E</td>
<td>1408</td>
<td>(\omega(\text{CH}_2))</td>
</tr>
<tr>
<td>F</td>
<td>1432</td>
<td>(\omega(\text{CH}_2))</td>
</tr>
<tr>
<td>G</td>
<td>1462</td>
<td>(\delta_1(\text{CH}_2))</td>
</tr>
<tr>
<td>H</td>
<td>1472</td>
<td>(\delta_1(\text{CH}_2))</td>
</tr>
<tr>
<td>I</td>
<td>1486</td>
<td>(\delta_{as}(\text{N-CH}_3))</td>
</tr>
<tr>
<td>J</td>
<td>1614</td>
<td>(\nu(C_{16}))</td>
</tr>
<tr>
<td>K</td>
<td>1657</td>
<td>(\nu(C_{16}))</td>
</tr>
</tbody>
</table>

**Table 3.4:** Description of all peaks depicted in Figure 3.24b for CTAB. \(\delta\) scissoring (in plane bending), \(\omega\) wagging (out of plane bending), \(\nu\) stretching, as asymmetric, s symmetric.

The full spectra of all CTAB stabilised samples have been plotted (along with CTAB for comparison) in Figure 3.25a to assess the changes in structure which occur after adsorption of CTAB on the AuNP surface. Due to the complexity of the spectra, three important sections have been enlarged for analysis. Wavenumbers 750-1250 cm\(^{-1}\) (section A) are presented in Figure 3.25b.

As previously noted, the three peaks in Figure 3.25b (denoted by green stars) represent \(\nu(C-N)\) bonds. The apparent absence or weakening and shifting of these peaks is generally indicative of the formation of a CTAB bilayer capping the AuNPs with bonds formed between the CTA\(^+\) cations and NP surface. Peaks in the range of 1000-1250 cm\(^{-1}\) and below \(\leq 900\) cm\(^{-1}\) marked by black stars demonstrate the new position of these peaks.
Figure 3.25: Full FTIR spectra of CTAB, AuNS (CTAB), AuNR (CTAB), AuNPr (CTAB) and AuNC (CTAB) (a) as well as the region of 750-1250 cm\(^{-1}\) (area A) resized (b).

Wavenumbers between 1250-1750 cm\(^{-1}\) are shown for all spectra in Figure 3.26a. The analysis of CTAB peaks are covered above, however new bands arising at \(~1600\) cm\(^{-1}\) denoted by black stars can be assigned to \(\delta_s(OH)\), or rather movement of water around the bound headgroups. The FTIR data thus clearly indicates that the surfactant is bound to the AuNS, AuNR and AuNC and that it acts to stabilize the particles by forming an interdigitated bilayered structure. While AuNPr (CTAB) does not contain prominent peaks in this area, the enlarged spectra of AuNPr (Figure 3.26b) shows a weak band in this region also.

Figure 3.26: FTIR spectra of for CTAB, AuNS (CTAB), AuNR (CTAB), AuNPr (CTAB) and AuNC (CTAB) between 1250-1750 cm\(^{-1}\) (area B) (a) as well as further enlarged spectra of AuNPr (CTAB) between 1250-1750 cm\(^{-1}\) for better visualisation of observed peaks (b).
Figure 3.27 shows higher wavelengths between 2750-3100 cm\(^{-1}\), and confirms the presence of CTAB due to the preservation of \((\text{CH}_2)\) and \(\nu_{\text{as}}(\text{CH}_2)\) stretching bonds at 2850 and 2945 cm\(^{-1}\) in all samples (green and orange stars). The shifting to higher wavenumbers for AuNR and AuNC samples is generally indicative of less ordered methylene chain structures arranged on the AuNPs surface.

![Figure 3.27: FTIR spectra of for CTAB, AuNS (CTAB), AuNR (CTAB), AuNPr (CTAB) and AuNC (CTAB) between 2750-3100 cm\(^{-1}\) (area C).](image)

3.3.4.6 X-ray Photoelectron Spectroscopy Analysis of Distinct Shaped Gold Nanoparticles

XPS measurements were conducted on the four distinct shaped AuNPs as well as for pure CTAB. For the sake of brevity, one spectrum for each element representative of the group of AuNPs is presented in Figure 3.28. The spectra confirm the presence of CTAB on all AuNPs chiefly from the persistence of nitrogen and bromide in all AuNP samples. Figure 3.28a and c show the nitrogen spectra for CTAB and AuNPs respectively with a single peak resolved in each case. The nitrogen peak for CTAB at 403.9 eV is shifted to 402.5 eV for AuNP indicative of the formation of a CTAB bilayer capping the AuNPs with bonds formed between the CTA\(^+\) cations and NP surface. Additional shifts are observed in the Br\(^-\) signal from 68.7 and 69.6 eV to 67.1 and 68.6 eV for CTAB and AuNPs respectively in Figure 3.28b and d. This shift is due to the removal of bromine from the head group region, and the formation of Br-C and Br-H bonds.
Additionally, Figure 3.28e shows the Au 4f spectra for each AuNP can be resolved to the characteristic doublet indicative of elemental gold [79]. Figure 3.28f shows the iodide signal detected on AuNPs. This peak demonstrates the inclusion of iodide on the surface of the particles and is consistent with Millstone et al [56]. AuNS, AuNR and AuNC did not exhibit a detectable iodide signal.

Figure 3.28g shows the C 1s core spectra decomposed to 2 energy levels, the lower at 285.0 eV due to C-C and C-H bonds, while the higher at 286.0 eV is due to C-N bonds. These results are consistent with those gained from FTIR and support the idea that CTAB forms bilayers on the surface of AuNPs.

![Figure 3.28: XPS spectra showing N 1s and Br 3d spectra from pure CTAB (a), (b) and AuNP (CTAB) (c), (d) as well as Au 4f (e) and I 3d (f) and C 1s from AuNP (CTAB)(g).](image-url)
3.3.4.7 Zeta Potential Measurements of Distinct Shaped Gold Nanoparticles

As expected, all particles synthesised with CTAB generated positive zeta potential measurements, regardless of shape, as presented in Table 3.5. This is in agreement with literature, and is the expected result for the analysis of particles stabilised with a cationic surfactant [80, 81].

<table>
<thead>
<tr>
<th>Nanoparticle</th>
<th>Zeta Potential</th>
</tr>
</thead>
<tbody>
<tr>
<td>AuNS (CTAB)</td>
<td>+44.9</td>
</tr>
<tr>
<td>AuNR (CTAB)</td>
<td>+38.1</td>
</tr>
<tr>
<td>AuNPr (CTAB)</td>
<td>+52.2</td>
</tr>
<tr>
<td>AuNC (CTAB)</td>
<td>+30.4</td>
</tr>
</tbody>
</table>

**Table 3.5**: Zeta potential measurements for all distinct shaped AuNPs.

3.4 Summary

From the extensive characterisation carried out in this Chapter, it has been determined that two distinct groups of AuNPs have been created for subsequent biological testing. The first set of AuNPs, comprised of four spherical particles (citrate, tryptophan, tyrosine and CTAB/citrate AuNS), were confirmed to be of comparable size and shape using UV-vis, DLS and TEM despite having surface properties which differ significantly. XRD analysis confirms consistent crystalline structures commonly attributed to fcc bulk gold, and FTIR, XPS and zeta potential measurements confirm that the surface of particles differ depending on the chemical stabiliser used during AuNP synthesis.

The second set of AuNPs, comprise of four CTAB stabilised particles of differing shape were confirmed to be spherical, rod shaped, prismatic and cubic by TEM and supported by UV-vis and DLS. Similarly to the first group of AuNPs, XRD analysis confirms consistent crystalline structures commonly attributed to fcc bulk gold. Due to the use of a common chemical surfactant (CTAB) employed during the synthesis of this set of AuNPs, XPS, FTIR and zeta potential measurements provide evidence that the surface characteristics of these AuNPs are analogous.
3.5 References


35. Daima, H.K., *Towards fine-tuning the surface corona of inorganic and organic nanomaterials to control their properties at nano-bio interface*. 2013, RMIT University.


Chapter 4: Viability and Uptake Studies - Materials & Methods
4.1 Introduction

The drive behind such great interest in gold nanoparticle toxicity stems from the promise of gold nanoparticles (AuNPs) for diverse biological applications in fields such as drug delivery, biosensing, biological imaging, gene therapy and photothermal therapy [1-5]. Many of these applications utilise various shaped AuNPs because of their interesting optical properties. However, the future of these applications hinges on the ability of researchers to declare the safety of these AuNPs for therapeutic use.

While there have been many studies conducted showing negligible toxicity to cells after exposure to gold nanoparticles [6-9], there are also studies showing conflicting results [10-12].

While most research has been conducted on the uptake of spherical AuNPs, there is a smaller body of work detailing the uptake of rod shaped AuNPs of varying aspect ratio, surface charge and surface functionalisation [10, 13, 14]. The uptake of alternate shapes such as cubic and prismatic AuNPs is studied to a lesser extent and remains largely unexplored.

Gaining attention more recently is the importance of the cell line selected for uptake experiments [15, 16]. Endocytosis or cellular uptake is a complex process which encompasses many endocytotic pathways including both pinocytosis and phagocytosis, which are generally responsible for the uptake of smaller and larger bodies respectively [17]. Within these two pathways are a number of more specific processes which account for the routes of cellular uptake for materials ranging from viruses to the extracellular fluid. The specialised and diverse nature of cells means that not all cells possess the means necessary to perform uptake through all possible endocytotic pathways [18]. Such cells are unsuitable for uptake studies and would provide exceptionally poor points of comparison. Furthermore, researchers have found that the rate and extent of uptake can vary dramatically between cell lines [19]. Such evidence suggests that comparison of uptake levels between different cell lines must be done with caution.

What is evident from these studies is that comparison between research groups is not a simple task with diverse cell lines used, various cell viability assays employed, and different chemical routes
utilised to synthesise AuNPs of different shapes and sizes impacting results leading to conflicting results.

4.2 Rationale

By using the set of chemically diverse AuNS detailed in Chapter 3, the effect of chemical composition on cellular toxicity can be explored. By maintaining a comparable size (8 -12 nm) the effect of using different chemical species (citrate, tryptophan, tyrosine and CTAB/citrate) to reduce and stabilise AuNPs allows us to determine the importance of surface chemical species on cellular toxicity.

By performing the toxicity and uptake tests in the three environments of varied serum levels, the importance of a protein corona in mediating cellular uptake and toxicity has been determined, in addition to the stability of the AuNPs in conditions of varying protein being confirmed.

It has been demonstrated in the previous Chapter that gold nanoparticles of various shapes can be synthesised using chemically similar processes, thus allowing for the comparison of their effect in terms of cytotoxicity. Additionally AuNP uptake has been compared as a function of shape. There exists no published study to date which details the synthesis of spherical, rod, prismatic and cubic shaped AuNPs using chemically similar routes, to subsequently test and compare their uptake and toxicity to mammalian cells. This systematic toxicity and uptake study provides evidence for interesting shape dependant trends in AuNP-cellular behaviour. These trends include the impact of specific AuNP geometric properties on cellular viability and the influence of AuNP facet size on cellular uptake.
4.3 Experimental

4.3.1 Materials

The following items were purchased and used as received; RPMI 1640 (Invitrogen 11875-093), Foetal Bovine Serum (Bovogen SFBS-F), Penicillin Streptomycin (Invitrogen 15070-063), TrypLE™ Express (Invitrogen 12404-021), Dulbecco’s Phosphate Buffered Saline, (Invitrogen 14200-166), dimethyl sulfoxide (DMSO) (Sigma D4540), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Life Technologies M6494), Trypan Blue stain 0.4% (Gibco 15250) and ethanol (Sigma E7023).

The following labware was used in the subsequent experiments; 75 cm² canted neck, vented, sterile tissue culture flasks (Greiner 658175V) and 175 cm² angled neck vented sterile tissue culture flasks (Corning CORN431080), 6 well sterile cell culture plates (CORN3516) and 96 well flat bottom sterile cell culture plates (CORN3599).

4.3.2 Cell Culture Techniques and Protocols

4.3.2.1 General Cell Culture Conditions

All cell culture work was performed in a biohazard hood (Labconco Purifer Logic+ Class II, Type A2), sterilised with a UV light for 15 minutes prior to use. Cells were discarded after a maximum of 10 passages to minimise the occurrence of genetic drift and other abnormalities which occur with increasing passage number [20]. All equipment was sterilised using 70% ethanol before being placed inside the biohazard hood. Solutions used for cell culture were warmed to 37 °C prior to use in a temperature controlled water bath unless otherwise noted.

PC-3 cells were cultured with RPMI-1640 media that had been supplemented with 10% (v/v) FBS and 1% (v/v) penicillin-streptomycin - hereafter referred to as cell culture media.

PC-3 cells were cultured in 75 cm² and 175 cm² flasks (depending on cell number required) with a seeding density of 30,000 cells/mL and allowed to grow to 75% confluency. At this point the
exhausted cell culture medium was discarded and the cells washed with DPBS, before being treated with 2-3 mL of TrypLE™. The flasks were re-incubated for a further 3 minutes to facilitate faster detachment of cells and minimise dwell time. The cells were checked under an optical microscope at 10X magnification to ensure that they had lifted from the surface of the flask before being resuspended in 8 mL of cell culture medium and centrifuged at 1000 RPM for 5 minutes. Following centrifugation, the supernatant was discarded and the cell containing pellet resuspended in 1 mL of cell culture media. A cell count was performed using a haemocytometer and viability calculated using Trypan Blue exclusion assay. The cells were transferred into a new flask (30,000 cells/mL) containing cell culture media and incubated (37 °C, 5% CO₂, 85% relative humidity (RH)) until they reached 75% confluency.

**4.3.2.2 Cell Line**

PC-3 cells, (human prostate cancer cells) derived from bone metastasis of a grade IV prostatic adenocarcinoma isolated from a 62-year-old male Caucasian were used for all cell culture studies described in this chapter. PC-3 cells have been well characterised since their isolation in 1979 and have demonstrated suitability in viability assays for various compounds since this time [10, 21, 22]. Generally speaking, macrophage cells, cancer cells and/or epithelial cells are employed for uptake studies [23] making them suitable for both purposes.

**4.3.2.3 Optimisation of Time Point for Cytotoxicity Experiments**

In regular cell culture conditions, cells are maintained in an environment of 10% serum (most commonly foetal bovine serum (FBS)) to promote growth and enhance the survival rate of cells [24]. The serum is rich in proteins and growth factors which makes it effective in replicating the protein rich environment of the blood. This study is intended to probe the behaviour of gold nanoparticles in protein rich and protein free environments in the hope that it will help to elucidate the role of the protein corona in mediating a cellular response. For this study, 3 environments will be investigated for cytotoxicity and nanoparticle uptake measurements as detailed in Table 4.1.
<table>
<thead>
<tr>
<th>Condition 1</th>
<th>Media</th>
<th>Gold nanoparticles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum free</td>
<td>RPMI 1640 media without supplementation</td>
<td>Pristine gold nanoparticles</td>
</tr>
<tr>
<td>Condition 2</td>
<td>RPMI 1640 media without supplementation</td>
<td>Pristine gold nanoparticles incubated for 3 hours in 5% foetal bovine serum</td>
</tr>
<tr>
<td>Serum incubated particles</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Condition 3</td>
<td>RPMI 1640 media supplemented with 10% foetal bovine serum</td>
<td>Pristine gold nanoparticles</td>
</tr>
<tr>
<td>Serum supplemented media</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 4.1: Conditions of varied protein levels utilised for cell culture experiments.

While Condition 3 is consistent with normal cell culture conditions, in the absence of FBS, Conditions 1 and 2 are potentially subjecting the cells to a state of starvation. When attempting to assess the toxicity of AuNPs, firstly it must be ensured that any effect of toxicity observed is primarily due to the presence of nanoparticles, and not the absence of serum. Thus a time course study of PC-3 cells was carried out to determine the maximal time point of unaffected viability in serum free media.

Figure 4.1: Optical microscope images of PC-3 cells after (a) 16 hours in serum supplemented medium, (b) 16 hours in serum free medium, (c) 24 hours in serum supplemented medium and (d) 24 hours in serum free medium.
The cells were plated out as described in the following Section 4.3.2.4, with both serum supplemented and serum free media. The percentage of viable cells was determined as the relative absorbance from serum free medium treated cells versus the absorbance of cells treated with serum supplemented medium (control). Figure 4.2 shows viability measurements which were collected at two hour intervals from 14-24 hours. To assess the morphology of cells, photographs were collected at 16 and 24 hour time points as shown in Figure 4.1.

It can be observed in Figure 4.1a and c, PC-3 cells growing in supplemented media at all time points are generally spindle shaped, possessing 2-3 long projections (pseudopodia) that extend from the cell body. Under conditions of prolonged serum depletion (Figure 4.1d), this morphology alters and the cells become further extended, thinner, and develop additional pseudopodia (up to 5 per cell) extending from the cell body. This change in morphology is a signal of cellular stress and is observed in cells after 24 hours of serum depletion. In addition to the changes in morphology, cells also show reduced cell number (which may be a function of decreased growth rate or loss of adhesion). These markers are not observed after 16 hours in serum free medium where normal morphology and expected cell number is observed in Figure 4.1b.

This is supported from a cytotoxicity perspective using MTT (Figure 4.2), where viability is not significantly altered until approximately 18 hours of serum depletion. A 16 hour time point was chosen for all further experiments to ensure cells are not experiencing stress after no significant difference was observed in cellular viability, or morphology.

It is interesting to note the possible initiation of a cell survival mechanism at approximately 24 hours post nutrient deprivation (Figure 4.2). Increased levels of reactive oxygen species (ROS) are generated by cells subject to suboptimal conditions – nutrient deprivation, oxygen deprivation etc. While high levels of ROS are detrimental – causing lipid peroxidation and cellular damage, there is also a danger associated with low levels of ROS as they are utilised by the immune system to destroy early cancer cells [25]. The balance of these reactive oxygen species is critical, however a sign of
slightly increased ROS levels is an increase in cellular growth rate [26]. This cell survival mechanism may be responsible for the slight recovery in cellular viability post 22 hours.

![Figure 4.2: MTT assay results for time point optimisation experiment comparing viability of serum supplemented and serum free media treated PC-3 cells.](image)

**4.3.2.4 MTT Assay**

MTT or 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is a yellow tetrazole which is capable of being reduced by living organisms into a purple formazan product [27, 28]. The MTT assay is used to assess viability by spectrophotometrically measuring the amount of insoluble formazan crystals which are produced when a viable cell cleaves the MTT tetrazolium ring using mitochondrial dehydrogenase.

The assay is performed by seeding PC-3 into a 96 well cell culture plate in 100 µl of cell culture medium at a density of 10,000 cells/mL. The plate were incubated (37 °C, 5% CO₂, 85% RH, 24 hours) before the media was removed and replaced with 90 µL fresh warmed media in fulfilment of the conditions outlined in Table 4.1;

**For condition 1 - Serum free:** RPMI 1640 media without any supplementation.

**For condition 2 - Serum incubated particles:** RPMI 1640 media supplemented with 0.5% foetal bovine serum used for control wells. This was required to normalise FBS levels across all wells.
due to the subsequent addition of AuNPs preincubated in 5% FBS (which provides a final concentration of 0.5% FBS in the well)

RPMI 1640 media without any supplementation was used for all wells containing AuNPs.

**For condition 3 – Serum supplemented media:** RPMI 1640 media supplemented with 10% foetal bovine serum was used.

Following the replenishment of media, 10 μL of nanoparticle solution or vehicle control (Milli-Q water) was added to the wells in triplicate. The plate was incubated (37 °C, 5% CO₂, 85% RH, 16 hours) before the addition of 10 μl of 5 mg/mL MTT solution, diluted in DPBS solution. The plate was then wrapped in aluminium foil to protect the light sensitive dye and re-incubated for a further 4 hours.

Following this period, the contents of the plate were carefully removed without disturbing the formazan crystals which had formed within the cells. To each well, 100 μL of DMSO was added and the contents resuspended 4 times to ensure adequate mixing and dissolution of the formazan crystals before being read using a Perkin Elmer Multimode Spectrophotometer at 595 nm. The percentage of viable cells was determined as the relative absorbance of AuNP treated cells compared with the absorbance of untreated control cells, averaged over the triplicate wells. Each assay was performed 3 times independently.

MTT assay was used to assess the toxicity of AuNPs to PC-3 cells in conditions of varied serum as outlined in Table 4.1. To assess toxicity, the cells were dosed with AuNPs to provide a final concentration range from 0.1 μM – 100 μM (concentration equivalent of Au ions).

Prior to AuNP toxicity testing, the four stabilising precursor chemicals were tested in Condition 1 and Condition 3. Condition 2 was not investigated due to the absence of a nanoparticle upon which a protein corona could form. Trisodium citrate, L-tryptophan, L-tyrosine and CTAB were tested in concentrations ranging from ranging from 0.1 μM – 100 μM (Figure 4.3). As predicted, trisodium citrate, L-tryptophan and L-tyrosine are biocompatible at high concentrations in both
conditions, while CTAB showed initial signs of toxicity at concentrations above 1 μM. In serum free conditions, almost total loss of viability was observed at 10 μM, while in 10% serum, this occurred at 100 μM. It is important to note that while some level of toxicity is associated with CTAB, the point at which this occurs is dependent on the cell type being tested. For comparison, Conner and co-workers tested CTAB on K562 cells (a human myelogenous leukaemic cell line) finding a total loss of viability at concentrations < 0.1 μM (in conditions equivalent to Condition 3 in this thesis.) With a toxic concentration which varies over 2 orders of magnitude between different cell lines, this highlights the danger of comparing toxicity results for CTAB stabilised AuNPs between groups.

![Graphs showing cytotoxicity profiles for free trisodium citrate, tryptophan, tyrosine and CTAB](image)

**Figure 4.3:** Cytotoxicity profiles for free trisodium citrate, tryptophan, tyrosine and CTAB as determined using MTT assay to assess the toxicity of the chemical species employed to stabilise AuNS.
4.3.2.5 Nanoparticle Preparation

To prepare AuNPs for biological testing, all nanoparticles were thoroughly washed (via dialysis or centrifugation) as outlined in Chapter 2. These purified AuNPs are referred to as pristine AuNPs hereafter.

After purification, MP-AES was performed on each nanoparticle solution to determine the concentration of gold for accurate dosing. To prepare the samples for MP-AES, a set of 4 gold standards (5 ppm, 10 ppm, 15 ppm, 20 ppm) as well as one blank solution were used to calibrate the instrument. Following this calibration, each sample was introduced to obtain a concentration in ppm which was then used to calculate the concentration of gold atoms in µM (or mg/mL).

To perform dose dependant toxicity experiments, AuNP dilutions were carried out approximately 3 hours prior to use. Serial dilutions were performed on each AuNP solution using Milli-Q water as the diluent. As Condition 2 required nanoparticles preincubated in 5% FBS v/v, the dilutions were modified such that the first dilution (1000 µM) contained 5% FBS v/v and all subsequent dilutions were performed with Milli-Q water supplemented with 5% FBS. The AuNP solutions were left to incubate at 37 ºC until required.

4.3.2.6 Determination of Gold Nanoparticle Uptake by ICP-MS

The extent of nanoparticle uptake was determined using ICP-MS following treatment of PC-3 cells with AuNPs in conditions of varying protein. To perform uptake experiments, PC-3 cells were seeded into multiple 75 cm² flasks at a seeding density of 100,000 cells/mL. The flasks were incubated (37 ºC, 5% CO₂, 85% RH, 24 hours) before the media was removed and replaced with fresh media to fulfil the conditions described in Table 4.1.

Following the replenishment of media, AuNPs were added to the flask. The concentration of AuNPs used was determined using the results from cytotoxicity testing presented in Section 5.1. The highest common tolerated dose among each group of AuNPs was used (i.e. a final concentration of 1 µM for chemically different AuNS and 10 µM for different shaped AuNPs). The flask was
reincubated (37 °C, 5% CO₂, 85% RH, 6 hours), which allowed for sufficient time for AuNP uptake to occur [29, 30].

Following this period the cells were washed three times with DPBS to ensure removal of free nanoparticles before treatment with TrypLE™ to dislodge the cells. Cells were resuspended in cell culture media and centrifuged at 3500 RPM for 10 minutes.

Following centrifugation, the supernatant was discarded and the cell containing pellet redispersed in cell culture medium before a cell count was performed. The sample was centrifuged again, with the supernatant discarded and the pellet resuspended in 70% ethanol before a final centrifugation. The resulting pellet was resuspended in 1 mL absolute ethanol, and placed in an oven at 80 °C for 8 hours to remove moisture.

To determine the gold content of the sample, gold standards were created at concentrations of 0, 5 ppb, 10 ppb, 20 ppb and 100 ppb dissolved in a solvent of 2% HNO₃ solution.

Each dried cell sample was dissolved in aqua regia for 20 minutes before making up to 5 mL with 2% HNO₃ solvent.

Using a 0.45 µm sterile filter, each sample was filtered into ICP-MS tubes (washed with aqua regia and rinsed with Milli-Q water) prior to being read. To ensure all samples were within the detection range of the machine, further dilutions were performed as necessary to account for different levels of AuNP uptake. Bismuth of 10 ng/mL was regarded as an internal standard.

Using the recorded cell count and correcting for dilution, the amount of gold per sample was determined in each case. Further calculations were performed to relate this value to the number of gold nanoparticles uptaken. Each sample was collected independently, in duplicate.

4.3.2.7 TEM Sample Preparation

Cells were exposed to nanoparticles for 6 hours, before being washed thoroughly with DPBS to remove free AuNPs. Cells were then fixed in a solution of 1% osmium tetroxide + 1.5% potassium
ferrocyanide for 60 minutes before being dehydrated in solutions of increasing ethanol concentration before finishing in absolute acetone. After dehydration, the cells were transferred to a resin-acetone mixture, moved to 100% resin and placed under vacuum before being embedded at 70 °C overnight for polymerisation. To image cells, a microtome was used to cut thin sections which were placed on TEM grids.

4.3.2.8 Statistics

MTT data was gathered from three independent experiments conducted in triplicate wells. Uptake data was determined from two independent samples. Data from both experiments are presented as mean ± SD. Statistical analysis was performed in Excel, with differences determined using two-tailed t tests or one-way ANOVA assuming significance at P=0.05 (indicated by asterisk).
4.4 References


Chapter 5: Viability and Uptake Studies – Results and Discussion
5.1 Cellular Viability Studies Following AuNP Treatment

5.1.1 Effect of Chemical Composition and Corona Formation on Cell Viability

Figure 5.1 shows the cell viability results for the chemically different nanoparticles in the different environments described in Table 5.1. In the case of citrate and tyrosine AuNS, results did not statistically vary until the highest dose (100 μM) was reached, while tryptophan showed significant difference at doses of 10 μM or higher (Figure 5.1). At the highest [Au] dose, the high serum environment provided in Condition 3 showed the highest viability in all 3 cases, with no toxic effect observed. In the case of citrate and tryptophan AuNS, no difference was observed between conditions 1 and 2 (77.2/73.8% and 85.2/89.4% respectively), highlighting that 3 hour incubation of these AuNS in serum prior to dosing created no effect. In the case of tyrosine AuNS, condition 1 showed the lowest degree of cell viability (52.2%) followed by condition 2 (79.9%) and condition 3 (106.1%) demonstrating that serum mediates toxicity, however to some extent, tyrosine AuNS are able to confer a protein corona during incubation.

<table>
<thead>
<tr>
<th>Condition 1 Serum free</th>
<th>Media</th>
<th>Gold nanoparticles</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>RPMI 1640 media without supplementation</td>
<td>Pristine gold nanoparticles</td>
</tr>
<tr>
<td>Condition 2 Serum incubated particles</td>
<td>RPMI 1640 media without supplementation</td>
<td>Pristine gold nanoparticles incubated for 3 hours in 5% foetal bovine serum</td>
</tr>
<tr>
<td>Condition 3 Serum supplemented media</td>
<td>RPMI 1640 media supplemented with 10% foetal bovine serum</td>
<td>Pristine gold nanoparticles</td>
</tr>
</tbody>
</table>

Table 5.1: Conditions of varied protein levels utilised for cell culture experiments.
In the case of CTAB/citrate AuNS, the highest dose tolerated was 1 μM, after which a total loss of viability was observed. As mentioned in Chapter 3, particle instability dictated a short dialysis period, producing particles which contained a high level of free CTAB in the surrounding solution which could not be removed. It is thought that for this reason, high levels of toxicity are observed in these particles. This hypothesis is supported by Figure 4.3 which shows the toxic effects of free CTAB. Analysis of results at 1 μM return statistically different results for each environment, with negligible toxicity observed in high serum (96.2%) as compared with the high levels of toxicity observed for Conditions 1 and 2 (22.6% and 25.8% respectively).

Considering each condition separately (Figure 5.2) allows the direct examination of the effect of chemical composition. Most obviously, the effect of high levels of CTAB within the CTAB/citrate
AuNS generated the highest level of toxicity in all environments with a total loss of viability in all environments at 10 μM. Viability is maintained up until 1 μM in 10% serum before dropping to zero (Figure 5.2c), whereas in serum free conditions, CTAB/citrate particles are significantly different to all other AuNS at the lowest dosage 0.1 μM (Figure 5.2a).

**Figure 5.2:** Cytotoxicity profiles for chemically different AuNS in different environments as determined using MTT assay. Shown above (a) Condition 1 – serum free, (b) Condition 2 – serum incubated particles, (c) Condition 3 serum supplemented media.

Comparison of citrate, tryptophan and tyrosine AuNS (all produced using chemicals generally deemed biocompatible) in typical cell culture conditions (Figure 5.2c) shows no significant difference with all groups failing to generate a cytotoxic response at 100 μM (99.6, 101.9 and 106.2% for citrate, tryptophan and tyrosine respectively). The results in serum supplemented medium are in agreement with reported values [1-4]; however the less explored area of serum free testing (Figure 5.2a) yielded different results. Most remarkably, tyrosine particles, which were previously deemed biocompatible in
serum, cause significant loss of viability (52.2%) in serum free conditions while citrate and tryptophan cause small declines in viability. There was no significant difference between the effects of the three particles after incubation with serum (Figure 5.2b), however a similar decline in cell viability is also observed at 100 μM.

In can be concluded from these observations that in typical cell culture conditions, citrate, tryptophan and tyrosine AuNS can be regarded as biocompatible. In serum free conditions, increasing toxicity is seen as tryptophan < citrate < tyrosine. Preincubating the AuNS in 5% serum prior to testing (Condition 2) had no significant effect in citrate or tryptophan AuNS, however in tyrosine (100 μM) and CTAB/citrate AuNS (1 μM) it proved to be an intermediate between Conditions 1 and 3.

5.1.2 Effect of Nanoparticle Shape on Cell viability

Figure 5.3 demonstrates that AuNS (CTAB) are reasonably well tolerated in all conditions up to a concentration of 10 μM, before a complete loss of viability is observed. A similar profile is created by AuNPr, however in this case, there is no statistical difference between all conditions at any concentration, while AuNS show increased viability in serum free conditions. This may be explained by the initiation of a cellular survival response due to mildly stressful stimulus (low levels of CTAB, serum free conditions) [5]. This response may also explain the increased viability observed in Figure 5.2a in the case of CTAB/citrate AuNPs at 0.1 μM.

While ultimately the AuNS (CTAB) cause total cellular death, at low levels the cellular defence mechanism can promote increased proliferation, which appears as increased viability using the MTT assay.
AuNR are reasonably well tolerated in all conditions, even at 100 μM dosage levels (84.8, 95.1 and 90.9% for Conditions 1, 2 and 3 respectively). While many groups have previously tested AuNR (CTAB) and determined them to be cytotoxic [6-8], our results are in agreement with other groups, who after thoroughly washing their AuNR, found them to be well tolerated by cells [2, 7]. Hauck et al. demonstrates similarly that comparable results are obtained in serum containing and serum free conditions, with only slight improvement in viability seen in Condition 3 at 100 μM [9].

Figure 5.3: Cytotoxicity profiles for various shaped AuNPs stabilised by CTAB, determined using MTT assay.

AuNC show no statistical difference between conditions at 100 μM dosage, and also appear to be well tolerated by the cells in all environments (92.7, 92.4 and 89.7% for Conditions 1, 2 and 3 respectively).
In comparison to the group of AuNS, the CTAB stabilised shapes show less variation between conditions. It is hypothesised that this may be reflective of the extent of protein corona formation on AuNPs stabilised with CTAB. Cifuentes-Rius and co-workers investigated the impact of CTAB on the binding of surface proteins, finding that a CTAB concentration < 1000 μM created particles with negligible protein corona formation [10]. The group found that higher CTAB concentrations allowed protein corona formation to occur, however it did not occur linearly due to the surfactant nature of CTAB. As concentrations of CTAB exceeded the critical micelle concentration the molecules formed micellar structures which also attracted proteins, lowering the amount available from within the solution [10]. It is estimated that CTAB concentrations are well below the CMC, and are indeed <1000 μM in the AuNP solutions tested due to repeated centrifugation and dilution with Milli-Q water.

With no remarkable difference observed between Conditions 1-3, the comparison of CTAB stabilised AuNPs within each environment is presented in Figure 5.4. AuNR and AuNC appear to be well tolerated by the cells, while AuNS and AuNPr are toxic at concentrations >10 μM. The toxicity observed by AuNS (CTAB) may be in part explained in the next section where it is shown that cells dosed with AuNS take up far higher levels of gold as compared to AuNR, AuNPr and AuNC. The same reasoning however does not explain the toxicity observed in AuNPr which are taken up in significantly lower levels.
This trend suggests that the shape of prismatic particles may be responsible for the cytotoxic response observed. While a handful of papers exist on the biocompatibility of biologically synthesised gold nanotriangles [11, 12], the effect of CTAB synthesised AuNPr remains largely unknown. In contrast, the antibacterial effect of silver triangles has been explored in greater detail, with multiple groups finding that the shape of prismatic (or triangular) particles plays a vital role in the particle’s antibacterial activity. While the antimicrobial effect of silver has been explored for many years, much of this work relates to bulk silver, and more recently nanoparticulate silver – most commonly in the form of spherical nanoparticles [13, 14]. Kasbohm and co-workers compared the antibacterial effect of spherical and prismatic silver nanoparticles, finding that the edges and vertices inherent to prismatic particles are the causative factor for the significant cellular damage that was observed. While spherical particles are known to enter the cell preferentially and in higher numbers, the geometry of prismatic particles have been hypothesised to cause higher levels of toxicity due to
disruption and damage to the cellular structure which occurs after ingestion of the particles [15]. This idea was maintained by Qureshi et al. after working with both Gram positive and negative bacteria [16]. While the likelihood that mechanical intracellular damage could be caused by slowly diffusing AuNPs remains to be proven, further work must be performed focusing on the impact of particle geometry on intracellular structures. It is also possible that this effect relates to the crystallography of prismatic particles and their influence on AuNP-protein interactions, however this remains unconfirmed.

5.2 Cellular Uptake Studies Following Treatment with AuNPs

To measure cellular uptake of AuNPs, cells were grown and subjected to nanoparticle treatment as described in Section 4.3.2. To compare the extent of AuNP uptake, data is first presented as the amount of gold (pg) taken up per cell - by dividing the total amount of gold detected (determined using ICP-MS) by the total number of cells collected (determined by a cell count performed after washing the cells thoroughly post treatment). Secondly, the number of AuNPs taken up per cell (P_{cell}) is presented by calculating the average weight of a single nanoparticle from each sample based on TEM measurements using the following equations.

For spherical AuNPs the total number of atoms per particle (N_{particle}) is given by;

\[ N_{\text{particle}} = V \frac{\rho \cdot N_A}{M} \]

Where \( V \) is the volume of a sphere, defined by the density (Au \( \rho = 1.93 \cdot 10^7 \text{ g/m}^3 \)), molar mass (Au \( M = 196.97 \text{ g/mol} \)) and Avogadro’s constant (\( N_A \)). Which can be simplified to;

\[ N_{\text{particle}} = \frac{\pi \rho \cdot N_A}{6M} D^3 = 30.89 \cdot D^3 \]

Where \( D \) is the average diameter of the spherical particle (determined via TEM measurements).
For cubic, rod shaped and prismatic particles the total number of atoms per particle \( N_{\text{particle}} \) was determined similarly after calculating the volume \( V \) of each particle using the relevant volume equation for each shape.

To determine the weight of each particle, the weight of a singular gold atom was calculated;

\[
m_{\text{atom}} = \frac{M}{N_A} = \frac{196.97}{6.022 \cdot 10^{23}} = 3.27 \cdot 10^{-22} \text{g}
\]

Thus particle weight \( m_{\text{AuNP}} \) was determined as follows and summarised in Table 5.2;

\[
m_{\text{AuNP}} = m_{\text{atom}} \cdot N_{\text{particle}}
\]

<table>
<thead>
<tr>
<th>AuNP</th>
<th>Average measurement</th>
<th>( m_{\text{AuNP}} ) (*10^{-18} \text{g})</th>
<th>Volume (*10^{-25} \text{m}^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrate AuNS</td>
<td>12.5 (diameter)</td>
<td>19.7</td>
<td>10.2</td>
</tr>
<tr>
<td>Tryptophan AuNS</td>
<td>8.4 (diameter)</td>
<td>5.9</td>
<td>3.1</td>
</tr>
<tr>
<td>Tyrosine AuNS</td>
<td>9.9 (diameter)</td>
<td>9.8</td>
<td>5.1</td>
</tr>
<tr>
<td>CTAB/citrate AuNS</td>
<td>8.9 (diameter)</td>
<td>7.1</td>
<td>3.7</td>
</tr>
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<td>AuNS (CTAB)</td>
<td>77.9 (diameter)</td>
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<td>2475.2</td>
</tr>
<tr>
<td></td>
<td>15.3 (width)</td>
<td>270</td>
<td>137.6</td>
</tr>
<tr>
<td>AuNR (CTAB)</td>
<td>58.8 (length)</td>
<td>1900</td>
<td>970.8</td>
</tr>
<tr>
<td>AuNPr (CTAB)</td>
<td>94.7 (side length)</td>
<td>2100</td>
<td>1071.7</td>
</tr>
<tr>
<td>AuNC (CTAB)</td>
<td>47.5 (side length)</td>
<td>2100</td>
<td>1071.7</td>
</tr>
</tbody>
</table>

**Table 5.2:** Dimensions of AuNPs (determined using TEM) as well as calculated weights and volumes of all AuNPs.

5.2.1 Effect of Chemical Composition, Protein Corona Formation and Shape on Cellular Uptake

Most notable from Figure 5.6 is that increased uptake is observed in serum free conditions. In all cases, AuNPs are taken up in greater numbers in Condition 1 – Serum Free, as compared with Condition 3 – Serum Supplemented Media. Testing in Condition 2 – Serum Incubated Particles - had varied results. In most cases (citrate, tryptophan, CTAB/citrate, AuNC and AuNPr), no significant difference between Condition 1 and 2 was observed, while in the case of tyrosine and AuNS (CTAB) an intermediate result between Conditions 1 and 3 was obtained. A two tailed t test indicated no significant difference between conditions affecting the uptake of rod shaped particles, despite results suggesting a slight increase of uptake in serum free conditions.
The effect of serum on uptake has divided the research community. Earlier papers comparing nanoparticle uptake in serum positive and negative conditions commonly found a trend of increasing uptake in serum positive media [17-20]. Interestingly, more recently published papers indicate the opposite trend showing enhanced uptake in serum free media, with some groups changing their stance over time [20-24].

It is generally agreed that nanoparticles are internalised via receptor-mediated endocytosis (also known as clathrin-mediated endocytosis) [6, 25-27]. This process usually involves recognition of specific proteins by receptors localised on the cellular surface, however Smith and co-workers have shown that this mechanism may still proceed in the absence of serum proteins via non specific binding [27]. After associating with the cellular surface, nanoparticles are internalised into vesicles, formed by invagination of the cellular membrane [26]. As the vesicles age, they transform first into endosomes, then further into lysosomes which are known to be acidic in nature due to their importance as cellular digestion machines. The various stages of this process can be observed in Figure 5.5. It is these vesicles which are responsible for the intracellular transport of nanoparticles as well as proteins, fluids, and nutrients. In normal circumstances, removal of intracellular material or exocytosis can proceed in the opposite manner whereby vesicles move toward the cellular membrane, fuse and transfer their contents to the extracellular environment [28].

For receptor-mediated endocytosis to occur, cells generally seek out specific serum proteins to bind to, corresponding to cellular receptors on the surface of the cell [6]. While there are over 3,000 proteins identified in human serum, it is estimated that any nanoparticle may only bind to several dozen of these [29, 30]. For uptake of AuNPs coated in serum proteins to occur, not only must they contain the specific species of protein that the cell is inclined to uptake, but the protein must be oriented on the nanoparticle surface such that the correct portion is exposed to allow binding to occur.

It is hypothesised that these factors actually work to reduce uptake of protein coated AuNPs by reducing membrane interactions [27, 31], an idea which is supported by the findings presented in this Chapter.
Figure 5.5: The endocytosis of AuNC (CTAB) is shown in various stages, with particles (1) localising on the cellular surface, (2) invagination of a portion of the cellular membrane and (3) particles localised inside vesicles.

Furthermore, it has been suggested that competition for binding sites may occur between bound and unbound proteins [18]. While further work must be done to substantiate this idea, it is plausible considering finite receptor sites are available on the cellular surface. Given this fact, unbound proteins with no restriction on their orientation may be more likely to bind to the available sites, blocking AuNP bound proteins, therefore limiting uptake.

In addition to the effect of serum on endocytosis, it appears that serum proteins affect exocytosis of AuNPs, which may enhance intracellular concentration. Panyam and Labhasetwar investigated this relationship finding that in serum free conditions, exocytosis of nanoparticles was almost totally prevented [32]. Conversely, nanoparticle bound serum was critical in the movement of nanoparticles back across the plasma membrane [33]. Thus in addition to increased uptake observed in serum free conditions, it is also expected that decreased removal of AuNPs creating higher levels of internalised AuNPs at a given time occurs in comparison with serum supplemented media.
In addition to this trend, it is observed that while the small spherical AuNPs are taken up in significantly higher numbers, the amount of gold internalised is much lower compared with the larger CTAB stabilised shapes. From Table 5.2 it can be seen that the volumes of the particles differ by several orders of magnitude. While the small AuNS range from $3.1 \times 10^{-25}$ m$^3$ for tryptophan and citrate respectively, the larger CTAB AuNPs range from $137.6 \times 10^{-25}$ m$^3$ for AuNR (CTAB) and AuNS respectively (CTAB). Thus the average weight of the small AuNS (citrate, tryptophan, tyrosine and CTAB/citrate) is over 200 times less than the average weight of the larger CTAB stabilised AuNPs. Therefore calculation of particle uptake for CTAB stabilised AuNPs equates to a much lower number of particles.
The tendency of smaller particles to be taken up in higher numbers as seen in Figure 5.6b as compared with Figure 5.6d is common among research groups. Generally speaking, particles in the range of 25 - 50 nm have been found to have the highest uptake while particles >50 nm show lower levels of uptake [34-36]. This concept may be explained using the principles which underpin particle wrapping. Before interaction with AuNPs, receptors are thought to be evenly spaced on the cellular surface, however after contact with AuNPs, receptors rearrange on the surface, localising under the AuNP to produce sufficient free energy to facilitate particle wrapping [35]. Logically, smaller particles require less energy to be internalised and will connect with a lower number of cellular receptors as compared with larger particles. Larger particles require a greater number of cellular receptors such that over time, there is insufficient coverage of receptors on the cell surface which may limit (and slow) the internalisation of additional particles [37].

Furthermore, this principle could explain why AuNPr (CTAB) are taken up in the lowest numbers comparatively. While AuNS (CTAB) have the largest surface area of all the particles, the point of contact a sphere makes with the cell is significantly less than a shape with flat sides resting on top of the cellular surface. The surface area of AuNPr > AuNC >AuNR which inversely correlates with the number of particles internalised. This provides evidence that cellular receptors sites may significantly diminish after repositioning to internalise large particles highlighting the effect of shape on cellular uptake of AuNPs.

Figure 5.6 a and b demonstrate higher uptake levels for amino acid stabilised tryptophan and tyrosine AuNS as compared with citrate and CTAB/citrate AuNPs. It has long been known that certain amino acids influence the binding and uptake of extracellular material into the cell. The importance of aromatic residues at cellular receptor sites (namely from tryptophan and tyrosine) in the uptake of lipoproteins was highlighted by Goldstein et al. in 1987, however the relevance of this finding in relation to the uptake of AuNPs required further evidence [38]. More recently, Yang et al. uncovered the importance of aromatic groups on the endocytosis of AuNPs. By surface modifying AuNPs with aromatic group bearing amino acids (including tryptophan and tyrosine) as well as non aromatic amino acids (leucine, isoleucine and valine), they discovered that the aromatic ring had
strong affinity for certain regions of the cellular membrane where it induced binding and AuNP wrapping to generate more efficient internalisation of AuNPs [39].

To allow further comparison between both groups of AuNPs, Figure 5.7 presents the particle uptake in terms of the percentage of the original dose that the cells internalised. When performing uptake studies, cells were dosed at the highest sub toxic concentration common to the group, thus AuNS and AuNP (CTAB) were subjected to 1 μM and 10 μM doses, respectively. While the trends within the groups remain the same, what becomes more obvious at this level is the enhanced uptake of spherically shaped particles. While the decreased uptake of CTAB/citrate stabilised AuNS cannot be explained, with the exception of these particles, the remaining spheres are taken up in higher numbers as compared with AuNR and AuNPr.

![Figure 5.7: Uptake normalised for percentage of original dose taken up per million cells.](image)

Cubic shaped AuNPs present an interesting result, taken up in comparable amounts in relation to citrate and tryptophan AuNS. In this case, some explanation may be gained by considering the geometry of a cube in relation to a sphere (particularly given the rounded edges observed in TEM imagery – Figure 3.22d) whereby for both shapes, all three dimensions share a common measurement. A recent simulation study performed by Liu et al. showed that when considering shape alone, this was an important factor in endocytosis, with the energy required to internalise different shaped particles
(and consequently the time required for particle engulfment and internalisation) was found to be in the order of spheres < cubes < rods < disks [40].

5.2.1.1 Microscopy

The uptake of CTAB stabilised AuNPs was confirmed by TEM, presented in Figure 5.8. Cells were prepared as detailed in Section 4.3.2.7.

Figure 5.8: TEM images of PC-3 cells after exposure to CTAB stabilised AuNPs (a) AuNS, (b) AuNR, (c) AuNPr and (d) AuNC (AuNPs indicated by red arrows).

While ICP-MS was performed for quantification of AuNP uptake, TEM was used to obtain clear imagery. In order to have a reasonable number of AuNPs to allow visualisation of the uptake process, cells were subjected to lower levels of AuNPs (0.1 μM equivalent gold concentration).
In each case, the CTAB stabilised AuNPs were internalised, but did not appear to enter the nucleus. Instead AuNPs appear to be localised within vesicles, with multiple AuNP containing vesicles present inside a given cell. Inspection of the AuNPs shows unagglomerated particles present within the vesicles. Higher magnification images are provided for AuNR and AuNPr samples whereby the original morphology of the particles appears to be preserved after internalisation (Figure 5.9). While ICP-MS cannot discern between particles associated with the cellular membrane and those actually internalised, TEM images show that AuNPs are predominantly located within the cell, while a small percentage exist on the surface as in Figure 5.5 (most likely in the process of endocytosis or exocytosis).

**Figure 5.9:** (a) AuNR and (b) AuNPr, shows preservation of AuNP morphology after internalisation. Red arrow indicates individual prismatic particle, blue arrow indicates prismatic particles with clipped edges located inside a vesicle. Inset for (a) and (b) show enlarged areas to better illustrate AuNP morphology.
5.3 Summary

This study highlights several important trends which relate AuNP chemical structure and shape with cellular uptake and toxicity. Firstly, by observing the cytotoxicity of AuNS it has been demonstrated that it is possible for chemical stabilisers to elicit cytotoxic effects. It is apparent that to some extent, the presence of serum can mediate this toxic effect; however similar trends are generally observed in both serum supplemented and serum free conditions. Pre-incubation of the nanoparticles in serum was only effective in providing protection (as compared with serum free conditions) for two AuNPs (tyrosine and CTAB/citrate), while the remaining six AuNPs afforded no appreciable protection from a protein corona generated from pre-incubated proteins. CTAB stabilised shapes showed minimal variation in varied serum conditions, which is speculated to be due to insignificant protein corona formation in serum supplemented media.

Probing the effect of shape demonstrated that thoroughly washed AuNR (CTAB) and AuNC (CTAB) were well tolerated by the cells, while toxicity was observed in AuNS (CTAB) and AuNPr (CTAB). It is hypothesised that the toxicity caused by AuNS is an indirect effect of shape. While spherical particles are generally found to be biocompatible, they have been demonstrated in this study, to be taken up in high numbers by cells. While levels of CTAB on the surface of an individual AuNS (CTAB) are low, it is proposed that after high levels of uptake, an accumulative effect of internalised CTAB may cause toxicity. On the other hand, the mechanism underlying the toxicity caused by AuNPr remains unknown. While this may be a direct effect of its angular shape on cellular structure – this hypothesis remains unproven.

When considering uptake, observing some level of uptake in all conditions, provides validation for toxicity data by dispelling the possibility that nanoparticles were not sufficiently stable in conditions of varied serum levels to remain at a size that would allow cellular uptake or cause toxicity. This follows preliminary stability studies of AuNPs in varied serum conditions (not shown) that support this fact.
It also appears that the uptake study highlights a complex relationship between AuNP shape and cellular uptake with at least two competing principles determining the uptake of different shapes. While AuNP surface area impacts on the availability of cellular receptors – as it appears that shapes with broad flat faces diminish the availability of surface receptors (causing less efficient uptake of these particles), the energy required for cellular uptake differs based on geometry, and thus certain shapes require fewer cellular receptors for endocytosis, which can occur more rapidly. It can be concluded based on these facts that spherical particles are taken up preferentially as their geometry makes contact with fewer receptors on the cellular surface, and also requires less free energy for particle wrapping and subsequent endocytosis.

The level of serum has a great affect in the amount of uptake that occurs (though less so on the overall profile generated by the nanoparticles). It is proposed that decreased uptake in the presence of serum is due to decreased AuNP-membrane interactions in the presence of serum. In this environment, free serum proteins may compete for binding sites, preventing AuNPs from binding and subsequently being internalised.

Increased uptake of amino acid stabilised AuNS – stabilised with tryptophan or tyrosine, provided evidence that chemical stabilisers can elicit an effect on cellular uptake. It is thought that the geometry of the aromatic rings induces particle wrapping after contacting the cellular surface. In total these results demonstrate that AuNP chemical structure and shape can influence particle uptake and toxicity though future work is necessary to conclusively prove the mechanism behind some of these interesting observations and trends.
5.4 References


Chapter 6: Assessing the Stability of Gold Nanoparticles and their Behaviour at the Biological Interface
6.1 Stability of Gold Nanoparticles

6.1.1 Introduction

As the use of nanoparticles in mainstream therapies increases, the behaviour and stability of AuNPs over extended time periods will be an important factor to consider. If AuNPs with varied geometric profiles such as gold nanorods are employed, the preservation of such features could be vital to their intended use.

Typical AuNP synthesis procedures employ a stabilising agent to stop the growth process and deter the particles from forming agglomerates in solution [1]. Common stabilisers include sodium citrate, polyethylene glycol (PEG), tetraoctylammonium bromide (TOAB) and cetyltrimethylammonium bromide (CTAB) [2, 3]. The dual nature of the stabilisers role means that the synthesis of larger particles requires a lower concentration of the stabiliser present, though this has obvious implications on the stability of the resulting particles. A further complication arises when considering AuNPs intended for human internalisation. While all AuNP solutions must be purified prior to use, in the case of AuNPs stabilised with CTAB, a more thorough washing technique must be adopted to avoid toxic effects generated from the stabiliser itself while leaving sufficient CTAB present to preserve particle stability.

Variables which impact on the stability of AuNPs over time include surface coating, zeta potential and concentration, as well as storage conditions such as temperature, lighting, the composition of the surrounding medium and the vessel used to contain them [4, 5].

In this study, the stability of each AuNP synthesised in Chapter 3 was monitored using UV-visible spectroscopy and DLS over a period of 6 months. The combination of these techniques will uncover changes in AuNP size and shape over the time course.
6.1.2 Experimental

The AuNP samples utilised in this study were used after undergoing synthesis and purification as described in Chapter 3. Approximately 2 mL of each AuNP solution was stored in a 10 mL tube at room temperature in dark conditions for the course of the experiments. At one month intervals, each AuNP solution was transferred to a quartz cuvette for UV-visible spectroscopic reading, before being transferred to a glass DLS tube for DLS readings. The solutions were then returned to the original 10 mL tube, ensuring the same solution was used for subsequent tests.

6.1.3 Results and Discussion

Figure 6.1 shows the UV-visible spectroscopy profiles of citrate, tryptophan, tyrosine and CTAB/citrate AuNS over a 6 month period. In the case of all AuNPs, some degree of particle agglomeration occurred as evidenced by the decrease in peak intensity over time [6]. This is most pronounced for citrate AuNS which show a significantly weaker signal with each subsequent month, and is least evident for CTAB/citrate AuNS.

In addition to the weakened signal, citrate AuNPs also exhibit significant red shift after 6 months, as demonstrated by a shift in peak position from 520 nm to 530 nm. This is caused by the coupling of surface plasmons as the particles draw closer together [7]. Tryptophan, tyrosine and CTAB/citrate AuNPs did not demonstrate this trait.

These findings are supported by the DLS results presented in Figure 6.2. While the position of the peaks remain very close for all samples over 6 months - evidence that the greater population of AuNPs within the sample remain relatively unchanged in size - the peak width broadens in the case of tyrosine, tryptophan and most significantly, citrate AuNS.

This broadening indicates a wider size distribution within the sample. Additional peaks at higher radii values also present over time in the case of most AuNPs, most notable in the case of tryptophan AuNS. This represents a small population of large particles forming within the sample.
Figure 6.1: UV-Visible spectroscopy measurements of (a) citrate, (b) tryptophan, (c) tyrosine and (d) CTAB/citrate AuNS over a 6 month period.
Figure 6.2: DLS measurements of (a) citrate, (b) tryptophan, (c) tyrosine and (d) CTAB/citrate AuNS over a 6 month period.

Figure 6.3 shows the UV-visible spectroscopy profiles of CTAB stabilised AuNS, AuNR, AuNPr and AuNC. All shapes show some degree of agglomeration evidenced by a weakening signal over time, although this is quite minimal in the case of AuNS. AuNR show initial blue-shift after the first measurement which may be indicative that the synthesis had not fully concluded when the initial measurement was taken and the particles became further stabilised in the days (or weeks) following synthesis. The preservation of both peaks in the case of AuNR and AuNPr provides evidence that the shape of both particles is maintained over time.
Figure 6.3: UV-visible spectroscopy measurements of (a) AuNS, (b) AuNR, (c) AuNPr and (d) AuNC over a 6 month period.

Figure 6.4 shows the DLS measurements for the CTAB stabilised particles, and supports the findings deduced from UV-visible measurements. The AuNS sample shows minimal changes over the course of 6 months and were the most stable of the particles studied. As mentioned in Chapter 3, the interpretation of DLS measurements on non-spherical shapes is a complex procedure, beyond the scope of this Thesis, however some information can be gained by comparing these measurements over time. In the case of AuNR, the initial peak obtained in the first measurement (centred at 1 nm) provides further proof that the synthesis may not have run to completion. Particles of this size may be seed particles remaining from the growth process which could later form particles in the solution. Measurements taken from the second month onwards do not indicate significant changes within the
sample in terms of shape and size, despite a small degree of agglomeration. This is true for AuNPr and AuNC where in both cases, changes are observed after the first measurement, after which the samples remain fairly unchanged with only small amounts of broadening in the signal.

Figure 6.4: DLS measurements of (a) AuNS, (b) AuNR, (c) AuNPr and (d) AuNC over a 6 month period.
6.2 Interaction of Different Shaped Gold Nanoparticles with Human Serum Albumin

6.2.1 Introduction

The most abundant protein found in human blood [8], human serum albumin (HSA) has many roles including the regulation of blood pH and colloid osmotic pressure and the transport and circulation of various compounds including fatty acids, hormones and drugs [8, 9]. Preservation of certain parts of the complex structure of a protein such as HSA is vital to ensure it continues to behave as expected [10], however exposure to various ligands including AuNPs have been shown to induce conformational changes [11, 12]. These changes may alter or inhibit the functioning of the protein which could potentially yield serious health effects.

It has been shown that differences in the shape and size of AuNPs produce varied effects on the structure and function of certain proteins [13, 14], hence, these factors must be considered when designing therapies and medicines which will come into contact with a protein rich environment such as the blood.

Fluorescence spectroscopy is one of many techniques which can be employed to study the changes that proteins undergo when exposed to AuNPs [13, 15, 16]. Some proteins possess intrinsic fluorescent properties due to the presence of tryptophan, tyrosine or phenylalanine in their structure [17]. In the case of HSA, its amino acid sequence contains a single tryptophan residue which is contained in the hydrophobic cavity of sub domain IIA (Sudlow I) [18].

After excitation at 295 nm, HSA produces an emission peak - attributed to its fluorophore tryptophan - between 300 and 500 nm. Conformational disturbances in the protein can be detected by measuring changes in the intensity of this peak after interaction with other compounds including AuNPs.
The IIA sub domain has been shown to play an important role in the binding of various ligands including AuNPs [19, 20]. Furthermore, the single tryptophan in HSA is known to be very sensitive to changes in the surrounding environment and as such, provides a good opportunity to study the interaction between HSA and AuNPs [13].

6.2.2 Materials

The AuNP samples used in this study were obtained after synthesis and purification as described in Chapter 3. Human serum albumin (HSA) was used as purchased from Sigma-Aldrich and diluted using Milli-Q water as required.

6.2.3 Experimental

To study the interaction of AuNPs with HSA, fluorescence measurements were performed using a Horiba FluorMax 4 – Compact Spectrofluorometer which utilises a 150W Xenon arc lamp, Czerny-Turner monochromator and a R928P photon counting PMT detector with internal temperature control. In accordance with the fluorescent behaviour of tryptophan, spectra were generated by exciting each sample at 295 nm and collecting the resulting emission spectra from 300 – 500 nm.

Due to the intrinsic fluorescence of tryptophan and tyrosine, these AuNPs were unsuitable for fluorescence studies and thus excluded from these experiments.

A series of concentration (2.5, 25, 50, 100 and 250 μM) and temperature (20, 30, 40, 50 and 60 °C) dependent readings were obtained by incubating the AuNPs with a fixed (final) concentration of HSA (200 μg/mL) for 5 minutes. This time point was determined via a time optimisation study to be sufficient to allow AuNP-HSA binding to occur and stable measurements to be obtained after temperature stabilisation.
6.2.4 Results and Discussion

The relationship between fluorescence quenching (Q) and fluorescence intensity (F) can be assessed using Equation 6.1 [13]

\[ Q = \left( \frac{F_0 - F}{F_0} \right) \]

Equation 6.1

To quantify this relationship, fluorescence measurements were performed on HSA in the absence \( F_0 \) and presence \( F \) of AuNPs. While the final concentration of HSA remained the same in each experiment (200 μg/mL), the concentration AuNPs was varied from 2.5 to 250 μM.

The results shown in Figure 6.5 demonstrate that quenching of HSA’s fluorescence does occur in the presence of AuNPs as shown by the diminishing peak maxima with increasing nanoparticle concentration. It should also be noted that the AuNP solutions were not inherently fluorescent.
Broadly speaking, there are two main mechanisms by which fluorescence quenching occurs; viz. dynamic or static [21]. Dynamic quenching stems from collisions between the fluorophore and the fluorescence quencher and results in a loss of excited state energy. In the case of dynamic quenching, the collisions must occur while the fluorophore is in an excited state. On the other hand, static quenching occurs as a result of a non-fluorescent complex forming between the fluorophore and the quencher, a process which can occur before the fluorophore is excited.
Interactions can be categorised as being either static or dynamic, or a combination of both by employing the Stern-Volmer equation (Equation 6.2) [22].

\[
\frac{F_0}{F} = 1 = k_q \tau_0 [Q] = 1 + K_{SV} [Q]
\]  
Equation 6.2

Where \( k_q \) is the biomolecular quenching rate constant, \( \tau_0 \) is the lifetime of the fluorophore (approximately equal to 4.9 ns for HSA [23]) \([Q]\) is the concentration of AuNPs and \( K_{SV} \) is the Stern-Volmer constant.

By plotting \( \frac{F_0}{F} \) versus \([Q]\), Stern-Volmer plots were generated for all AuNPs as shown in Figure 6.6. The Stern-Volmer constant is calculated as the slope of the linear regression fit of the data points.

Linear agreement to the equation usually signifies either static or dynamic quenching while positive deviation can signify a combination of both mechanisms. Figure 6.6 shows that AuNS (CTAB), AuNR and AuNC show linear agreement across all concentrations measured, while AuNPr, Citrate AuNS and CTAB/citrate AuNS show linear fit up until a concentration of 100 μM. Given this fact, it is possible that some AuNPs may exhibit different mechanisms of quenching at different concentrations. To distinguish the quenching mechanism, multiple temperature measurements are required to differentiate the quenching as being either static or dynamic [21]. Thus, the full concentration range was used to determine the quenching mechanism for AuNS (CTAB), AuNR and AuNC, while the linear portion (0-100 μM) was used to determine the quenching mechanism for AuNPr, Citrate AuNS and CTAB/citrate AuNS.
Figure 6.6: Stern-Volmer plots for (a) AuNS (CTAB), (b), AuNR (CTAB), (c) AuNPr (CTAB), (d) AuNC (CTAB) (e) Citrate AuNS and (f) CTAB/citrate AuNS. Measurements were collected at 20 °C.

In the case of dynamic quenching which relies on collisions, $K_{SV}$, is expected to increase with temperature as the diffusion rate increases. On the other hand, static quenching is inversely related to temperature with stronger complexes formed at lower temperatures.

To determine the method of binding between each AuNP and HSA, Stern-Volmer plots were generated over a range of temperatures from 20 – 60 °C. As an example, Figure 6.7 shows the Stern-Volmer plot for AuNS (CTAB) at temperatures from 20- 60 °C while the results for all AuNPs are presented in Table 6.1.
Figure 6.7: Stern-Volmer plots for HSA quenching by AuNS (CTAB). Measurements were taken from 20-60 °C and show decreased $K_{SV}$ with increasing temperature.

<table>
<thead>
<tr>
<th>Temperature</th>
<th>$K_{SV}$</th>
<th>R</th>
<th>$K_{SV}$</th>
<th>R</th>
<th>$K_{SV}$</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 °C</td>
<td>3.447</td>
<td>0.997</td>
<td>5.114</td>
<td>0.995</td>
<td>2.063</td>
<td>0.992</td>
</tr>
<tr>
<td>30 °C</td>
<td>3.491</td>
<td>0.997</td>
<td>4.870</td>
<td>0.996</td>
<td>2.220</td>
<td>0.926</td>
</tr>
<tr>
<td>40 °C</td>
<td>3.292</td>
<td>0.997</td>
<td>4.674</td>
<td>0.995</td>
<td>1.500</td>
<td>0.933</td>
</tr>
<tr>
<td>50 °C</td>
<td>3.259</td>
<td>0.997</td>
<td>4.553</td>
<td>0.994</td>
<td>1.560</td>
<td>0.861</td>
</tr>
<tr>
<td>60 °C</td>
<td>2.938</td>
<td>0.997</td>
<td>4.259</td>
<td>0.998</td>
<td>0.926</td>
<td>0.949</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Temperature</th>
<th>$K_{SV}$</th>
<th>R</th>
<th>$K_{SV}$</th>
<th>R</th>
<th>$K_{SV}$</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 °C</td>
<td>2.306</td>
<td>0.980</td>
<td>5.098</td>
<td>0.975</td>
<td>11.672</td>
<td>0.991</td>
</tr>
<tr>
<td>30 °C</td>
<td>2.122</td>
<td>0.989</td>
<td>4.724</td>
<td>0.982</td>
<td>11.656</td>
<td>0.989</td>
</tr>
<tr>
<td>40 °C</td>
<td>1.914</td>
<td>0.985</td>
<td>3.509</td>
<td>0.996</td>
<td>10.361</td>
<td>0.997</td>
</tr>
<tr>
<td>50 °C</td>
<td>1.603</td>
<td>0.983</td>
<td>3.051</td>
<td>0.940</td>
<td>9.520</td>
<td>0.995</td>
</tr>
<tr>
<td>60 °C</td>
<td>1.931</td>
<td>0.985</td>
<td>2.426</td>
<td>0.987</td>
<td>10.076</td>
<td>0.996</td>
</tr>
</tbody>
</table>

Table 6.1: Calculated Stern-Volmer constants for the quenching of HSA by each AuNPs from 20-60 °C. $R$ is the linear correlated coefficient for $K_{SV}$ values. $K_{SV}$ values are expressed as $\times 10^3 \text{ L mol}^{-1}$.

The $K_{SV}$ values presented in Table 6.1 establish that all AuNPs tested exhibit static binding.

In the case of dynamic quenching, $K_{SV}$ values provide an indication on the accessibility of the fluorophore to the quencher. On the other hand, for static quenching, $K_{SV}$ values provide an indication
of the association constant for the newly formed complex between the fluorophore and the quencher [8, 24].

Thus it can be said that strength of binding between the AuNPs and HSA is strongest in the case of CTAB/citrate AuNS, followed by AuNR > citrate AuNS > AuNS > AuNC AuNPr. While there are very limited studies performed in this area as a point of comparison, one example of such a study was performed by Nandi and co-workers who compared the binding of 40 nm AuNPs (AuNPr (CTAB), AuNR (CTAB), AuNS (CTAB) and AuNS (Citrate)) with BSA [25]. Providing weight to the argument that shape plays an important role in protein AuNP interactions, the ranking of shapes obtained above are in agreement with those found by that group with AuNR exhibiting stronger binding as compared with AuNPr in both cases.

The ordering of binding strength, which ranks the two smallest AuNS among the strongest binders indicated that these nanoparticles may have greater ease at accessing the site of the fluorophore and may have less steric inhibition in binding as compared with the larger particles. Notably, along with the small spherical AuNPs, AuNR also show the high level of interaction with HSA. While the AuNR have a large dimension longitudinally, their transverse ends are approximately 15 nm and possibly able to bind with high affinity near the tryptophan site within the HSA structure. It is hypothesised that serum albumins would preferentially bind to the ends of CTAB stabilised rods, as they are thought to be free from CTAB (which is present in a bilayer along the longitudinal faces) [16, 26].

It is also worth noting that when comparing AuNPs of the same concentration which differ in size, the number of particles as well as the available surface area differ. This point was also made by Liu et al. who concluded that smaller silver nanoparticles had stronger interactions and exhibited more efficient binding with HSA as compared to larger AgNPs at the same concentration [27]. Table 6.3 shows the calculated surface areas for all AuNPs together with the biomolecular quenching rate constant values.
<table>
<thead>
<tr>
<th>AuNP</th>
<th>Average measurement (nm)</th>
<th>( m_{AuNP} ) ((\times 10^{-18} \text{ g}))</th>
<th>No. of particles in 1 Mole AuNPs ((\times 10^{16}))</th>
<th>Surface area of particles in 1 Mole AuNPs ((\times 10^{-3} \text{ m}^2))</th>
<th>( K_{SV} ) ((20^\circ \text{C}) ) ((\times 10^3 \text{ L mol}^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>AuNS (CTAB)</td>
<td>77.9 (diameter)</td>
<td>4800</td>
<td>4</td>
<td>3.1</td>
<td>3.447</td>
</tr>
<tr>
<td>AuNR (CTAB)</td>
<td>58.8 (length) 15.3 (width)</td>
<td>270</td>
<td>73</td>
<td>3.0</td>
<td>5.114</td>
</tr>
<tr>
<td>AuNPr (CTAB)</td>
<td>94.7 (side length)</td>
<td>1900</td>
<td>10</td>
<td>1.5</td>
<td>2.063</td>
</tr>
<tr>
<td>AuNC (CTAB)</td>
<td>47.5 (side length)</td>
<td>2100</td>
<td>9</td>
<td>1.3</td>
<td>2.306</td>
</tr>
<tr>
<td>Citrate AuNS</td>
<td>12.5 (diameter)</td>
<td>19.7</td>
<td>1000</td>
<td>19.6</td>
<td>5.098</td>
</tr>
<tr>
<td>CTAB/citrate AuNS</td>
<td>8.9 (diameter)</td>
<td>7.1</td>
<td>2774</td>
<td>27.6</td>
<td>11.672</td>
</tr>
</tbody>
</table>

Table 6.2: Calculated surface area values for each AuNP.

There is some correlation between increased surface area and increased \( K_{SV} \), making it possible that the effects seen in this report are in part due to the increased surface area of citrate and CTAB/citrate AuNPs, however the increase cannot be fully explained by this trend. While the smaller spherical particles indeed have the largest surface area and are among the particles with the highest \( K_{SV} \) values, the trend does not hold for the larger varied shaped particles.

To further probe the binding action of each AuNP to HSA, the binding constant \( k_b \) and number of binding sites per HSA were calculated using Equation 6.3 [27].

\[
\log \frac{F_0 - F}{F} = \log k_b + n \log \left[ Q \right]
\]

Equation 6.3

Figure 6.8 displays plots for \( \log \frac{F_0 - F}{F} \) versus \( \log \left[ Q \right] \) in the case of each AuNP whereby the slope and intercept of each plot represent the number of binding sites (n) and \( \log k \) respectively.
Figure 6.8: Determination of the binding constant $k_b$ and number of binding sites per HSA for (a) AuNS (CTAB), (b), AuNR (CTAB), (c) AuNPr (CTAB), (d) AuNC (CTAB) (e) Citrate AuNS and (f) CTAB/citrate AuNS.

It can be observed from Figure 6.8 that the values deviate significantly from linearity at $\log[Q] = -5.59$ for AuNPr, citrate AuNS and CTAB/citrate AuNS. For this reason, linear fitting was applied to the upper 4 points from the plots in Figure 6.8 for these AuNPs, while fitting of the full region was applies to AuNS, AuNR and AuNC (CTAB). Results for all AuNPs are provided in Table 6.4. An example of the linear regression applied in each case is shown in Figure 6.9.
Table 6.3: Binding constant, $k_b$, and number of binding sites, $n$, for each AuNP as determined by linear fit of plots of $\log \frac{F_0 - F}{F}$ versus $\log [Q]$. $R$ is the linear correlated coefficient for $k_b$ and $n$ values. Measurements were performed at 20 ºC.

<table>
<thead>
<tr>
<th>AuNS</th>
<th>AuNR</th>
<th>AuNPr</th>
<th>AuNC</th>
<th>Citrate AuNS</th>
<th>CTAB/citrate AuNS</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_b$ (L mol$^{-1}$)</td>
<td>68.78</td>
<td>1702</td>
<td>63.56</td>
<td>14.6</td>
<td>1727.85</td>
</tr>
<tr>
<td>$n$</td>
<td>0.55</td>
<td>0.88</td>
<td>0.49</td>
<td>0.37</td>
<td>0.83</td>
</tr>
<tr>
<td>$R$</td>
<td>0.978</td>
<td>0.968</td>
<td>0.910</td>
<td>0.975</td>
<td>0.975</td>
</tr>
</tbody>
</table>

Figure 6.9: Determination of the binding constant $k_b$ and number of binding sites per HSA for (a) AuNC and (b) CTAB/citrate AuNS with linear regression applied.

Approximately speaking for the majority of AuNPs examined, the number of binding sites, $n$, can be approximated to 1, which indicates that there is one unique binding site for the AuNPs to bind to HSA. However, the number of binding sites can also be considered as a measure of association cooperativity [13]. For $n > 1$ (as calculated for CTAB/citrate AuNS) it is thought that after binding of an AuNP to a HSA molecule occurs, the protein increases its affinity for the bound AuNP developing a stronger bond over time. For $n < 1$ (as calculated for AuNS, AuNC and AuNPr) the opposite is true such that bound AuNPs have decreased affinity with HSA as additional proteins bind. Therefore values less than one can imply that the AuNP may only be partially bound to the HSA molecule [8]. For $n \approx 1$ (as calculated for AuNR and citrate AuNS) the binding of AuNPs is independent of the binding of other species [27].
Furthermore the binding constant, $k_b$, and number of binding sites, $n$ are intrinsically linked, with higher $k_b$ values gained where increased affinity is observed [28]. Commonly, $k_b$ values follow the same approximate ranking obtained from ordering $K_{SV}$ values which holds true in this study.

Equation 6.4 was used to examine the thermodynamics of the reaction between each AuNP and HSA

$$
\Delta G = -RT\ln k_b
$$

Equation 6.4

Where $\Delta G$ is the free energy change after the binding of AuNPs to HSA, $R$ is the gas constant, $T$ is the temperature in Kelvin and $k_b$ is the binding constant calculated using Equation 6.4. The results for all AuNPs are compiled in Table 6.5.

<table>
<thead>
<tr>
<th></th>
<th>$\Delta G$ kJ mol$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>AuNS</td>
<td>-10.31</td>
</tr>
<tr>
<td>AuNR</td>
<td>-18.12</td>
</tr>
<tr>
<td>AuNPr</td>
<td>-10.11</td>
</tr>
<tr>
<td>AuNC</td>
<td>-6.53</td>
</tr>
<tr>
<td>Citrate AuNS</td>
<td>-18.16</td>
</tr>
<tr>
<td>CTAB/citrate AuNS</td>
<td>-28.83</td>
</tr>
</tbody>
</table>

Table 6.4: Change in free energy, $\Delta G$ which occurs after binding of HSA to AuNPs at 20 °C.

While positive $\Delta G$ values are used to describe reactions requiring energy to proceed, negative $\Delta G$ values represent spontaneous reactions. All of the reactions presented in Table 6.5 are deemed to be spontaneous, meaning that energy is released after the binding of AuNPs to HSA bind and the transition to a more stable, lower energy state [29]. This finding is consistent with other studies investigating the thermodynamics of AuNPs with serum proteins [30, 31]. From these results it can be determined that CTAB/citrate AuNPs release the greatest amount of energy upon binding to HSA while AuNC release approximately three times less energy per mole of AuNP bound. This again may be in some part due to the increased numbers (and greater surface area) of smaller AuNPs per mole as compared with larger AuNPs.
6.3 Summary

It can be determined from the results provided in this Chapter, that shape and size indeed plays an important role in AuNP-protein interactions. The clear indication is that HSA will bind with greater efficiency and affinity to AuNPs of smaller sizes (when calculated on the basis of molar concentration), and with least efficiency and affinity to shapes with large flat planes like AuNC and AuNPr. The reasons for this remain unknown and further modelling experiments may provide insight into this complex relationship.
6.4 References


Chapter 7: Conclusion and Future Outlook
7.1 Summary

Increasing the multidisciplinary nature of scientific research will be the key to solving complex issues not yet understood. While researchers continue to investigate novel medical applications of AuNPs, we are currently stalled at the periphery of understanding the forces which govern critical nano-bio interactions. By applying the strengths of materials science, biophysical techniques and cellular biology, this Thesis attempts to develop a deeper understanding of the behaviour of nanoparticles at the biological interface.

One key aspect of such a study involves carefully controlled AuNP synthesis procedures. It is imperative, when comparing the biological activity of nanoparticles, to ensure that relevant parameters are held constant, while investigating the effects of changing a single variable. In this thesis, the AuNP size, shape and surface chemistry were controlled and finely tuned, and then thoroughly characterized. The resulting particles provided a solid foundation for the biological studies which followed. This allows the unambiguous determination of which properties are responsible for differences in cellular uptake and toxicity, something that is not possible in much of the literature, due to the fact that differences (e.g. in shape) are often accompanied by chemical differences, and so the two effects cannot be disentangled.

This Thesis highlights several important trends which relate AuNP size, shape and chemical structure to trends observed in cellular uptake and toxicity.

Firstly, when examining the effect of AuNP size on cellular uptake, a trend for increased uptake of smaller particles is common among research groups, and consistent with the findings in this Thesis. It can be understood by the notion that smaller particles require less energy to be internalised and will connect with a lower number of cellular surface receptors compared with larger particles. Larger particles require a greater number of cellular receptors such that over time, there is insufficient coverage of receptors on the cell surface which may limit (and slow) the internalisation of additional particles. In the case of toxicity, in the limited scope for comparison with the AuNPs used, no size dependant trends were observed.
When examining the effect of AuNP surface chemistry on cellular uptake, increased uptake levels were observed for amino acid stabilised tryptophan and tyrosine AuNS compared with citrate and CTAB/citrate AuNPs, highlighting the importance of surface chemistry in the process of AuNP uptake. It has long been known that certain amino acids influence the binding and uptake of extracellular material into the cell and the findings in this Thesis support this research, which demonstrates that the presence or absence of aromatic groups within an amino acid alters cellular uptake by affecting affinity and binding between the AuNP and the cellular surface. Furthermore, it was demonstrated that it is possible for chemical stabilisers alone to elicit varied cytotoxic effects.

When considering the effect of AuNP shape on cellular uptake, the results presented here suggest that shapes with broad flat faces diminish the availability of surface receptors—causing decreased efficiency in the uptake of these particles. When considering the energy required for cellular uptake, this too was found to differ based on geometry, and thus certain shapes required fewer cellular receptors for endocytosis, which thus could occur more rapidly. It can be concluded that spherical particles are taken up preferentially, as their geometry makes contact with fewer receptors on the cellular surface, while also requiring less free energy for particle wrapping and subsequent endocytosis. The effect of AuNP shape on cytotoxicity was also examined, and it was found that rod and cubic shaped particles were well tolerated if washed thoroughly to remove free CTAB. Toxicity was observed in the case of CTAB stabilised spherical particles, the cause of which may indirectly be a result of shape. Due to the preference for cells to uptake spherical particles, a high degree of internalisation was observed in the case of CTAB stabilised spherical particles relative to the other shapes which may have led to a build-up of CTAB within the cell, eventually leading to cell death. Of particular note were the effects of AuNP on toxicity which were found to be toxic at the same dose observed for AuNS (CTAB) despite markedly low levels of uptake. It is possible that this may be a direct effect of its angular shape on cellular structure, however this hypothesis remains unproven and requires further work.

The role of serum was shown to be important in determining AuNP uptake. Generally speaking, lower levels of uptake were observed in the presence of serum. From these results, it can be
proposed that decreased AuNP/membrane interactions occur in the presence of serum. In this environment, free serum proteins may compete for binding sites, preventing AuNPs from binding and subsequently being internalised. Conversely, the proposed role of serum in mediating toxic effects is not supported by this study, where similar trends were generally observed in both serum supplemented and serum free conditions.

Furthermore, when using HSA, the most abundant protein found in human blood, to model the interactions which occur between AuNPs and various proteins, the importance of shape and size on these interactions was highlighted. After ranking the binding strength of all particles with HSA, the two smallest spherical particles ranked among the strongest binders, thus it can be concluded that HSA binds with increased efficiency and affinity with particles of smaller sizes. This can be justified as these nanoparticles may have greater ease at accessing the site of the fluorophore within HSA and may have less steric inhibition in binding as compared with the larger particles. Conversely it was shown that HSA binds with least efficiency and affinity to shapes with large flat planes such as AuNC and AuNPr. The exact mechanism for this remains unknown, but it likely related to morphology.

Overall, the combination of techniques demonstrated in this Thesis has contributed to elucidate the role of AuNP shape, size and surface corona on chemical uptake and toxicity.

7.2 Future Outlook

Many of the areas covered in this thesis; protein corona formation, toxicity of AuNPs, and AuNP-cellular interactions, are intensely active areas of research. Relative to other scientific fields, research in this area is still in its infancy and as such, due to the various complexities of this research, we are yet to fully explain many aspects of nano-bio interactions.

While significant progress has been made in the area of nanoparticle synthesis and characterisation, further attention is required to understand the biological effects of these newly synthesised materials.
As suggested within the pages of this Thesis, a more targeted study to show the effect of certain shapes (those possessing acute angles and ‘sharp’ features) on internal cellular structures may be necessary to fully conclude if shape plays a critical role in cytotoxicity. The broader role of shape may also be illuminated by studying shapes similar to those produced in this Thesis, synthesised from alternative materials such as silver or silica.

While studies presented in this Thesis were performed with a cancerous mammalian cell line, it would be interesting to perform similar studies on additional cell lines to determine the scope of variation which may arise between cancerous and non-cancerous cells, in addition to those originating from various areas of the body.

As one of the most rapidly expanding areas of research, protein corona research may deliver large advances in the understanding of nano-bio interactions in the near future. To expand on the protein interaction studies presented in this Thesis, additional techniques may be employed to assess the composition of proteins which are included in the corona for each AuNP. These studies may indicate if AuNP shape and size dictate the species of proteins which bind, and further circular dichroism studies would demonstrate the effect that these parameters may have on the structure of the proteins which do bind.

While it is evident that there is much to be learned in this area, each layer of knowledge brings us one step closer to realising the full potential for nanoparticles as therapeutic and diagnostic agents. It is therefore expected that the outcomes presented in this Thesis will contribute to the ongoing evolution of research in this area.
Appendix 1 - Publications

The research in this thesis will be published in several articles, detailed below:

Chapter 1

“Size, shape and surface chemistry of nano-gold dictate its cellular interactions, uptake and toxicity”
Catherine Carnovale, Gary Bryant, Ravi Shukla, Vipul Bansal.
Submitted to Progress in Materials Science – Under Review.

Articles under preparation

Chapter 5

“Identifying trends in gold nanoparticle toxicity and uptake; size, shape and surface corona”
Catherine Carnovale, Gary Bryant, Ravi Shukla, Vipul Bansal.
To be communicated to ACS Nano.

Chapter 6

“Interaction of Different Shaped Gold Nanoparticles with Human Serum Albumin”
Catherine Carnovale, Gary Bryant, Ravi Shukla, Vipul Bansal.
To be communicated to Physical Chemistry Chemical Physics.