Understanding the effect of phytochemical coated silver nanoparticles on mammalian cells and the protein interactions with the surface corona of these nanoparticles.

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.

Amanda Nicole Abraham

28th September 2016
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Abstract

Silver nanoparticles (AgNPs) are currently the most common nanomaterial in commercial products; however, there is insufficient evidence on the long term toxicity of AgNPs on mammalian cells. There is also no information on the extent to which the surface coating on the AgNPs influences the toxic effects produced in cells. Yet another area lacking information is the impact the surface coating might have on the interaction of these AgNPs with proteins found in blood serum, which would be useful information if these AgNPs are to be used for medical purposes. To shed light on these aspects, this project used silver nanoparticles (AgNPs) synthesised with bi- and polyphenolic compounds viz. curcumin (cur) and epigallocatechin gallate (EGCG), phytochemicals from turmeric and green tea, respectively to study the effects on cancer cells and on the interaction with human serum albumin (HSA). L-Tyrosine (tyr), a monophenolic amino acid was used as a control since it is found in all organisms and is non-toxic.

The results of this project suggest that the phytochemical coating on the AgNPs can work synergistically with the silver ions generated from the AgNPs to cause toxicity. It also provided an understanding of the residual effects AgNPs can have on mammalian cells even after only 24h of exposure. The results further suggest that the concentration of the phytochemicals on the AgNPs can affect the mechanism of action of these AgNPs. For example, the CurAgNPs which acted as pro-oxidant agents in the cells and caused an increase in ROS levels and cell adhesion and migration, possibly due to the low quantity of curcumin on the surface. Hence these CurAgNPs would be unsuitable as anticancer agents, however they would be useful for other applications such as wound healing. With regards to the interaction of these AgNPs with HSA, once again the surface coating plays a role in dictating the extent to which the interaction occurs. The CurAgNPs showed the greatest interaction with serum proteins, while EGCGAgNPs were capable of increasing the α-helix of HSA. These results suggest that the surface coating on the AgNPs could possibly be the determining factor when deciding the purpose for which the AgNPs will be used.
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<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>AAS</td>
<td>Atomic absorption spectroscopy</td>
</tr>
<tr>
<td>Ag</td>
<td>Silver</td>
</tr>
<tr>
<td>AgNPs</td>
<td>Silver nanoparticles</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>Cur</td>
<td>Curcumin</td>
</tr>
<tr>
<td>CtNPs</td>
<td>Citrate capped silver nanoparticles</td>
</tr>
<tr>
<td>cDNA</td>
<td>Complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>DLS</td>
<td>Dynamic light scattering</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EGCG</td>
<td>Epigallocatechin–3–gallate</td>
</tr>
<tr>
<td>FBS</td>
<td>Foetal bovine serum</td>
</tr>
<tr>
<td>FTIR</td>
<td>Fourier transform infrared</td>
</tr>
<tr>
<td>HSA</td>
<td>Human serum albumin</td>
</tr>
<tr>
<td>HUVEC</td>
<td>Human umbilical endothelial cells</td>
</tr>
<tr>
<td>MMP</td>
<td>Mitochondrial membrane potential</td>
</tr>
<tr>
<td>MTT</td>
<td>3–(4,5–dimethylthiazol–2–yl)–2,5–diphenyltetrazolium bromide</td>
</tr>
<tr>
<td>NPs</td>
<td>Nanoparticles</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PC-3</td>
<td>Prostate cancer cells</td>
</tr>
<tr>
<td>PCNA</td>
<td>Proliferating cell nuclear antigen</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Institute</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Real time quantitative reverse transcription polymerase chain reaction</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>Tyr</td>
<td>Tyrosine</td>
</tr>
<tr>
<td>UV–vis</td>
<td>Ultraviolet–visible</td>
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Chapter 1: Literature review

1.1. Historical use of silver

In nature silver occurs as a soft metal or as a white or grey to black powder [1]. The symbol Ag arises from Argentum, which is the Latin word for silver. The earliest reported usage was as currency, jewellery and as a water purifier [2]. Figure 1.1 provides a brief timeline of the usage of silver throughout. As early as 335 BC, the Romans and Greeks used silver vessels to store water [2, 3]. Ambrose Pare, a battlefield surgeon who served the French royalty from 1510-1590 advocated the use of silver clips for facial reconstruction [2, 3]. By the 1800s, when it became known the microorganisms were the cause of certain diseases, silver was incorporated in various medical applications since it possessed the ability to retard bacterial growth. One of the more unique applications was by Dr. Georgia Fix who used of a silver coin, hammered into a thin silver sheet using a hammer and a piece of rail iron. She then used the silver sheet to close an open skull injury resulting from an accident [2, 3]. William Halsteat, MD, the first Chief of Surgery at Johns Hopkins Hospital in 1889, used silver wire sutures and utilised silver foil as a method to prevent postoperative infections [1-3].

Silver has also been used to treat burn injuries for over 200 years and until today, 0.5% silver nitrate solution is used as a topical antimicrobial agent in burn medication. In the last two decades, there has been an increase in silver-coated fabric for wound and burn dressings. This first commercial product to be used as a topical treatment for burns was Flammacerium®, which is 1% silver sulfa-diazine, a combination of sulphonamide and silver [1, 2].

Apart from burn and wound dressing, nosocomial infections, such as urinary tract and bloodstream infections are common issues faced by hospitalised patients. This is due to the fact that catheters and central venous lines are prone to biofilm formation, which makes the microorganisms resistant to antimicrobial agents. Silver coated catheters and endotracheal tubes have shown a significant decrease in infection rates and have been commercially available for over 10 years [4-7].

Silver nitrate (AgNO₃) has been used since the 1840s, to treat dental cavities. Howe’s ammoniacal silver nitrate solution (AgNH₂NO₃) was used from 1917 up to the 1950s. However, it was discontinued due to damage to the dental pulp. By the 1970s, it was replaced with the usage of silver diammine fluoride, the
usage of which continues until today [8-10]. This is due to its efficacy against *Streptococcus mutans* and *Lactobacillus acidophilus*, two of the most common bacteria associated with the formation of cavities [8, 10].

The first reported synthesis of silver nanoparticles (AgNPs) was probably in 1889 by M. C. Lea, who synthesised silver nanoparticles by citrate stabilisation [11]. Collargol in 1894, Aryronin in 1895 and Protargol in 1897 have been some of the commercially available nanosilver formulations marketed as across-the-counted medications for bacterial infections and syphilis [12, 13]. By 1970, with the onset of antibiotic resistance, the usage of silver increased and by 1990, AgNPs were incorporated into wound dressings [3]. By 1912, there were at least 52 nanosilver formulations available on the market [13].

![Figure 1.1: Timeline depicting the usage of silver products throughout history](image)

### Figure 1.1: Timeline depicting the usage of silver products throughout history

#### 1.2. Silver nanoparticles and their applications

The word ‘nano’ comes from the Greek language and it means something very small. It depicts one billionth (10⁻⁹) of a unit [14, 15]. Nanoparticles (NPs) are substances that have one or more dimensions in the nanometer range (1-100nm) and can be metals, ceramics, polymers or composites [15, 16]. They also exhibit uniquely different physical, chemical and mechanical properties compared to bulk materials, such as surface plasmon resonance and surface-enhanced Raman scattering [17, 18]. Due to their unique properties, nanoparticles have found applications in a wide range of areas such as electronics, biotechnology, textile engineering and water treatment [15]. In the field of medicine, they are widely used in catheters and wound dressings. Various personal care products such as cosmetics, toothpastes, shampoos, air sanitiser sprays, bedding and clothing now contain AgNPs [19-28]. It is also finding its way...
into household appliances, such as a washing machine manufactured by Samsung Electronics, a refrigerator by LG Electronics and a dishwasher by Hitachi. All these devices contain silver nanoparticles which generate silver ions for their antimicrobial properties. According to the Project on Emerging Nanotechnologies, as of 2013, of the 762 products containing nanomaterials in the Health and Fitness category, products containing silver were the most frequently listed (435 products, 24%) [29]. According to the Danish Consumer Council, the number of products containing AgNPs has increased from 192 in 2012 to 331 in 2015 [30]. This would suggest that the production and consumption of AgNP containing products will continue to increase in the future.

One aspect that makes silver nanoparticles such an excellent choice in numerous consumer products is the high surface-to-volume ratio [16] making them excellent antimicrobial agents [31, 32]. Studies have now shown that AgNPs can be toxic to many cancer cell lines, as evident in Table 1.1. Mohammed Fayaz, A., et al. 2012 are even advocating the development of AgNP-coated polyurethane condoms since their investigation indicated that AgNPs could inactivate human immunodeficiency virus type 1 (HIV-1) and herpes simplex virus (HSV) [33]. Other researchers have also found that AgNPs can be effective against viruses (Table 1.2).

Table 1.1: In vitro acute toxicity of AgNPs on mammalian cells

<table>
<thead>
<tr>
<th>Surface coating/method of synthesis</th>
<th>TEM size (nm)</th>
<th>Mammalian cell line</th>
<th>Exposure time (hours)</th>
<th>Notes</th>
<th>Reference</th>
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<td>Polyethylene glycol conjugated to nuclear targeted peptides</td>
<td>35</td>
<td>Human oral squamour cell carcinoma (HSC-3), human keratinocytes (HaCat)</td>
<td>24</td>
<td>Oxidative stress and G$_2$ phase accumulation of cell cycle</td>
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<td>Citrate capped</td>
<td>29.57</td>
<td>Human keratinocytes (HaCat), human cervical adenocarcinoma (HeLa)</td>
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<td>[35]</td>
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<tr>
<td>Commercial</td>
<td>40 – 90</td>
<td>Human lung carcinoma (A549)</td>
<td>24 – 48</td>
<td>Oxidative stress, S phase cell cycle arrest.</td>
<td>[36]</td>
</tr>
</tbody>
</table>
Citrate capped NR Macrophage (RAW 264.7, J774.1), human lung carcinoma (A549), hepatocellular carcinoma (Hep G2), neurolan (Neuro 2A) 24 – 72 Uptake though cell surface receptors and toxicity due to silver ion generation. [37]

Citrate capped NR Human lung carcinoma (A549) 24 – 48 Dose dependent toxicity [38]

Proprietary preparation 7-20 Human skin carcinoma (A431), human fibrosarcoma (HT-1080) 24 Apoptosis through DNA fragmentation. [39]

Polyethylenimine 7 – 10 Hepatocellular carcinoma (HepG2) 24 Increase cell proliferation at low doses (<0.5mg/L). Cytotoxicity observed above 1.0 mg/L was due to DNA damage. [40]

Commercial 68.9 Mouse peritoneal macrophage (RAW 264.7) 24 – 96 Trojan horse mechanism causing oxidative stress and increase in matrix metalloproteinases (MMP-3, MMP-11 and MMP-19). [41]

Commercial 20 – 80 Human epidermal keratinocytes (HEK) 24 Dose-dependent toxicity and increase of inflammatory cytokines such as interleukins 6 and 8 (IL-6 and IL-8) [42]

NR – Not reported, TEM – Transmission electron microscopy

<table>
<thead>
<tr>
<th>Method of synthesis</th>
<th>TEM size (nm)</th>
<th>Application</th>
<th>Exposure time (hours)</th>
<th>Notes</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Commercial</td>
<td>20 and 110</td>
<td>Mycobacterium tuberculosis infect human monocyte-derived macrophages (MDM)</td>
<td>24</td>
<td>AgNPs supressed the M.tb induced immune responses of the cell.</td>
<td>[43]</td>
</tr>
</tbody>
</table>

*Table 1.2: Effectiveness of AgNPs against microorganisms and viruses.*
<table>
<thead>
<tr>
<th>Capping Agent</th>
<th>Size (nm)</th>
<th>Bacteria/Viruses</th>
<th>Treatment Duration</th>
<th>Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrate capped</td>
<td>5</td>
<td><em>Candida albicans</em> and <em>Candida glabrata</em></td>
<td>2 and 24 days</td>
<td>Inhibition of biofilm formation. [44]</td>
</tr>
<tr>
<td>Citrate and hydrazine hydate capped</td>
<td>8 – 50</td>
<td><em>Escherichia coli</em>, <em>Pseudomonas aeruginosa</em> and <em>Staphylococcus aureus</em></td>
<td>24 days</td>
<td>Smaller particles showed higher antibacterial activity. [45]</td>
</tr>
<tr>
<td>Sodium borohydride</td>
<td>13.4</td>
<td><em>E.coli</em> and <em>S.aureus</em></td>
<td>24 days</td>
<td>AgNP accumulation in the cell membrane caused cell death. Free radicals could also contribute to the toxicity. [46]</td>
</tr>
<tr>
<td>Foamy carbon polyvinyl pyrrolidone (PVP)</td>
<td>1–10</td>
<td>HIV-1</td>
<td>3-5 days</td>
<td>Foamy carbon showed greater interaction than PVP and BSA, possibly due to capping agents. [47]</td>
</tr>
<tr>
<td>Bovine serum albumin (BSA)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Commercial AgNPs coated with PVP</td>
<td>30-50</td>
<td>HIV-1</td>
<td></td>
<td>AgNPs were effective irrespective of viral tropism or resistance profile. [48]</td>
</tr>
<tr>
<td>Plasma gas synthesised AgNPs coated with polysaccarhides</td>
<td>25, 55, 80</td>
<td><em>Monkeypox virus</em></td>
<td></td>
<td>25nm exhibited significant, dose-dependent toxicity. [49]</td>
</tr>
<tr>
<td>Tannic acid</td>
<td>11.4</td>
<td>Adenovirus type 3 (Ad3)</td>
<td></td>
<td>AgNPs decreased DNA loads in a dose-dependent manner. [50]</td>
</tr>
<tr>
<td>Fungal synthesised</td>
<td>5 – 30</td>
<td><em>HSV types 1 and 2</em> and human parainfluenza virus type 3</td>
<td></td>
<td>7 – 20nm particles showed better inhibition of viral activity. [51]</td>
</tr>
</tbody>
</table>

NR – Not reported, TEM – Transmission electron microscopy
1.3. **Risks associated with the usage of silver nanoparticles**

Argyria is a condition that occurs from the deposition of silver in the body, as a result of the consumption of copious amounts of silver containing products. It usually presents as a bluish or bluish-grey discolouration of the skin and the whites of the eyes. The skin discolouration also appears to be exacerbated by exposure to sunlight. In extreme cases, silver deposition occurs in the liver, kidneys and heart [52, 53]. There have been numerous reports of argyria, as early as 1935 to as recent as 2013 [53-58] and many have been reported due to the consumption of colloidal silver products [1, 52, 59]. While there have been no reported fatalities resulting from argyria, there is no known treatment and the pigmentation is permanent.

By 1974, when it was found that silver ions in silver sulfadiazine were responsible for the toxic effects on microorganisms [60]. This has since been corroborated by similar reports on silver containing products [61, 62]. In bacteria, silver ions cause damage to the cell membrane [62, 63], condensation of the deoxyribonucleic acid (DNA) [62, 64] and inactivation of proteins [64], which is possibly due to binding of silver ions to the thiol groups [62]. Greulich, C., *et al.* 2012 have also shown that silver ions affect bacteria and mammalian cells at the same concentrations [65]. This has increased uncertainties regarding the effect these silver ions might have on normal cells in the human body, when exposed to products that contain silver.

While studies indicate that silver nanoparticles are less toxic than silver ions, evidence suggests that both cause similar effects on cells. Previous reports have shown an increase of inflammatory cytokines such as interleukin–6 (IL–6) [66], interleukin–8 (IL–8) [66, 67], tumour necrosis factor alpha (TNF-α) [67], and superoxide dismutase (SOD) which leads to an increase in oxidative stress in the cells [68]. Oral exposure of rats to AgNPs and silver ions showed similar results; with silver deposition mainly in the liver and the spleen up to 8 weeks post exposure, however less deposition with AgNPs than with silver ions [69, 70]. The ability to cross the placental barrier into the pups in rats has also been demonstrated with AgNPs [71] and silver ions from the AgNPs can cause cytotoxicity to the developing embryo [72]. This has raised concerns over the use of products containing AgNPs.

However, whether the toxicity of AgNPs is solely due to silver ions has been the topic of much debate. Reports suggest silver nanoparticles employ a Trojan–horse mechanism, by generating silver ions after
endocytosis into the cells [41] and the toxicity to mammalian cells subsequently increases [69, 70, 73]. Conversely, studies indicate that toxicity of AgNPs does not arise solely from silver ions [16, 74, 75]. Beer, C., et al. 2012 also reported that at lower concentrations, the silver ions and AgNPs appear to work synergistically in producing toxic effects on mammalian cells [16]. Even in bacteria, reports indicate that silver ions are not the only cause of toxicity from AgNPs [76].

With regards to the biodistribution of AgNPs in the body, in vivo studies indicate that highest accumulation of AgNPs occurs in the liver and spleen after 24 hours [77-79]. However, with increase in exposure time, the concentration in the kidneys and brain increased after 28 days [77]. Dziendzikowska, K., et al. 2012 also found that smaller AgNPs (20nm) show higher distribution in tissues than larger AgNPs (200nm) [77] and Ashraf, A., et al. 2015 indicate that the surface coating on the AgNPs can dictate the retention time of the AgNPs in the blood stream [79].

On the plus side, AgNPs have been shown to have a higher removal rate from the body, through excretion, than silver ions [69, 70]. Evidence also suggests that sample preparation; such as sonication in the presence or absence of ice, centrifugation and filtration, can affect the amount of silver ions generated from the AgNPs [16]. Reports further indicate that surface modification can play a role in decreasing the generation of silver ions from the AgNPs [80]. On the other hand, surface modification can work synergistically with the AgNPs, thereby enhancing the toxic effects of the NPs [81]. This suggests that the method of synthesis can impact the effect the AgNPs have on mammalian cells. This also suggests that the mechanistic aspects of AgNPs are not fully understood and more research is required if these NPs are to be used extensively in commercial products.

1.4. Synthesis of nanoparticles

Figure 1.2 provides a schematic representation of the two main approached to nanoparticle synthesis. Typically, production of NPs in general can occur through a top-down approach, wherein bulk material is broken down to nanoparticles or a bottom-up approach which involves reduction of the metal salt to nanoparticles [82]. The top-down approach can occur through evapouration–condensation, which involved vapourising the bulk material into a carrier gas in a tube furnace, at atmospheric pressure [14, 83]. However, tube furnaces are bulky, require several kilowatts of energy to power it and can be a time consuming process. Another method of breaking down bulk material to nanoparticles is through laser
ablation [14, 83, 84]. While AgNPs synthesised by this method have shown antibacterial activity [84], it is difficult to control the size of the particles and the equipment can be expensive [85].

![Diagram](image)

**Figure 1.2: Schematic representation of the two approaches to nanoparticle synthesis**

As observed in tables 1.1 and 1.2, most AgNPs are synthesised by the bottom – up approach. This involves the use of reducing agents, such as sodium citrate [35, 37, 38, 86], sodium borohydride [86-88], PVP [47, 48, 86, 87, 89-93] or hydrazine [45] to reduce metal ions (Ag⁺) to nanoparticles (Ag⁰) [83]. Often, the use of a stabilising agent is necessary to prevent the nanoparticles from aggregating. However, when considering the use of nanoparticles for biological purposes, the toxic nature of these reducing and stabilising agents needs to be taken into consideration. For example, hydrazine and sodium borohydride, powerful reducing agents currently used in the synthesis of metal NPs, are highly toxic to living organisms and the environment [94]. This is a major problem when considering the use of NPs for application and consumption by living organisms, since these toxic substances get adsorbed onto the surface of the NPs [31].

Living organisms such as bacteria, fungi and algae are also capable of synthesising nanoparticles [95]. Microorganism that naturally exist in metal rich environments, develop inherent defence mechanisms to
resist the high metal concentrations, and this can be exploited for nanoparticles synthesis. For example, *Pseudomonas stutzeri* AG259, a bacterial strain isolated from silver mine, is capable of producing silver nanocrystals up to 200nm in size [22, 96]. *Lactobacillus* strains found in buttermilk could produce AgNPs from 15nm to 500nm in size [23]. However, one drawback is these silver crystals remain mostly embedded within the matrix of the bacteria [22, 23]. On the other hand, the bacterial species belonging to the genus *Morganella*, which are silver resistant bacteria, are capable of extracellular production of AgNPs [97]. Ramanathan, R., *et al.* 2011 have also demonstrated that altering the temperature conditions at which *Morganella psychroterans* were grown could result in the production of different shaped AgNPs [98]. It is also possible to use cell extract for NP synthesis, as demonstrated by Singh, G., *et al.* 2014, who used a cell extract of the cyanobacterium *Anabaena dolioïdum* [28].

Apart from bacteria, some species of algae, such as *Spirogyra varians* [26] have demonstrated the ability to synthesise AgNPs. Priyadharshini, R. I., *et al.* 2014 used an extract from macro-algae *Gracilaria edulis* to produce 55-99nm AgNPs [99]. Fungi, such as *Verticillium sp.* [100], *Fusarium oxysporum* [101], *Aspergillus fumigatus* [102] and *Fusarium semitectum* [103] have been used to synthesise AgNPs. Even a silver resistance stain of yeast MKY3 has demonstrated the ability to produce extracellular AgNPs [104]. However, these nanoparticles produced by microorganisms are not free of cellular components, such as the presence of enzymes and proteins on the surface [99, 105], which can hinder the use of these NPs for biological applications.

Recently, the use of plant extracts for synthesis and stabilisation of nanoparticles are gaining popularity since these substances already exist within living organisms and these nanoparticle systems have shown improved solubility, stability and bioavailability in the living organism [94, 106]. Plant extracts such as Neem [27], cinnamon [21], *Aloe vera* [19], *Chenopodium album* [107] and edible mushrooms [24] have been used to synthesise AgNPs. A brief summary of AgNPs synthesised by plant extracts has been provided in Table 1.3.

The use of plant extracts has proven to be an easier and more cost effective method than physical and chemical methods of synthesis [108]. The extracts act as simultaneous reducing and stabilising agents, thereby eliminating the use of harsh chemicals in the synthesis process [108]. The AgNPs synthesised from these plant extracts have shown higher antibacterial properties than the crude extracts [109]. Different parts
of plants can give different shaped AgNPs [110] or result in different sized AgNPs [111]. Many of these AgNPs have also shown antibacterial properties, as evident from table 1.3 and demonstrated biocompatibility with mammalian cells [20, 110]. Studies that examined the surface coating on NPs synthesised from plant extracts found that the NPs are coated with polyols and phenolic compounds from the plant extracts, which suggests that the phytochemicals present in these plant extracts were responsible for the reduction of the metal ions to nanoparticles [19, 21, 24, 107, 110, 112].

Table 1.3: Use of plant extracts for the synthesis of silver nanoparticles.

<table>
<thead>
<tr>
<th>Plant</th>
<th>Plant part</th>
<th>TEM size (nm)</th>
<th>NP shape</th>
<th>Applications</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cinnamon</td>
<td>Leaf</td>
<td>55-80</td>
<td>Quasi-spherical</td>
<td></td>
<td>[21]</td>
</tr>
<tr>
<td><em>Chenopodium album</em></td>
<td>Leaf</td>
<td>10-30</td>
<td>Spheres, some triangles</td>
<td></td>
<td>[107]</td>
</tr>
<tr>
<td>Mushroom</td>
<td>Whole</td>
<td>15-30</td>
<td>Spheres, some ellipsoidal</td>
<td></td>
<td>[24]</td>
</tr>
<tr>
<td><em>Nyctanthes arbortristis</em></td>
<td>Flower</td>
<td>5-20</td>
<td>Spheres, ovals</td>
<td>Antioxidant and antibacterial activity against <em>Escherichia coli</em> while showing biocompatibility with mouse fibroblast cells (L929)</td>
<td>[20]</td>
</tr>
<tr>
<td><em>Hibiscus rosa sinensis</em></td>
<td>Leaf</td>
<td>5-50</td>
<td>Anisotropic</td>
<td></td>
<td>[113]</td>
</tr>
<tr>
<td><em>Mangifera indica</em></td>
<td>Leaf</td>
<td>20-30</td>
<td>Triangles and hexagons</td>
<td></td>
<td>[114]</td>
</tr>
<tr>
<td>Neem</td>
<td>Leaf</td>
<td>5-35</td>
<td>Spheres</td>
<td></td>
<td>[27]</td>
</tr>
<tr>
<td><em>Ocimum tenuiflorum</em></td>
<td>Leaves and peel</td>
<td>22.3-28.4</td>
<td>Anisotropic</td>
<td></td>
<td>[115]</td>
</tr>
<tr>
<td><em>Solanum tricobatum</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Syzgium cumini</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Sesbania grandiflora</em></td>
<td>Leaf</td>
<td>22</td>
<td>Spherical</td>
<td>Anticancer properties against human breast cancer (MCF-7) cells.</td>
<td>[116]</td>
</tr>
<tr>
<td>Plant Name</td>
<td>Part(s)</td>
<td>Dimensions</td>
<td>Shape(s)</td>
<td>Properties</td>
<td></td>
</tr>
<tr>
<td>------------</td>
<td>---------</td>
<td>------------</td>
<td>----------</td>
<td>------------</td>
<td></td>
</tr>
<tr>
<td>Euphorboa helioscopia Linn</td>
<td>Leaf</td>
<td>5-14</td>
<td>Spheres</td>
<td>Catalytic properties</td>
<td>[117]</td>
</tr>
<tr>
<td>Ziziphora tenuior</td>
<td>Leaf</td>
<td>8-40</td>
<td>Spheres</td>
<td></td>
<td>[118]</td>
</tr>
<tr>
<td>Trianthema decandra</td>
<td>Root</td>
<td>36-94</td>
<td>Spheres</td>
<td>Antimicrobial activity against <em>Streptococcus faecalis</em>, <em>Staphylococcus aureus</em>, <em>Enterococcus faecalis</em>, <em>E. coli</em>, <em>Pseudomonas aeruginosa</em>, <em>Proteus vulgaris</em>, <em>Bacillus subtilis</em>, <em>Yersinia enterocolitica</em> and the fungus <em>Candida albicans</em></td>
<td>[112]</td>
</tr>
<tr>
<td>Cucurbita maxima</td>
<td>Petals</td>
<td>30-70</td>
<td>Spheres</td>
<td>Antimicrobial activity against <em>P. aeruginosa</em>, <em>B. subtilis</em>, <em>E. coli</em> and <em>Vibrio cholerae</em>. Anticancer properties against epidermoid carcinoma (A431) cell line</td>
<td>[110]</td>
</tr>
<tr>
<td>Moringa oleifera</td>
<td>Leaf</td>
<td></td>
<td>Rectangles</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acorus calamus</td>
<td>Rhizome</td>
<td></td>
<td>Spheres</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tribulus terrestris L</td>
<td>Fruit</td>
<td>20-70</td>
<td>Spheres</td>
<td>Antibacterial activity against <em>Streptococcus pyrogen</em>, <em>E. coli</em>, <em>P. aeruginosa</em>, <em>B. subtilis</em> and <em>S. aureus</em></td>
<td>[119]</td>
</tr>
<tr>
<td>Pinus densiflora</td>
<td>Pine cones</td>
<td>40-70</td>
<td>Mostly spheres, some triangles</td>
<td>Antibacterial activity against <em>Brevibacterium linens</em>, <em>Propionibacterium acnes</em>, <em>Bacillus cereus</em> and <em>Staphylococcus epidermidis</em></td>
<td>[120]</td>
</tr>
<tr>
<td>Cocos nucifera</td>
<td>Inflorescence</td>
<td>22</td>
<td>Spheres</td>
<td>Antibacterial activity against <em>Klebsiella pneumoniae</em>, <em>Plesiomonas shigelloides</em>, <em>Vibrio alginolyticus</em>, <em>Salmonella paratyphi</em></td>
<td>[109]</td>
</tr>
<tr>
<td>Basil (Ocimum sanctum)</td>
<td>Stem</td>
<td>10</td>
<td>Spheres</td>
<td></td>
<td>[111]</td>
</tr>
<tr>
<td></td>
<td>Root</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ficus talboti</td>
<td>Leaf</td>
<td>11.9</td>
<td>Spheres</td>
<td>Antibacterial activity against <em>V. cholera</em>, <em>P. aeruginosa</em>, <em>Salmonella typhi</em> and <em>E. coli</em></td>
<td>[121]</td>
</tr>
<tr>
<td>Camellia sinensis</td>
<td>Leaf</td>
<td>47x10</td>
<td>Triangular nanoplates</td>
<td></td>
<td>[122]</td>
</tr>
</tbody>
</table>

TEM – Transmission electron microscopy
1.5. Benefits of phytochemicals

Phytochemicals (‘phyto’ comes from the Greek word meaning plant) are bioactive non-nutritive plant components found in fruits, vegetables and grains. They have been strongly associated with reduced risk of cardiovascular diseases, cancer, diabetes, Alzheimer’s disease and age-related functional declines [123]. The main reason for the beneficial effects of phytochemicals has been attributed to their antioxidant properties. Reactive oxygen species (ROS), such as superoxide anions, hydroxyl radicals and hydrogen peroxide are normal by-products of cellular metabolism. However, exposure of cell to ROS for extended periods or overproduction of ROS results in oxidative stress, which can further leads to diseases such as cancer and neurodegenerative disorders. Phytochemicals have been shown to act as antioxidants, thereby working with the natural antioxidant mechanisms in the cells, to restore the ROS balance [124].

1.5.1. Curcumin

Curcumin is a phytochemical from turmeric (Curcuma longa), which is a spice often used in Indian and Thai cooking [125]. It is a hydrophobic phytochemical consisting of two phenol groups and has a predominant enol form in alkaline pH and a keto form in acidic and neutral pH [126, 127]. In traditional Indian medicine it is used for its anti-inflammatory and anti-septic properties [128, 129]. It exhibits good anticancer properties, especially to prostate cancer cells. Hilchie, A. L., et al. 2010 have demonstrated that curcumin caused apoptosis of prostate cancer (PC-3) cells through the inhibition of glutathione (GSH) and mitochondrial damage [130]. Killian, P. H., et al. 2012 have shown that curcumin can decrease proinflammatory cytokines such as CXL1 and CXL2. These cytokines can increase metastasis by promoting metastatic factors such as cyclooxygenase–2 (COX–2). Hence by inhibiting these cytokines, curcumin can decrease metastasis of PC-3 cells [131]. It can further prevent metastasis and angiogenesis by downregulation of matrix metalloproteinase 9 (MMP 9) and osteopontin (OPN), respectively [132]. Chendil, D., et al. 2004 have also shown that it can sensitise PC-3 cells to radiation therapy by altering the Bcl2 : Bax ratio[133]. Apart from anticancer properties, curcumin has shown the ability to regulate Wnt signalling, which can alleviate renal injury as a result of diabetes [134]. It can exhibit neuroprotective effects in cases of cerebral ischemia [135]. Hence curcumin is a versatile phytochemical that can be used to treat a vast array of diseases.
1.5.2. Epigallocatechin–3–gallate (EGCG)

Epigallocatechin-3–gallate (EGCG) is a polyphenolic compound derived from green tea (*Camellia sinensis*). Ahmed, N. A., *et al.* 2016 have found that EGCG is capable of attenuating the oxidative stress caused by exposure to electromagnetic radiation (EMR) generated from cell phones [136]. It is also known for its antioxidant effects against various forms of cancer, especially prostate cancer. It is capable of inducing the extracellular signal-regulated kinase (ERK 1/2) pathway in prostate cancer cells without activating this pathway in normal prostate cells [137]. It is capable of inhibiting COX-2 expression in PC-3 cells [138] and MMP 9 [139] which suggests that it can inhibit migration. Apoptosis in PC-3 cells is promoted through the upregulation of caspase 9 and it can enhance the activity of anticancer drugs like cisplatin [140]. Application of EGCG to the skin has been shown to reduce inflammation in cases of psoriasiform dermatitis [141]. It can reduce the toxicity of the ricin toxin, which is produced by the castor bean (*Ricinus communis*) and can be lethal to humans [142]. Aggregation of the alpha-synuclein (SNCA) protein is one of the hallmarks of Parkinson’s disease and EGCG has demonstrated the ability to prevent the aggregation of this protein, which suggests it could potentially be used to treat the disease [143]. It can also decrease the formation of kidney stones by reducing calcium oxalate monohydrate (COM) crystal deposition in renal cells [144]. Thus EGCG has also proven to be a useful therapeutic agent for various diseases.

1.6. Stability of phytochemicals

In spite of these good health benefits, these phytochemicals have low absorption rate in the body, undergo rapid elimination and are most often degraded to inactive by-products [123, 127, 128]. Many of these phytochemicals have low solubility in aqueous solutions, such as curcumin, which is insoluble in water [128] and this results in low uptake in the gastrointestinal (GI) tract. This was demonstrated by Wahlstrom, B., *et al.* 1978 who found that curcumin orally administered to rats at a dose of 1g/kg was excreted 75% in the faeces and poorly absorbed from the gut. They also found that 90% of curcumin added to liver microsomes was metabolised within 30 minutes [145]. Similarly, Ravindranath, V., *et al.* 1980 found that oral administration of 400mg of curcumin to rats resulted in less than 60% absorption, with only trace levels found in portal blood 15 minutes later. Approximately 38% of the dose administered was found in
the caecum after 24 hours of treatment. They also discovered that treatment with higher doses of curcumin does not result in higher absorption [146].

Similarly, when humans were orally administered 2g/kg of curcumin for 1 hour, either undetectable or extremely low levels were found in the serum [128]. Pan, M. H., et al. 1999 found that oral administration of curcumin resulted in peak plasma levels after 1 hour, whereas intraperitoneal (i.p) administration resulted in peak plasma levels within 15 minutes. This indicates that the mode of administration can impact the rapidity with which curcumin degrades [147].

Holder, G. M., et al. 1978, Asai, A., et al. 2000 and Ireson, C., et al. 2001 have found that oral administration of curcumin results in metabolism into glucuronide and sulfate conjugates. Figure 1.3 represents the metabolic pathway of curcumin following oral administration. The predominant metabolites in the plasma were glucuronides and sulfates of curcumin. The bile in the liver resulted in glucuronides of dihydrocurcumin, tetrahydrocurcumin and hexahydrocurcumin which were found in the plasma 1 hour after ingestion. Dihydroferulic acid and ferulic acid were minor biliary metabolites [148-150]. Ireson, C., et al. 2001 also found that metabolism of curcumin resulted in a reduction in the inhibition of COX–2 expression [150] and Sandur, S. K., et al. 2007 found that tetrahydrocurcumin produced lower antiproliferative and anti-inflammatory effects in comparison to curcumin [151]. While further studies are required on the effects of the curcumin metabolites, the reports so far suggest that the rapid metabolism of curcumin results in a reduction in the beneficial effects of the compound.
Similarly when rats were orally administered with 50mg of EGCG dissolved in 2ml water (25mg/ml), it was found in plasma after 1 hour, but quickly disappeared within the next 4 hours [123]. Unno, T., et al. 1995 hypothesised that EGCG was possibly decomposed or metabolised after distribution to tissues. This was based on their findings which were similar to Li, Z., et al. 2015, in that EGCG levels in plasma reached a peak 1 hour after oral administration and thereafter rapidly declined, with no traces found 4 hours later [152]. Figure 1.3 depicts a schematic representation of the metabolism of EGCG. Takagaki, A., et al. 2010 found that EGCG is first broken down into epigallocatechin (EGC) and gallic acid, after which EGC is rapidly metabolised into numerous by-products. The metabolites highlighted in red in figure 1.4 were found in rat faeces following oral administration, suggesting these are the main metabolites of EGCG [153]. van’t Slot, G., et al. 2009 also corroborated these results using bacteria found in pig caeca [154]. Thus the clinical efficacy of phytochemicals are diminished following ingestion into the body due to the rapid metabolism into less effective by-products.
Figure 1.4: Metabolism of EGCG by intestinal bacteria found in rats

One method of increasing the stability and uptake of these phytochemicals is through the use of NPs. It was found that the phytochemicals themselves can be used in the synthesis of metal NPs. The phytochemicals are coated on the surface of the nanoparticles, while simultaneously acting as reducing agents and surfactants, thus preventing the NPs from aggregation. This was demonstrated by Shukla, R., et al. 2012 who used EGCG to synthesize biocompatible gold nanoparticles (AuNPs) [155]. Hussain, S., et al. 2014 used EGCG to cap cetyltrimethylammonium bromide (CTAB) AgNPs [122] and Chandra, G. K., et al. 2012 used EGCG to study the interaction with lysozyme-conjugated AgNPs [156].

Similarly, Sindhu, K., et al. 2014 demonstrated the synthesis of biocompatible AuNPs using curcumin [157]. Bettini, S., et al. 2014 synthesized AgNPs using curcumin and demonstrated the ability of these AgNPs to chelate nickel ions from aqueous solution. Yang, X. X., et al. 2016 synthesized curcumin AgNPs which showed antiviral properties against respiratory syncytial virus and biocompatibility with mammalian cells [159]. Curcumin has also been used to cap PVP-AgNPs for sensing bacterial nuclei acid [160], it was capped on ascorbic-acid-citrate-AgNPs which demonstrated antibacterial properties [161].

1.7. Rationale behind this thesis

With the availability of less toxic methods of synthesising nanoparticles and the increasing use of AgNPs in commercial products, there is need to better understand the extent to which these AgNPs can affect mammalian cells. Most studies look only at acute toxicity, such as those listed in tables 1.1 and 1.3, where viability studies are performed for a maximum of 72 hours. However, this raises the question whether 72
hours is sufficient to determine whether NPs are not toxic to mammalian cells [94, 155, 159, 162-165]. Thus there is a need for more long term or chronic studies, which will enable an understanding of realistic exposure scenarios.

Evidence also suggests that the surface coating on the NPs can also influence the cellular responses produced [166], there is no evidence that explains the extent to which the metal core and the surface coating influence the cellular responses elicited by the NPs. Many phytochemicals and AgNPs studied individually show similar effect on mammalian cells, such as causing oxidative stress, DNA damage and disruption of membrane integrity. Phytochemical coated AgNPs have also shown these same effects [116, 167-169], hence, there is no information on which component causes these reactions in the cells or if it is a synergistic effect. Thus, a better understanding of these aspects will enable safer usage of AgNPs in commercial products.

Thus, this projects aims to determine the role surface coating on AgNPs plays in interaction with mammalian cells in a biological scenario. Tyrosine, curcumin and EGCG capped AgNPs are used in this study. Tyrosine is an amino acid capable of synthesis of AgNPs [170] and their antibacterial activity has been demonstrated [171]. These tyrosine AgNPs serve as a control in comparison to curcumin and EGCG AgNPs. Prostate cancer (PC-3) cells have chosen as the mammalian cell line since the effects of curcumin and EGCG have been been well established on this cell line, as evident in sections 1.5.1 and 1.5.2.

1.8. Outline of thesis

Chapter 1 provides an introduction to the wide usage of silver and AgNPs in commercial products and stresses the importance for a better understanding of the interaction of AgNPs in a biological scenario.

Chapter 2 provides the method of synthesis of the AgNPs and details the different methods used to characterise the AgNPs. It provides a brief explanation of the principles of the instruments to provide a better understanding of the various aspects of the AgNPs.

Chapter 3 investigates the influence of the surface coating on the AgNPs might have on interaction with serum in the blood. Since in an in vivo scenario, the AgNPs would first come into contact with serum in the blood before reaching the target organ.
Chapter 4 deals with the development of a chronic toxicity method to enable testing for longer than 72 hours. Using this method, the long term toxicity of the AgNPs on mammalian cells is determined and the role the surface coating plays in the toxicity is investigated.

Chapter 5 further investigates the effect of the AgNPs on cellular adhesion and migration to shed light on the extent to which AgNPs can affect cellular functioning. The extent to which the surface coating on the AgNPs can affects these aspects was also determined.

Chapter 6 provides a summary of the conclusions and scope of future work possible based on the results obtained from this thesis.

An appendix of publications produced during the course of this PhD thesis.

1.9. References


Chapter 2: Synthesis and characterisation of phytochemical coated silver nanoparticles

2.1. Introduction

This Chapter deals with the synthesis and characterisation of the silver nanoparticles (AgNPs) that will be used for in vitro testing on mammalian cells. As evident from Figure 2.1, Tyrosine (tyr) is a monophenolic amino acid, curcumin (cur) is a diphenolic phytochemical from turmeric (Curcuma longa) [1] and epigallocatechin-3-gallate (EGCG) is a polyphenolic compound from green tea (Camellia sinensis) [2]. The AgNPs were synthesised using eqimolar concentrations of silver salt and respective phenolic compounds and were characterised for size, shape, metal content, surface characteristics and stability. This was achieved by Ultraviolet-visible (UV-vis) absorbance spectroscopy, dynamic light scattering (DLS), zeta potential, atomic absorption spectroscopy (AAS), transmission electron microscopy (TEM) and Fourier transform infrared spectroscopy (FTIR).

![Figure 2.1: Chemical structures of (A) Tyrosine, (B) Curcumin and (C) EGCG molecules.](image)

2.2. Materials and methods

2.2.1. Chemicals and materials

Silver sulphate (Ag₂SO₄), L-tyrosine, curcumin, epigallocatechin-3-gallate (EGCG), potassium hydroxide (KOH) and human serum albumin (HSA) were purchased from Sigma-Aldrich. Dialysis tubing cellulose membrane (12 kDa cut-off) was purchased from Sigma-Aldrich. Foetal bovine serum (FBS) was purchased from Interpath Services. Phosphate buffered saline (PBS) was purchased from Life Technologies.
2.2.2. Synthesis of the silver nanoparticles (AgNPs)

The method of synthesis for the tyrosine silver nanoparticles (TyrAgNPs) was followed as described by Selvakannan, P. R., et al. 2004 [3], with the use of equimolar concentrations of the phenolic compounds to silver content in the silver salt. To boiling water, aqueous silver sulphate solution was added followed by eqimolar aqueous tyrosine solution and 0.1 M KOH to obtain pH 10.5. Similarly, the method was adapted for the synthesis of curcumin silver nanoparticles (CurAgNPs) and EGCG silver nanoparticles (EGCGAgNPs). Curcumin was freshly dissolved in 0.1 M KOH and added to boiling water after adding aqueous silver sulphate solution to obtain eqimolar final concentrations of silver ions and curcumin. For the EGCGAgNPs, equimolar aqueous silver sulphate and aqueous EGCG solutions were added to boiling water followed by 0.1 M KOH to obtain pH 10.5. All solutions were freshly made just prior to use to avoid potential degradation.

The samples were heated with constant stirring until the colour changed from colourless to yellowish brown, indicating the formation of nanoparticles (NPs). The samples were allowed to rest for 24 hours before concentration by rotary evaporation and purification by dialysis.

2.2.3. Rotary evaporation of the AgNPs

Rotary evaporation is a common method used to remove the solvent and concentrate NPs after synthesis [4-6]. The rotary evaporator works at low pressure, which lowers the temperature at which a solvent evaporates [7]. The evaporation of the solvent can be aided by simultaneously heating the sample, but due to the low pressure, much lower temperatures are sufficient to reach the boiling point [7, 8]. Thus rotary evaporation allows faster solvent removal while reducing heat damage to the sample. As shown in Figure 2.2, the flask containing the sample is also rotated by an electric motor to provide a thin film of solution, enabling better evaporation of the solvent. The condenser has a coolant flowing through it, allowing condensation and collection of the evaporated solvent in the receptacle below [8]. After synthesis, the AgNPs were evaporated using a Heidolph rotary evaporator, operated at 60 °C, under 60 hPa (45 Torr) of pressure for 30 minutes. The concentrated NPs were then dialysed immediately, to prevent aggregation.
Figure 2.2: Rotary evaporation was used for concentration of the AgNPs. The temperature of the water bath heating the sample was 60 °C and the pressure used for the process was 60 hPa.

2.2.4. Dialysis of the AgNPs

Dialysis is a separation method most often used to remove salts and small molecules during the purification of proteins. It involves the use of a semipermeable membrane, known as a dialysis membrane, which retains molecules larger than the pore diameter inside the bag and smaller molecules and ions pass through the pores into the dialysate on the outside [9]. Dialysis can also be used to remove unreacted ions and surfactants after the synthesis of NPs [10, 11]. The dialysis membrane was processed by boiling in MilliQ water followed by addition of sodium bicarbonate for 15 minutes. The membrane was then washed well with MilliQ water and used immediately. The process was set up as shown in Figure 2.3. After rotary evaporation, the AgNP samples were loaded into the membranes and secured using dialysis clips. The samples were dialysed, at room temperature, against MilliQ water for 24 hours, with constant stirring on a magnetic stirrer. The water was changed 3-4 times during the process to prevent saturation of the dialysate. Henceforth, all analyses and characterisations were performed with dialysed AgNPs, unless stated otherwise. The pH of the AgNPs after dialysis was found to be 7.8.
Figure 2.3: Schematic representation of dialysis. Nanoparticles (orange) remain within the dialysis membrane, while unreacted ions (purple) diffuse out into the dialysate.

2.3. Characterisation of the AgNPs

2.3.1. Ultraviolet–visible (UV-vis) absorbance spectroscopy

Ultraviolet–visible (UV-vis) absorbance spectroscopy was used to characterise the unique surface plasmon resonance (SPR) features of the AgNPs. These SPR features refer to the interaction of incident light with the free electron cloud (plasmons) found on the surface of metal nanoparticles [12, 13]. When these surface plasmons (SPs) oscillate with the same frequency as the photons of the incident light, SPR occurs, producing unique absorption spectra [13]. The SPR band is also highly sensitive to changes in the surrounding medium [13], hence a shift or broadening of the absorption spectra can signify a change in shape or state of aggregation of the NPs. Therefore, UV-vis absorbance spectra were used to not only determine the formation of AgNPs after synthesis but also to monitor the stability of the AgNPs in the presence of phosphate buffered saline (PBS) and foetal bovine serum (FBS), as described in Section 2.3.7, to ensure their suitability for in vitro experiments.

UV-vis spectra can also be generated when valence shell electrons in a molecule transition between different energy levels when exposed to incident light. The electrons in the hydroxyl group bonded to a benzene ring in a phenolic compound are capable of transition from bonding to antibonding orbital ($\pi -$
\(\pi\) 

However, the absorption is weak and the spectra produced are around 260 nm [14]. Since the AgNPs were synthesised with phenolic compounds, UV-vis spectra can be used to detect the presence of these phenolic compounds on the surface of the AgNPs.

The UV-vis spectra were observed using an Agilent Varian Cary 50 spectrophotometer, which consists of a xenon lamp as the light source, a monochromator, a beam splitter to create a reference beam and a sample beam and a detector (Figure 2.4). A quartz cuvette, with a path length of 1 cm was used for all measurements. The instrument was operated at room temperature, at a range of 200 – 800 nm, with a resolution of 2 nm.

As shown in Figure 2.5, the TyrAgNPs, CurAgNPs and EGCGAgNPs showed SPR bands at 409 nm, 412 nm and 406 nm, respectively. These AgNPs displayed characteristic SPR bands for spherical AgNPs, which are typically centred around 400 – 450 nm [15-18]. This indicates that all three AgNPs were quasi-spherical, which was further confirmed by transmission electron microscopy (Figure 2.12). The decrease in intensity of the SPR peaks after dialysis can be attributed to the dilution of the AgNPs with water on the removal of free ions and unreacted reagents from within the dialysis membrane. It should be noted that there is no shift in the SPR bands after dialysis, indicating no significant aggregation or change in morphology of the AgNPs as a result of the dialysis. The secondary peaks observed at 272 nm, 263 nm and 273 nm for TyrAgNPs, CurAgNPs and EGCGAgNPs, respectively are attributed to the tyrosine [19].
curcumin [20] and EGCG [21] coatings on the nanoparticles, since they exhibit absorption features at those respective wavelengths. The decrease in intensity of these peaks after dialysis is attributed to the removal of unbound molecules from the NP solutions.

![UV-visible absorbance spectra](image)

**Figure 2.5**: UV-visible absorbance spectra of the AgNPs showing peaks at (A) TyrAgNPs 409 nm and 270 nm, (B) CurAgNP 412 nm and 263 nm and (C) EGCGAgNPs 406 nm and 273 nm, after synthesis (Syn) and after dialysis (Dia). The peaks at 270 nm, 263 nm and 273 nm correspond to tyrosine, curcumin and EGCG, respectively.

### 2.3.2. Dynamic light scattering (DLS)

Dynamic light scattering (DLS), also known as Quasi-Elastic Light Scattering (QELS) was used to determine the hydrodynamic radii and particle size distributions of the AgNPs. When the AgNP sample is illuminated by a laser beam in the instrument, the fluctuations in scattered light caused by Brownian motion of the particles can be used to determine the size distribution and radii of the particles (Figure 2.6). This is obtained using the Stokes-Einstein relationship for spherical particles:

$$D = \frac{k_B T}{6 \pi \eta r}$$

Where $D$ is the diffusion constant, $k_B$ is Boltzmann’s constant, $T$ is the absolute temperature, $r$ is the radius of the particles and $\eta$ is the dynamic viscosity [22]. Since surface modification and any molecules in contact with the particles can affect the particle diffusion in the solution, DLS is a measure of hydrodynamic radii and is often larger than the size measure by electron microscopy [22].
DLS measurements were performed on an ALV – 5022 FAST DLS instrument. Dialysed samples were used without further dilution and the measurements were conducted at 22 °C, regulated by a temperature controlled sample holder fitted in the instrument. The particle distributions obtained are displayed in Figure 2.7.

The TyrAgNPs have a radius of 18.8 nm and a polydispersity index (PDI) of 0.63, CurAgNPs are 20.6 nm in radius with a PDI of 0.43 and the EGCGAgNPs showed a radius of 16.2 nm, with a PDI of 0.49. The PDI is a measure of NP size distribution and since the AgNPs have PDIs of 0.4 – 0.6, it indicates the
presence of different sized particles. This is further evident in the images and histogram obtained from transmission electron microscopy displayed in Figure 2.12.

### 2.3.3. Zeta potential

Surfactants, or in this case, phenolic compounds, can coat the surface of NPs, modifying the surface charge on the NPs. This surface charge may play an important role in the uptake and toxicity of nanoparticles in different types of cells [23], hence it is important to determine the charge on the NPs. Particles with larger zeta potential repel each other, leading to less aggregation, thereby remaining stable for longer periods [24]. Hence, the zeta potential can also provide information on the stability of the nanoparticles.

As described in Figure 2.8, the surface charge consists of two layers – an inner layer, known as the Stern layer, which is strongly associated with the particle surface and an outer diffused layer which is less firmly bound to the particle. At the boundary between the Stern and the diffused layers, is the slipping plane, inside which the ions form a stable entity with the particle in an electric field. The electric potential in this slipping plane is what constitutes the zeta potential on a particle [24].

![Figure 2.8: Schematic representation of the electric double layer found on the surface of a nanoparticle. Zeta potential is the electric potential in the slipping plane.](image)

The zeta potentials of the AgNPs were measured using a Malvern Nano-Zetasizer, operated at 25 °C. The samples were loaded into a folded capillary cell and the Smoluchowski methodology [25] for aqueous
media was employed, using the refractive index for silver and water as the dispersant. Apart from NP surface charge, zeta potential was also used to determine the interaction of the AgNPs with human serum albumin (HSA), as described in Chapter 3. The zeta potentials of the AgNPs were found to be –40.8 mV, –41.3 mV and –38.9 mV for TyrNPs, CurAgNPs and EGCGAgNPs, respectively. Since particles with larger zeta potential repel each other and do not aggregate easily, and since these AgNPs show zeta potentials beyond –20 mV, they can be considered stable formulations [24].

2.3.4. Atomic absorption spectroscopy (AAS)

Atomic absorption spectroscopy (AAS) was used to quantify silver content in the samples. This occurred by atomising the sample in a flame, then, light from a hollow-cathode lamp with a cathode of the same element (silver, in this case) being analysed, is passed through the flame containing the atomised sample (Figure 2.9). The light absorbed by the sample is used to quantify the amount of element present in the sample through the use of the Beer-Lambert’s law:

\[
\text{Abs} = \log \frac{I_0}{I} = \epsilon \cdot l \cdot c
\]

Where \(\text{Abs}\) is the absorbance, \(I_0\) is the intensity of the light from the lamp, \(I\) is the intensity of transmitted light or light absorbed by the sample, \(\epsilon\) is the absorption coefficient, \(l\) is the path length of the sample and \(c\) is the concentration of the element being analysed [26].

![Figure 2.9: Schematic representation of atomic absorption spectroscopy.](image)
A Varian PerkinElmer Atomic Absorption Spectrometer was used to determine the silver content in the AgNPs. Samples were prepared by dissolving the AgNPs in concentrated nitric acid and bringing it to a final concentration of 2% nitric acid, with MilliQ water, in 10 mL total volume. Silver standards ranging from 1 – 5 ppm in 2% nitric acid were employed. The standard curve obtained is shown in Figure 2.10, from which the concentrations of silver in the AgNPs were interpolated. Henceforth, all AgNP concentrations correspond to silver content of the AgNPs.

![Absorbance vs Concentration](image)

**Figure 2.10: Calibration curve of silver standards to determine the concentration of silver in the AgNPs.**

### 2.3.5. Transmission Electron Microscopy (TEM)

Transmission electron microscopy (TEM) was used to assess the morphology of the AgNPs. An illustration of the instrument is shown in Figure 2.11. The instrument functions by emitting an electron beam from a gun containing a LaB6 electrode. The electrons emitted from it travel through a vacuum and are focused on the specimen using electromagnets. The electron beam then travels through the specimen, is focused and magnified by an objective lens and is collected on a fluorescent screen below. Electrons that have been scattered by the specimen are removed through an aperture, allowing the generation of a ‘shadow image’ of the specimen with brighter areas where there was no electron scattering. The image is then captured using a charge-coupled device (CCD) camera [27].
The instrument used to characterise the AgNPs was a JEOL 1010 transmission electron microscope, operated at an accelerating voltage of 100kV. Samples were prepared by drop casting on strong carbon coated 200 mesh copper grids and were allowed to dry overnight at room temperature. The images obtained are displayed in Figure 2.12 A – C. Image analyses for size distributions were conducted using Image J software and histograms are displayed in Figure 2.12 D – F. The TyrAgNPs had an average diameter of 10.56 nm ±2.27, CurAgNPs had an average of 13.68 nm ±0.76 and the EGCGAgNPs were an average of 9.27 nm ±1.29. The images indicate that all three AgNPs were quasi-spherical and the average sizes indicate that they may be considered approximately of the same diameter for the purpose of investigations performed in this thesis. Figure 2.12 D – F also indicates a wide distribution of particle sizes, with few approaching up to approximately 40 nm in diameter.
Figure 2.12: TEM images of the (A) TyrAgNPs, (B) CurAgNPs and (C) EGCGAgNPs. Scale bar corresponds to 0.2 µm. Histograms depicting particle size distribution as obtained from Image J software after analysing 50 – 150 particles each of (D) TyrAgNPs, (E) CurAgNPs and (F) EGCGAgNPs.

2.3.6. Fourier transform infrared (FTIR) spectroscopy

Fourier transform infrared (FTIR) spectroscopy was used to examine the atomic vibrations that might occur in the organic coating on the surface of the AgNPs. When a sample is exposed to electromagnetic radiation equivalent to 4000-400 cm\(^{-1}\), energy can be transmitted or absorbed giving rise to vibrational movements within the molecules in the sample. Since specific functional groups can give rise to fingerprint vibrational bands, they can be used to identify the existence of those functional groups in a sample. An example of an FTIR spectrum is shown in Figure 2.13. Hence, FTIR is a useful tool in gaining information about the functional groups found on the surface coating of the NPs [28]. Since phenolic compounds were used to functionalise the AgNPs, FTIR can provide insight into the role the phenolic groups played in the reduction of silver ions to AgNPs and on the nature of interaction between the NPs and the oxidised molecules.
Figure 2.13: Example of a FTIR spectrum indicating the regions where specific functional groups can be found.

The FTIR measurements of the AgNPs were conducted on a Perkin Elmer Spectrum 100 instrument with a Spotlight 400 attachment. Spectra from 64 scans were collected and averaged. The data was collected from 4000–750 cm$^{-1}$ at a resolution of 2 cm$^{-1}$. The results shown in Figure 2.14 indicate that the spectra of the free phenolic compounds match with those found on the AgNPs, which would suggest that the phenolic compounds are coating the surface of the AgNPs. As reported earlier, phenol groups tend to reduce silver ions under alkaline pH and are oxidised into semi-quinone groups which have very strong interaction with the silver surface [3, 29]. In Figure 2.14 A, this is evident in the case of tyrosine, which exhibits carbonyl stretching vibrations at approximately 1607 cm$^{-1}$ due to the carboxylate ion. There is evidence of a shift to 1673 cm$^{-1}$ in the case of the TyrAgNPs, indicating possible formation of a quinone type structure of the phenolic group in tyrosine [3], this could suggest that the tyrosine molecule binds to the surface of the AgNPs by the oxidised phenolic group [29].

In Figure 2.14 B, the peak observed at 1626 cm$^{-1}$ corresponds to the carbonyl (C=O) vibration stretching and the neighbouring 1601 cm$^{-1}$ is attributed to the aromatic ring stretching vibration in curcumin. These peaks have been replaced by a broad peak at 1582 cm$^{-1}$ in the CurAgNPs. The peak assigned to C=O at 1507 cm$^{-1}$ [30] has also shifted to 1512 cm$^{-1}$. This could indicate that the carbonyl groups in curcumin are involved in the AgNP synthesis. This is similar to the results obtained by a previous report on gold NPs.
The enol C – O peak at 1276 cm\(^{-1}\) and the keto peak at 1233 cm\(^{-1}\) have both shifted in the CurAgNPs, which could indicate that both phenol groups might be involved in the AgNP synthesis.

In the case of EGCG (Figure 2.14 C), the characteristic peaks at 1343 cm\(^{-1}\) corresponding to the O – H in-plane bending vibration has disappeared in the EGCGAgNPs and the 1144 cm\(^{-1}\) peak corresponding to the O – H aromatic vibration is replaced by a broad peak. The peaks at 1691 cm\(^{-1}\) and 1614 cm\(^{-1}\) which correspond to the C=O have shifted to 1704 cm\(^{-1}\) and a broad peak at 1620 cm\(^{-1}\), respectively. This could indicate that possibly a number of phenol groups in EGCG are involved in the AgNP synthesis. The peak observed at 1707 cm\(^{-1}\) in the EGCGAgNPs is a characteristic ester band and this could further indicate an increase in C=O features on the surface of the EGCGAgNPs.

Since all three molecules used in the synthesis of the AgNPs possess phenol functional groups, the corresponding O–H stretching frequencies were observed at 3210 cm\(^{-1}\) in tyrosine, 3508 cm\(^{-1}\) and 3293 cm\(^{-1}\) in curcumin and 3350 cm\(^{-1}\) in EGCG. However, these peaks were replaced with intense, broad O–H stretching bands in all three AgNPs (Figure 2.14 D, E and F). This can probably be due to the strong intermolecular hydrogen bonding among the surface bound molecules due to their hydroxyl groups, which were not present as extensively in their pure forms [33, 34]. This hydrogen bonded network among the surface...
bound molecules not only protects the NPs from aggregation, but also provides a strong hydrophilic surface to the nanoparticles.

2.3.7. Stability studies

The stability of the AgNPs in phosphate buffered saline (PBS) and in foetal bovine serum (FBS) were assessed, since the AgNPs would come in contact with cell culture media typically containing PBS and 10% FBS. This was achieved by incubating the AgNPs with PBS and 10% (v/v) FBS in PBS. The mixtures were incubated for 1 hour and 24 hours at 37 °C to simulate an in vitro scenario. The stability was assessed by UV-vis absorbance spectroscopy and DLS as described in sections 2.3.1 and 2.3.2, respectively.

The UV-vis spectra (Figure 2.15) indicate that all three AgNPs showed aggregation in PBS alone. The TyrAgNPs (Figure 2.15 A) showed aggregation even after 1 hour incubation in PBS, while the CurAgNPs (Figure 2.15 B) appeared to show less aggregation compared to the other two AgNPs. When 10% FBS was present in the PBS, however, the AgNPs showed better stability. The TyrAgNPs (Figure 2.15 A) showed a decrease in absorbance, indicating the possibility of some aggregation, however, CurAgNPs (Figure 2.15 B) and EGCGAgNPs (Figure 2.15 C) showed good stability both at 1 hour and after 24 hours. There is evidence of a shift in the peaks in the presence of 10% FBS after 24 hours from 412 nm to 416 nm in CurAgNPs and from 406 nm to 412 nm in EGCGAgNPs. This is possibly due to the coating of proteins on the surface of the particles causing a change in size [35].

![Figure 2.15: Stability of (A) TyrAgNPs, (B) CurAgNPs and (C) EGCGAgNPs in pure PBS and PBS containing 10% FBS as observed by UV-vis absorbance spectroscopy.](image)
To further assess the stability of the AgNPs, hydrodynamic radii were determined by DLS as described in Section 2.3.2. The results shown in Table 2.1 support the results obtained from the UV-vis spectra wherein all three AgNPs showed aggregation in the presence of PBS. In the presence of 10% FBS in PBS, all three AgNPs showed an increase in hydrodynamic radius due to the formation of a protein corona on the NPs. This protein corona can be categorised into soft and hard coronas. The soft corona consists of proteins that initially bind to the NP surface, while the hard corona refers to the binding of proteins with greater affinity for the NP surface, which replace the proteins of lesser affinity in the soft corona [35-37]. The fact that there is marginal decrease in size after 24 hours in comparison to the size after 1 hour could indicate the formation of hard protein corona on the AgNPs after 24 hours. Since 10% FBS is the concentration used for cell culture experiments, these results indicate that these AgNPs are suitable for use in in vitro scenarios. The DLS data indicate that the hydrodynamic size of the AgNPs increases in the presence of FBS, possibly due to the formation of a protein corona on the surface of the AgNPs.

Table 2.1: Hydrodynamic radii (nm) of the AgNPs in the presence of pure PBS and PBS containing 10% FBS as determined by DLS.

<table>
<thead>
<tr>
<th>AgNPs</th>
<th>Water</th>
<th>PBS – 1h</th>
<th>PBS – 24h</th>
<th>10% FBS – 1h</th>
<th>10% FBS – 24h</th>
</tr>
</thead>
<tbody>
<tr>
<td>TyrAgNPs</td>
<td>23.7</td>
<td>210.0</td>
<td>180.0</td>
<td>30.0</td>
<td>26.0</td>
</tr>
<tr>
<td>CurAgNPs</td>
<td>23.8</td>
<td>47.5</td>
<td>49.9</td>
<td>49.9</td>
<td>38.8</td>
</tr>
<tr>
<td>EGCGAgNPs</td>
<td>18.5</td>
<td>620.0</td>
<td>867.0</td>
<td>24.9</td>
<td>23.6</td>
</tr>
</tbody>
</table>

2.4. Conclusions

The AgNPs obtained were quasi-spherical, negatively charged, approximately 10 nm in size with a hydrodynamic radius of 20 nm, coated with organic compounds and they are possibly quinone functional groups. Studies indicate spherical NPs less than 50 nm in diameter were optimal for uptake into mammalian cells [38-40], indicating that these AgNPs might be suitable for in vitro and in vivo applications. While there is much debate on whether negatively charged particles are more suitable for uptake into cells over positively charged particles [38, 41], studies indicate that the uptake of negatively charged particles possibly occurs through interaction with proteins in the serum [39, 40]. Stability studies in the presence of FBS indicate that all three AgNPs undergo protein corona formation with FBS and this
could indicate that they might be more favourable for cellular uptake than positively charged particles. Hence these AgNPs were used to study the interaction with serum proteins and assess the cellular effects on mammalian cells, as described in subsequent chapters.

2.5. References


Chapter 3: Role of surface corona on interaction with human serum

3.1. Introduction

The unique physiochemical and broad spectrum antibacterial and antifungal properties of silver nanoparticles (AgNPs) have made them the most incorporated nanomaterial in consumer and medical products to date [1, 2]. However, on injecting these nanoparticles (NPs) into the blood stream, they first come into contact with proteins found in the plasma [3]. These plasma proteins interact with the surface of the NPs forming a protein corona (PC) on the NPs [4], which can elicit or suppress the body’s natural immune response. This can alter the mechanism of interaction of the NPs with the target organs and in doing so, change the properties of the NPs [5]. Hence, distribution and biological responses of the NPs in the body are dictated by this PC, since this is what is perceived when the NPs first come in contact with the target cells [4, 6, 7]. The NPs in turn, can change the conformation of the plasma proteins they interact with, possibly altering the function of these proteins [1]. Hence, when considering the use of NPs for in vivo purposes, it is important to understand and control this interaction of NPs with protein in the blood stream.

Albumins are the most abundant proteins found in circulating blood [5, 8-10] and in the serum used in cell culture media for in vitro experiments. Human serum albumin (HSA) contains 585 amino acid residues and it serves as an important carrier for many substances such as fatty acids, bilirubins, hormones and exogenous and endogenous ligands [11, 12]. Since HSA possess so many important physiological functions, any changes to the structure can prove detrimental to its normal functioning in the body [1, 8, 9, 13]. Hence, it is important to study the interaction of NPs with this protein. HSA contains a single tryptophan (Trp-214) moiety, found in the hydrophobic cavity of sub-domain IIA (Sudlow I) of the HSA molecule. This Trp residue is capable of producing strong intrinsic fluorescence and has been used extensively as a reporter for ligand binding and conformational studies [1, 7, 9-18].

It has been observed that most studies that look at NP-protein interactions utilize bovine serum albumin (BSA) [3, 6, 19-22] instead of HSA, however, one important difference between BSA and HSA is the presence of two tryptophan residues in BSA while HSA has a single unique tryptophan residue [10, 14].
Gelamo, E. L., et al. 2000 have shown that in the presence of various ionic surfactants, BSA showed a quenching of fluorescence, while HSA showed an enhancement of fluorescence with the same ionic surfactants [10]. Manivel, A., et al. 2012 have also shown that BSA can exhibit higher binding with NPs than HSA [2]. This indicates that BSA is not a reliable substitute for HSA, especially when using fluorescence as the method of detection. Hence HSA was used to study the interaction with NPs in this study.

On the other hand, the nanoparticle surface i.e. biomolecules or phytochemicals present on the nanoparticle surface may affect the degree and type of protein interactions that occur with the nanoparticles. Evidence suggests that isolated phytochemicals, such as curcumin [23], epigallocatechin-3-gallate (EGCG) [24], piperine [25], various flavonoids [26] and pheophytins [27] show interaction with serum; however, there are no reports available on the interaction of phytochemical coated nanoparticles with HSA. Fourier transform infrared spectroscopy (FTIR) data on phytochemical coated nanoparticles suggest that the functional groups of the phytochemicals are modified during the process of synthesis and capping of nanoparticles [28-30] and Chapter 2, Section 2.3.6. This change in phytochemical structure could then alter the mode of interaction these nanoparticles undergo with serum proteins. However, there is a lack of information on how this change in phytochemical structure might affect the interaction these NPs with proteins in the blood.

This Chapter aims to use curcumin (cur) and epigallocatechin-3-gallate (EGCG) coated silver nanoparticles (AgNPs) to demonstrate the interaction with HSA. Since tyrosine (tyr) is also fluorescent in the same range as the tryptophan molecule in HSA [31], citrate-capped silver nanoparticles were used as a control instead of the TyrAgNPs. Ultra-violet visible (UV-vis) absorbance spectroscopy, fluorescence spectroscopy and circular dichroism spectroscopy were employed to elucidate the role of surface corona in nanoparticles-protein interactions.

### 3.2. Materials and methods

#### 3.2.1. Chemicals and materials

Human serum albumin (HSA), monosodium phosphate (NaH$_2$PO$_4$) and disodium phosphate (Na$_2$HPO$_4$) were purchased from Sigma-Aldrich (Castle Hill, NSW, Australia). Sodium phosphate buffer (pH 7.4) was
prepared by mixing 40.5 mL of 0.2 M Na$_2$HPO$_4$ with 9.5 mL of 0.2 M NaH$_2$PO$_4$ and made up to a final volume of 100 mL. MilliQ water was used for all experiments.

### 3.2.2. Synthesis of citrate–capped silver nanoparticles (CtNPs)

The citrate–capped silver nanoparticles (CtNPs) were synthesised using citrate reduction method [3]. 100 mL of silver nitrate (1 mM) was heated on a magnetic stirrer to 95 °C and 50 mL of 1% trisodium citrate solution was added. The formation of NPs was indicated by the colourless solution turning yellow. The solution was dialysed for 24 hours against distilled water, as described in Chapter 2, Section 2.2.4.

CurAgNPs and EGCGAgNPs were synthesised and all AgNPs were characterised as described in Chapter 2, Sections 2.2 and 2.3.

### 3.2.3. Spectroscopic studies of interaction of the silver nanoparticles (AgNPs) with human serum albumin (HSA)

To study the interaction of the phytochemical coated AgNPs with HSA, concentration dependent studies were performed by incubating varying concentrations of the AgNPs (0.1 to 240 µM) with 3 µM of HSA in sodium phosphate buffer for 2 hours at 4 °C. All solutions were brought to room temperature for 30 minutes before measuring UV-visible absorbance and fluorescent spectra. UV-vis absorbance spectroscopic studies were conducted on an Agilent Varian Cary 50 spectrophotometer as described in Chapter 2, Section 2.3.1. The dynamic light scattering and zeta potential was determined using a Malvern Nano-Zetasizer as described in Chapter 2, Sections 2.3.2 and 2.3.3.

Fluorescence measurements were performed on a Jobin Yvon Horiba Fluoromax 4 spectrophotometer. Since tryptophan produces an excitation peak between 300 to 350 nm when excited at 295 nm [32], fluorescence spectra were recorded in the range of 300 to 500 nm by exciting the reaction mixture at 295 nm at room temperature. Further, to determine the binding affinity of the AgNPs to the HSA, using the fluorescence spectra, the dissociation constant ($K_d$) was determined using the equation

$$Y = \frac{B_{\text{max}}X}{K_d + X}$$

Where $B_{\text{max}}$ refers to the binding maxima and $X$ and $Y$ refer to the corresponding values from the X and Y axes [32]. This equation was fitted using GraphPad Prism 5.02, Graph-Pad, La Jolla, CA.
For temperature dependent studies, fluorescence spectra were collected from 20 °C to 70 °C at intervals of 10 °C. From this data, the binding mechanism was elucidated using Stern-Volmer plots by plotting $F_0/F$ vs $[Q]$ at different temperatures [33]. The binding constant and number of binding sites were determined using the formula:

$$\log \frac{F_0}{F} = \log K + n \log [Q]$$

Where $F_0$ and $F$ denote the fluorescence intensities in the absence and presence of the quencher (AgNPs), respectively, and $[Q]$ is the concentration of the AgNPs. $K$ is the binding constant and $n$ is the number of binding sites.[3]

### 3.2.4. Circular dichroism (CD) spectroscopy

In circular dichroism (CD), the electric field of a beam of light, when polarised by suitable prisms or filters, oscillates sinusoidally in a single plane. This wave, when viewed from the front, is visualised as two vectors of equal length which trace a circle, one rotating clockwise and the other anticlockwise. The beam of light is hence referred to as circularly polarised light. If an asymmetric molecule were to interact with this light, it will absorb the left and right handed circularly polarised light to different extents. These vectors will then trace out an ellipse, hence the light is said to be elliptically polarised and the difference in absorbance is reported in degrees ellipticity.

This method can be used to determine the secondary structure of proteins. In this case, the protein will be the asymmetric molecule interacting with the circular polarised light and the different structural components of the protein produce characteristic CD spectra [34]. The $\alpha$-helical proteins have two negative bands at 208 nm and 222 nm and a positive band at 193 nm [10, 12, 16, 34]. Since this spectra is dependent on the conformation of the proteins, CD has been used to determine changes in protein structure [34].

The conformational changes to HSA on interaction with the AgNPs were studied using a Jasco J-815 spectropolarimeter at room temperature (25 °C). The path length of the quartz cell used was 0.1 cm. Protein concentration was 3 µM and AgNP concentration was 240 µM. The CD spectra were collected from 190-300 nm, using a scan speed of 50 nm/minute, under constant nitrogen flow. Three scans were averaged to improve signal to noise ratio. The ellipticity values are expressed in terms of mean residue
molar ellipticity (θ), in degree cm² dmol⁻¹. Appropriate baseline corrections using sodium phosphate buffer were made.

3.3. **Results and discussion**

3.3.1. **Characterisation of the citrate–capped silver nanoparticles (CtNPs)**

CtNPs showed a surface plasmon resonance (SPR) band at 416 nm. TEM images indicate that these NPs were approximately 20 nm in size (Figure 3.1). The DLS particles size is 55.61 nm with a zeta potential of -25.6 mV (Table 3.1). These results are similar to those obtained by the published protocol for the synthesis of these NPs [3].

![Figure 3.1: (A) UV-vis absorbance spectrum of CtNPs, after synthesis (Syn) and after dialysis (Dia). (B) TEM images of the CtNPs.](image)

3.3.2. **Dynamic light scattering (DLS) and zetapotential of the interaction of the AgNPs with HSA**

As a preliminary method of determining the interaction of HSA with the AgNPs, the DLS and zeta potential were monitored in the presence and absence of HSA. Table 3.1 depicts the results obtained. It indicates that the hydrodynamic diameters of the AgNPs were increased in the presence of HSA, accompanied by a decrease in zeta potential. The CtNPs and EGCGAgNPs show a 1.1x increase in size in the presence of HSA, while CurAgNPs show a 1.29x increase in size. This possibly indicates that CurAgNPs show a greater interaction with HSA than the other two NPs. Brewer, S. H., et al. 2005 have noted that a decrease in the zeta potential indicates the binding of BSA to the surface of the NPs [20].
Hence the decrease in zeta potential indicates that HSA binds to the surface of all three AgNPs. However, this data merely confirms that HSA forms a protein corona on the AgNPs. The mechanism of interaction is investigated further below.

Table 3.1: Hydrodynamic diameters and zeta potentials for CtNP, CurAgNPs and EGCGAgNPs in the presence and absence of HSA.

<table>
<thead>
<tr>
<th></th>
<th>CtNPs</th>
<th>CtNPS+HSA</th>
<th>CurAgNPs</th>
<th>CurAgNPs+HSA</th>
<th>EGCGAgNPs</th>
<th>EGCGAgNPs+HSA</th>
</tr>
</thead>
<tbody>
<tr>
<td>DLS (nm)</td>
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<td>61.6</td>
<td>44.8</td>
<td>57.9</td>
<td>61.5</td>
<td>67.7</td>
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<tr>
<td>Zeta potential (mV)</td>
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<td>-16.5</td>
<td>-41.7</td>
<td>-31.3</td>
<td>-38.9</td>
<td>-32.7</td>
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</tbody>
</table>

3.3.3. Absorption characteristics of the AgNPs interaction with HSA

An increase in intensity of HSA absorption spectra has been attributed to the complex formation between HSA and NPs and has been used as an indicator of the type of interaction between NPs and HSA or BSA [1, 3, 13]. Hence, the absorption spectrum of HSA at 279 nm was monitored in the presence and absence of the AgNPs. The results in Figures 3.2 A – C show an increase in intensity and blue shift in the HSA peak at 279 nm. This suggests the possibility of ground state complex formation, since dynamic collisions only affect the excited states of the quenching molecules and has no effect on absorption spectra [15, 19]. Similar effects of NPs on HSA absorption spectra were observed by other studies [1, 3, 15, 19]. Overall, the intensity of the interaction was greater with CurAgNPs and EGCGAgNPs than with the CtNPs. This evidence, coupled with the DLS and zeta potential results (Table 3.1), which could suggest that the phytochemical surface coating on the NPs plays a role in the interaction with proteins.

The absorption spectra of the AgNPs at approximately 400 nm were also monitored in the presence of HSA. The SPR peaks observed at 412 nm (CurAgNPs) and 406 nm (EGCG AgNPs) show a marginal increase in the intensity and a red shift to 413 nm and 412 nm for CurAgNPs and EGCGAgNPs, respectively, while the 416 nm CtNP peak shows no significant change (Figure 3.2 D). The shifts toward red end of spectrum indicate an increase in overall nanoparticle size, which further supports the DLS and
zeta potential measurements (Table 3.1). It should be noted that while the CtNP absorption spectrum did not show any change, the HSA spectrum with CtNPs indicates that there is a definite interaction between the two. It should also be noted that the spectra indicate no aggregation of the AgNPs in the presence of HSA. Overall, this data indicates that the interaction of HSA with the AgNPs possibly occurs through complex formation.

Figure 3.2: UV-vis absorbance spectra depicting the blue shift and increase in intensity of HSA spectra with dialysed (A) CtNPs, (B) CurAgNPs and (C) EGCGAgNPs respectively, indicating strong interaction of HSA molecules with the surface of the phytochemical coated AgNPs. The concentrations of the AgNPs range from 0.1-240 µM [Ag]. (D) The red shift and increase in intensity in the spectra of CurAgNPs and EGCGAgNP.

3.3.4. Fluorescence characteristics of the AgNPs interaction with HSA

The Trp-214 residue in HSA is very sensitive to changes in the microenvironment of the HSA molecule, hence the intrinsic fluorescence spectra of Trp is a good indicator of HSA interactions with other molecules [16]. Quenching or decrease in fluorescence can indicate a variety of interactions such as molecular rearrangement, collisional quenching or ground state complex formation [3, 7]. HSA showed an emission band at 346 nm with excitation at 295 nm, while the AgNPs showed no fluorescence at this
wavelength, indicating the absence of overlapping fluorescence spectra from the AgNPs. HSA concentration was maintained constant at 3 µM, while the nanoparticle concentration was increased from 0.1 to 240 µM. The reason these concentrations were chosen was to determine the effect of the AgNPs on HSA when the protein concentration was up to 30 times greater than that of the AgNPs as well as when the protein concentration was up to 80 time less than the AgNPs.

As shown in Figures 3.3 A – C, in the presence of increasing concentrations of AgNPs, the Trp fluorescence was quenched significantly, in a concentration dependent manner, with greater quenching observed with CurAgNPs, followed by EGCGAgNPs and the least quenching was seen with CtNP. Since efficiency of quenching depends on the proximity of the quencher to the chromophore [21], these results indicate that the AgNPs bind at or near the Trp moiety, in sub-domain IIA of the HSA molecule.

Another interesting aspect evident from Figure 3.3 A – C is an increasing blue shift in the fluorescence spectra as AgNP concentration increases. When the nanoparticle concentration was 30 times less (0.1 µM) than the HSA concentration (3 µM), the blue shift was a mere 2 nm. However, this continues to increase with increasing AgNP concentration, with a blue shift up to 5-6 nm when the AgNP concentration is greater than the HSA concentration. This shift in the fluorescence spectra has also been observed by others [1, 13, 18] and indicates a concentration dependent effect of the AgNPs on HSA. This also suggests that chromophore of the protein has been brought into a more hydrophobic surrounding [13], which signifies that the AgNPs are capable of altering the structure of the HSA molecule.

From the fluorescence data, the dissociation constant \(K_d\) was calculated using GraphPad Prism (Figure 3.3 D), which determined the affinity of the AgNPs for HSA [32]. The results indicated that affinity of CurAgNPs for HSA was highest, with a \(K_d\) of 2.9 µM, followed by EGCGAgNPs (8.55 µM) and CtNPs (28.99 µM). This could be attributed to the coating of the AgNPs by the phytochemicals. Since curcumin is a smaller molecule than EGCG, it is possible that curcumin does not cover the entire surface of the NP, allowing gaps where HSA can bind directly to the NP surface. Another possible explanation is discussed below.
Figure 3.3: Fluorescence spectra displaying concentration dependent quenching of HSA by (A) CtNPs, (B) CurAgNPs, (C) EGCGAgNPs. Blue shift in the spectra is evident at higher concentrations. The concentration of the AgNPs range from 0.1 to 240 µM [Ag] and HSA concentration is 3 µM. (D) Raw data used for calculating apparent dissociation constants using nonlinear regression in Graph-Pad Prism 5.02 (GraphPad, La Jolla, CA, USA).

While similar quenching of spectra have been observed by other studies that looked at the interaction of HSA and BSA with NPs [1-3, 13, 16, 19], they considered a single type of NP, and the role of the surface coating has not been considered. Free curcumin and EGCG show a concentration dependent increase in HSA quenching [35, 36]. However, Maiti, T. K., et al. 2006 showed a red shift in the HSA fluorescence spectra with EGCG, indicating a shift towards a more polar environment [36], as opposed to a blue shift observed with the AgNPs. Ishii, T., et al. 2011 have shown that the hydroxyl groups on the galloyl moiety and on the B-ring of EGCG are responsible for the interaction with HSA [37]. If the FTIR data of the CurAgNPs and EGCGAgNPs (Chapter 2, Section 2.3.6) are to be considered, a change in the phenol functional groups of the phytochemicals on the surface of the AgNPs was observed. This could indicate that the interaction of the free phytochemicals with HSA will not necessarily be the same as the interaction
of phytochemical-coated AgNPs. This also suggests that the surface coating on the AgNPs plays a significant role in determining the interaction with serum proteins.

### 3.3.5. Fluorescence quenching mechanism

There are two types of quenching mechanisms i.e. dynamic or collisional quenching and static quenching or complex formation. Dynamic quenching occurs when a fluorophore in an excited state is deactivated on contact with the quencher, while static quenching occurs when the quencher and fluorophore form a nonfluorescent ground state complex [3, 32]. These two types of quenching can be differentiated by studying the interaction at varying temperatures. When temperature is increased, diffusion occurs at a faster rate leading to the dissociation of weakly bound complexes and an overall increase in collisional quenching [3]. Fluorescence spectra of HSA with increasing concentrations of AgNPs were obtained at temperatures ranging from 20 °C to 70 °C (293 K to 343 K). In order to determine the mechanism of quenching, the data was analysed for the quenching constant using the Stern-Volmer equation:

\[
\frac{F_0}{F} = 1 + K_{sv} [Q]
\]

Where \(F_0\) and \(F\) denote the fluorescence intensities in the absence and presence of the quencher (AgNPs) respectively and \([Q]\) is the concentration of the AgNPs. \(K_{sv}\) is the Stern-Volmer quenching constant [3].

The Stern-Volmer quenching plots for the AgNPs are shown in Figure 3.4. The Stern-Volmer quenching constants are displayed in Tables 3.2 – 3.4. The \(K_{sv}\) values for CurAgNPs show a decrease with increasing temperature from 30 °C to 70 °C. This indicates that the quenching mechanism is static binding since a decrease in \(K_{sv}\) with increasing temperature indicates complex formation or static quenching [1, 3, 13]. The \(K_{sv}\) values for EGCGAgNPs, on the other hand, show an increase from 20 °C to 40 °C, followed by a decrease from 40 °C to 70 °C. This suggests that at physiological temperature of 37 °C, EGCGAgNPs exhibit static quenching. However, as temperature increases and diffusion occurs at a faster rate, there is evidence of dynamic quenching, with a decrease of \(K_{sv}\) with increase in temperature [1, 3, 13]. This indicates that while complex formation is seen at physiological conditions, the interaction of HSA with EGCGAgNPs is less stable. The CtNPs also showed an increase in \(K_{sv}\) with increasing temperature indicting static quenching, as similarly reported by others [3]. Since the AgNPs at higher concentrations
deviated from linearity range, only results that fall within the linearity were used for $K_{sv}$ and further calculations.

Using the Stern-Volmer equation it was possible to calculate the biomolecular quenching rate constant ($k_q$):

$$\frac{F_0}{F} = 1 + K_{sv} [Q] = 1 + k_q \tau_0 [Q]$$

Where $K_{sv}$ is the Stern-Volmer quenching constant, $k_q$ is the biomolecular quenching rate constant and $\tau_0$ is the average lifetime of the molecule without quencher. $F_0$ and $F$ denote the fluorescence intensities in the absence and presence of the quencher (AgNPs), respectively, and $[Q]$ is the concentration of the AgNPs [3].

The fluorescence lifetime of the biomolecule is $10^{-8}$ s ($\tau_0$) and $K_q$ was calculated using the formula $K_{sv}/\tau_0$ [3]. If dynamic quenching is present, the maximum scatter quenching collision constant of various quenchers with the biopolymer is $2.0 \times 10^{10}$ M$^{-1}$s$^{-1}$ [3, 22]. Since the $K_q$ values we obtained are in the order of $10^{13}$ M$^{-1}$s$^{-1}$ (Tables 3.2 – 3.4), which is above $10^{10}$ M$^{-1}$s$^{-1}$, this implies that the quenching resulted from static rather than dynamic quenching [13]. This, taken in conjunction with the above $K_{sv}$ values, confirms that CtNPs and CurAgNPs show static quenching, while EGCGAgNPs exhibit a combination of static and dynamic quenching [3].

![Figure 3.4: Stern-Volmer plots for (A) CtNPs, (B) CurAgNPs and (C) EGCGAgNPs.](image)

3.3.6. Binding constants and thermodynamic parameters

The double logarithmic regression curves of log($(F_0-F)/F$) versus log($Q$) were plotted for the AgNPs, the intercept of which gives $K$, the binding constant (Figure 3.5). The binding constants ($K$) for the
phytochemical coated AgNPs are shown in Tables 3.2 – 3.4. The K value for CtNPs shows an increase with temperature, indicating the binding capacity increases as temperature rises. The value of K decreases with increasing temperature for both phytochemical coated AgNPs and this indicates a reduction in binding capacity with rise in temperature. This in turn signifies the instability of the AgNPs-HSA complex [19], in both the cases of CurAgNPs and EGCGAgNPs.

Figure 3.5 Double logarithmic regression plots of log[(F₀-F)/F] versus log[Q] for binding of (A) CtNPs, (B) CurAgNPs and (C) EGCGAgNPs to HSA from which the binding constants (K) were calculated.

The thermodynamic forces responsible for the binding of these AgNPs to HSA were determined using following equation:

\[ \ln K = \frac{-\Delta H^\circ}{RT} + \frac{\Delta S^\circ}{R} \]

Where K is the binding constant at the corresponding temperature (T) in Kelvin, R is the gas constant, \( \Delta H^\circ \) and \( \Delta S^\circ \) correspond to the changes in enthalpy and entropy, respectively.[3] These can be determined from the slopes and intercepts of linear van’t Hoff plots (Figure 3.5).

The Gibbs free energy (\( \Delta G^\circ \)) was estimated from the equation:

\[ \Delta G^\circ = \Delta H^\circ - T\Delta S^\circ \]

As listed in Tables 3.3 and 3.4, both CurAgNPs and EGCGAgNPs exhibit negative \( \Delta H^\circ \) and negative \( \Delta S^\circ \) values.[3] This is indicative of van der Waals forces and hydrogen bonds [3, 22]. Free curcumin and EGCG also show the involvement of van der Waals forces and hydrogen bonds in the interaction with HSA [14, 36], which further indicates that surface coatings do play an important role in protein interactions. The negative \( \Delta H^\circ \) also suggests that the binding of the AgNPs to HSA is an exothermic
reaction.[19] The positive $\Delta G^0$ suggests a thermodynamically unfavourable process, which once again indicates the instability of the interactions between CurAgNPs and EGCGAgNPs and HSA. The CtNPs (Table 3.2), on the other hand, show a negative $\Delta H^0$ and positive $\Delta S^0$, which indicate electrostatic forces. The negative $\Delta G^0$ indicates a spontaneous, thermodynamically favourable interaction between the CtNPs and the HSA [3]. Thus, we can infer that the phytochemical coated AgNPs, while they do bind to HSA, the binding is weaker than the interaction between CtNPs and HSA.

**Table 3.2: Stern-Volmer constants and thermodynamic parameters for CtNPs.**

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<thead>
<tr>
<th>Temperature (K)</th>
<th>$K_{sv}$ $(10^4 \text{ M}^{-1})$</th>
<th>$K_q$ $(10^{13} \text{ M}^{-1} \text{s}^{-1})$</th>
<th>$K$ $(10^5 \text{ M}^{-1})$</th>
<th>$\Delta G^0$ kJmol$^{-1}$</th>
<th>$\Delta H^0$ kJmol$^{-1}$</th>
<th>$\Delta S^0$ Jmol$^{-1} \text{ K}^{-1}$</th>
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**Table 3.3: Stern-Volmer constants and thermodynamic parameters for CurAgNPs.**

<table>
<thead>
<tr>
<th>Temperature (K)</th>
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<th>$K_q$ $(10^{13} \text{ M}^{-1} \text{s}^{-1})$</th>
<th>$K$ $(10^5 \text{ M}^{-1})$</th>
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<td>$\Delta H^\circ$ kJmol$^{-1}$</td>
<td>$\Delta S^\circ$ Jmol$^{-1}$K$^{-1}$</td>
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### 3.3.7. Effect of the AgNPs on the secondary structure of HSA

Circular dichroism (CD) spectroscopy provides a convenient method for assessing the changes to secondary structure, conformation and stability of proteins in solution on interaction with NPs. Typically, the α-helix of proteins exhibit two negative bands at 208 nm and 222 nm, contributed by the π- π* and n-π* transition of the peptide bonds of the α-helix [1, 19]. The CD spectra of HSA in the presence and absence of the AgNPs are shown in Figure 3.6.

![Figure 3.6: CD spectra in the absence and presence of the AgNPs.](image-url)
The α-helix was calculated from the mean residue ellipticity (MRE) values at 208 nm using the following equation:

\[
\text{MRE} = \frac{\text{Observed CD (mdeg)}}{C_p n l \times 10}
\]

Where \(C_p\) is the molar concentration of the protein, \(n\) is the number of amino acid residues in HSA (585) and \(l\) is the path length (0.1 cm) [19]. The percentage of α-helix content was calculated using the equation:

\[
\alpha\text{-helix} (\%) = \frac{-\text{MRE}_{208} - 4000}{33000 - 4000} \times 10
\]

Where \(\text{MRE}_{208}\) is the observed MRE at 208 nm, 4000 is the MRE of the β-form and random coil conformation cross at 208 nm and 33000 is the MRE value of a pure α-helix at 208 nm [19].

The α helix content showed a reduction from 44.4% (free HSA) to 38.6% in the presence of CtNPs, 41.8% with CurAgNPs and 42.4% with EGCGAgNPs. This indicates that while all three AgNPs show a reduction in α helices, the reduction with CtNPs was the greatest. With the phytochemical coated AgNPs, CurAgNPs showed greater reduction than EGCGAgNPs, but both were less than the CtNPs. Since the highest concentration of AgNPs was used to obtain the CD spectra and the fluorescence data indicated a concentration dependent interaction with HSA, it is possible that at lower concentrations this difference in reduction in α helices will be even less pronounced. This evidence further suggests phytochemical coated AgNPs showed fewer changes to the secondary structure of the protein than citrate-capped NPs.

3.3.8. Effect of pH on the AgNP-HSA interaction

Since NPs may encounter a range of pH in in vitro and in vivo scenarios, which in turn could affect the interaction of the AgNPs with HSA, we studied the effect of pH on nanoparticle – protein interactions. The fluorescence spectra of HSA in the presence of EGCGAgNPs and CurAgNPs were recorded ranging from pH 3 to 10. The results (Figure 3.7) revealed that CurAgNPs interact with HSA only at neutral pH, however, EGCG AgNPs displayed interaction with nanoparticles at both neutral and alkaline pH.
Figure 3.7: Fluorescence spectra displaying pH dependent quenching of HSA by (A) CurNPs and (B) EGCGAgNPs. CurAgNPs are effective only at pH 7, while EGCGAgNPs are effective at alkaline and neutral pH, with an increase in intensity of the peak at acidic pH.

A possible explanation for this can be derived from the observation of Ishii, T., et al. 2011 who showed that the binding affinity of free EGCG to HSA was enhanced under alkaline conditions [37]. Further, at acidic pH there is significant increase in fluorescence intensity with EGCGAgNPs. It should be noted that the stability of free EGCG increases with decrease in pH [38, 39] and EGCG exhibits fluorescence when excited at 275 nm with emission between 350 and 400 nm [40] at acidic pH. Since Trp-214 is excited at 295 nm with emission between 330-345 nm, there is a possibility of interference from free EGCG at acidic pH, since the EGCGAgNPs aggregate at acidic pH. This could possibly have led to the release of the EGCG from the AgNPs, causing the fluorescence spectra of EGCG at acidic pH. These results suggest that the pH of the medium also influences the binding capacity of the AgNPs to proteins.

3.4. Conclusions

Studies that consider the interaction of serum proteins with AgNPs use sodium borohydride or citrate capped AgNPs [1, 3]. The ones that used plant extracts for NP synthesis did not use individual phytochemicals, but whole plant extracts [13, 19]. Since isolated phytochemical also show interaction with serum [24, 26, 41], it is not possible to determine, from previous studies, the role that the surface coatings play in protein interactions. Hence, this Chapter investigated the interactions of CurAgNPs and EGCGAgNPs with HSA, using CtNPs as a control.
The results of this Chapter revealed that all three AgNPs were capable of interacting with HSA in a concentration dependent manner, with CurAgNPs having a higher affinity for HSA than EGCGAgNPs and CtNPs having the least. CurAgNPs and CtNPs interacted through static quenching, while EGCGAgNPs showed a combination of static and dynamic quenching mechanisms with HSA. Van der Walls forces and hydrogen bonds played a key role in the reaction of both phytochemical coated AgNPs, while CtNPs involved electrostatic forces. Overall, the absorbance spectra and fluorescence spectra indicated that CurAgNPs showed the greatest interaction with HSA, while CtNPs showed the least. However, temperature dependent studies and thermodynamic parameters indicated that while the phytochemical coated AgNPs showed greater interaction, the complexes formed were not stable, when compared to the CtNPs. The CD spectra also indicated that the phytochemical coated AgNPs caused fewer changes in the secondary structure of HSA, than the CtNPs. pH of the medium is also an important condition for interaction of these AgNPs with HSA.

Recent studies have reported that free EGCG exhibits dynamic quenching through van der Waals interactions and hydrogen bonding with HSA [42], which is similar to the results obtained by the EGCGAgNPs. Free curcumin also showed interaction with HSA through similar interactions [35], which was also seen with the CurAgNPs. The CtNPs, on the other hand, interact through electrostatic forces [3]. This would suggest that the phytochemical coating plays a major role in determining the interaction with serum albumins. To further explain the role the phytochemical coating played in the HSA – AgNP interaction, the FTIR data from Chapter 2, Section 2.3.6 had to be taken into consideration. This data provided information on the changes the phytochemicals underwent during the process of synthesising and coating the AgNPs. It indicated the presence of large amount of hydrogen bonds in the case of both phytochemical coated AgNPs, which could account for the high affinity these NPs exhibited towards HSA, as evident in the UV-Vis absorbance and fluorescence spectra, when compared to the CtNPs. If the hydrodynamic and cores sizes of the AgNPs were to be considered (Chapter 2, Sections 2.3.2 and 2.3.5, respectively), the CurAgNPs have a hydrodynamic layer of 27.6 nm in diameter surrounding it, while the layer around the EGCGAgNPs was only 23.0 nm in diameter. Since the thermodynamic parameters indicated that the interaction with HSA occurs through hydrogen bond, this could indicate that the CurAgNPs were more prone to hydrogen bonding at the surface than the EGCGAgNPs. This could account for the greater interaction of CurAgNPs with HSA than the EGCGAgNPs. The involvement of
hydrogen bonds and van der Waals forces could also account for the instability of the HSA – AgNP complex and the lesser effect on the secondary structure of the HSA molecule, observed from the temperature studies, thermodynamic parameters and the CD spectra. This information explains the effect that altering the phytochemical structure during NP synthesis might have on the interaction with serum proteins.

These results indicate that by changing the surface coating, pH and temperature, it is possible to increase or decrease the interaction of AgNPs with serum proteins. It also suggests that phytochemical coated AgNPs might be useful for medical applications since the serum proteins appear to coat the surface of the NPs, thereby stabilising them, however the interactions do not appear to cause permanent damage to the structure of the proteins.

3.5. References


8. Ang, JC, Henderson, MJ, Campbell, RA, Lin, JM, Yaron, PN, Nelson, A, Faunce, T, and White, JW, Human serum albumin binding to silica nanoparticles--effect of


Chapter 4: Assessing the effects of phytochemical-stabilised silver nanoparticles on successive generations of mammalian cells

4.1. Introduction

Silver nanoparticles (AgNPs) are very versatile, mainly due to their physiochemical and antimicrobial properties. With the aim to extend the usage of these nanoparticles (NPs) to other areas of medicine, researchers have found that AgNPs can be toxic to some cancer cell lines, such as human oral squamous cell carcinoma (HSC-3) [1], human cervical adenocarcinoma (HeLa) [2], human lung carcinoma (A549) [3, 4], human kidney carcinoma (A498) [4] and human liver carcinoma (HepG2) [4], to name a few. However, these studies consider only acute or short term toxicity, for a maximum of 72 hours with a lack of evidence on long term or chronic toxicity of these NPs. The effect of AgNPs on normal mammalian cells is also limited to studies on skin penetration and effects on keratinocytes and lung cells [2, 5, 6], since these are the most likely routes of AgNPs interacting with normal cells. However, even these studies only consider acute or short term toxicity, for a maximum of 72 hours [2, 5, 6]. Therefore, the long term effects of AgNPs on mammalian cells are not fully understood.

The use of biological substances such as phytochemicals, amino acids and proteins for synthesis and stabilisation of NPs are also gaining popularity [7-12]. Since these substances already exist within living organisms, they are considered non-hazardous to the environment and living organisms. Nanoparticles synthesised using these biological moieties have shown improved solubility, stability and bioavailability [9, 13]. The syntheses of these phytochemical coated NPs have been demonstrated using the phytochemicals available in cinnamon, tea, soy and cumin, to name a few, and these NPs have shown toxicity to numerous cancer cell lines [9, 11, 12, 14]. However, there is a lack of information on which component is responsible for the toxicity observed – the phytochemical coating or the NP core, or if the phytochemicals work synergistically with the NP core to produce cellular responses.

This Chapter aims to investigate these aspects, by using AgNPs synthesised using tyrosine, curcumin and epigallocatechin-3-gallate (EGCG), which are mono-, bi- and polyphenolic compounds respectively. These AgNPs were of approximately uniform size, shape and surface charge (Chapter 2, Section 2.3) and these NPs were utilised to study the chronic effects of AgNPs on human prostate cancer (PC-3) cells. To assess
the effects of these AgNPs on mammalian cells, acute and chronic toxicity, reactive oxygen species (ROS) generation, mitochondrial membrane potential (MMP) and cell cycle analysis was studied. ROS and MMP are good indicators of oxidative stress in the cells and may serve as good tools to provide information on any antioxidant effects from the surface coatings [7, 15]. This is particularly useful, as the antioxidant effects of native phenols and polyphenols are well established [16-19]. In cancer cells, the increase in cell proliferation is due to mutations resulting in alterations to the genetic control of cell division. These mutation can disrupt the normal progression of cell cycle [20] and targeting cell cycle arrest has proven to be an effective method of killing cancer cells. Hence, studying the effect that the AgNPs had on cell cycle provided information on the potential anticancer effects these NPs have on the prostate cancer cells.

Acute and chronic toxicities were assessed by trypan blue cell exclusion assay, oxidative stress by fluorescent staining using carboxy H$_2$DFFDA for ROS and rhodamine 123 for MMP. Effect on cell cycle was analysed by flow cytometry, using propidium iodide (PI) to stain deoxyribonucleic acid (DNA) in the cells and by observing the gene expression profile of proliferating cell nuclear antigen (PCNA) and cyclin D1.

### 4.2. Materials and methods

#### 4.2.1. Chemicals and materials

Roswell Park Memorial Institute (RPMI) 1640 media, Penicillin-Streptomycin 10,000 U/mL, TrypLE Express, carboxy-H$_2$DFFDA, Rhodamine 123, Ultrapure DNase and RNase free water, High-Capacity cDNA Reverse Transcription Kits, TaqMan fast universal PCR master mix, FAM-MGB probe/primer set for proliferating cell nuclear antigen (PCNA) (HA00427214) and cycle D1 (HS00765553) were purchased from Life Technologies, Mulgrave, Victoria, Australia. A stock solution of 1 mM carboxy-H$_2$DFFDA was prepared in ethanol and stored under nitrogen, at -20 °C and in the dark. A stock solution of 1 mg/mL Rhodamine123 was prepared in methanol and stored at 4 °C and in the dark. Paraformaldehyde was purchased from Electron Microscopy Sciences, The Patch, Victoria, Australia. Working solution of 4% was prepared in PBS and chilled to 4 °C right before use. Dimethyl sulphoxide (DMSO), molecular grade ethanol, propidium iodide (PI), Triton-X 100, DNase free Ribonuclease A, molecular grade chloroform and molecular grade isopropanol were purchased from Sigma-Aldrich, Castle Hill, New South Wales,
Australia. Cell culture wares such as 25 cm$^2$, 75 cm$^2$ flasks and centrifuge tubes were purchased from Corning, Castle Hill, New South Wales, Australia. Tissue culture grade 6 well plates and 24 well plates were obtained from Nunc, Scoresby, Victoria, Australia. Pipette tips and eppendorf tubes were purchased from Eppendorf, North Ryde, New South Wales, Australia.

4.2.2. Cell line

Prostate cancer (PC-3) cells were acquired from American Type Cell Collection (ATCC), Manassa, Virginia, USA and maintained in RPMI media supplemented with 10% FBS, 100 units/mL penicillin and 100 µg/mL streptomycin, at 37 °C in the presence of 5% CO$_2$ and 95% humidified air.

The AgNPs used in this Chapter were synthesised and characterised as described in Chapter 2.

4.2.3. Acute toxicity assay

Evidence suggests that the coating on AgNPs such as citrate or carbon, can give false positive results with MTT assay [21-23], hence, trypan blue cell exclusion assay was chosen for assessing the cytotoxicity. This assay involves the use of trypan blue – a dye introduced by Pappemheimer in 1917 [24]. This dye has the capability to stain dead cells blue, due to the fact that dead cells do not have an intact cell membrane unlike live cells [24]. This method is used to determine cell viability by counting unstained live cells and stained dead cells on a haemocytometer. Cells were counted in the four corner squares of the haemocytometer and cell count determined using the formula:

$$\text{Cell count per mL} = \text{Average cell count from the four squares} \times 10^4 \times \text{dilution factor}$$

$$\% \text{Viability} = \frac{\text{Live cells per mL}}{\text{Total cells per mL}} \times 100$$

Where $10^4$ refers to the conversion factor to convert the volume of one haemocytometer square (10$^{-4}$ mL) to 1 mL and the dilution factor in this case is 2 (1:1 dilution with trypan blue).

In order to determine the toxicity of the phytochemicals, silver ions and the NPs, $5 \times 10^4$ cells per well was seeded into 24 well plates and allowed to grow for 24 hours. After which, cells were treated with increasing concentrations of silver ions, phytochemicals or dialysed NPs for 24 hours. Tyrosine and EGCG...
were directly dissolved in the media to obtain desired concentrations, while curcumin was dissolved in DMSO. Vehicle control using equivalent concentration of DMSO was used. Nanoparticle treatments were based on silver content of the NPs and only dialysed NPs were used to ensure that any effect observed were due to the NPs and not contributed by unreacted metal ions or phytochemicals in the solution. All treatments were performed using molar concentrations and each well received 1 mL of treatment in cell culture media containing 10% FBS and 1% penicillin and streptomycin.

After 24 hours of treatment, 800 µL of media was removed in order to include the dead cells in the cell count. Cells from the plate were removed using 200 µL TryplE express for 5 minutes at 37 °C. The TryplE express was neutralised with the media originally removed from the cells and centrifuged at 200x g for 10 minutes. Of the supernatant, 800 µL was discarded and cells were resuspended in the remaining media. From this, 20 µL of cell suspension was removed, mixed with equal volume of Trypan Blue and cells were counted using a haemocytometer as mentioned above. The cell counts were attempted to be confirmed using a Countess Automated Cell Counter (Invitrogen), however it was found that AgNPs were sometimes counted as dead cells, leading to erroneous results. Therefore, the data presented here corresponds to cell counts from haemocytometer. All treatments were performed in duplicates and cells were counted in both chambers of the haemocytometer for each replicate. Results are expressed as percentage viability (% viability) and total cell count with standard error bars.

4.2.4. Chronic toxicity assay

Further investigations using 100 µM, 250 µM and 500 µM of the NPs, based on silver content of the Ps were carried out on successive generations of PC-3 cells. These concentrations were used based on the results from the acute toxicity assay (Figure 4.3), since 100 µM, 250 µM and 500 µM of AgNPs showed good viability. Another reason for choosing these concentrations was to observe an effect on the cells within four to five cell generations, since increasing the number of passages can affect the behaviour of the cells [25]. The cells were treated for 24 hours with the respective nanoparticle concentrations and counted using a haemocytometer, as mentioned above. The cells were then reseeded into a 24 well plate at the same seeding density used for acute toxicity, 5 x 10^4 cells per well and allowed to grow for a period of 48 hours before subculturing. Of the reseeded cells, some cells received the same dose of NPs every 24 hours (T), or a single dose of NPs after 24 hours of allowing the cells to recover in treatment free media (24 hours).
allowed the cells to settle and recover for 24 hours before treatment and enable a better understanding of the long term toxicity of the AgNPs. Some cells were allowed to recover (R) after the first treatment, in order to study the recovery of the cells over consecutive passages. A schematic representation is shown in Figure 4.1. Pristine or untreated cells (UNT) that did not receive any treatment were used as controls.

This experiment was carried out for a maximum of four passages or until cell numbers declined preventing seeding density of $5 \times 10^4$ cells per well. All treatments were performed in duplicates and cells were counted in both chambers of the haemocytometer for each replicate. Three independent experiments were conducted and results are expressed as percentage viability ($\%$ viability) and total cell count with standard error bars, taking an average of all three experiments.

![Figure 4.1: Schematic representation of chronic toxicity experimental set up.](image)

4.2.5. **Role of silver ions on toxicity**

Cysteine has the ability to neutralise silver ions through its thiol groups [26], and has been used to determine the role that silver ions play in AgNP toxicity. As described by Zanette, C., *et al.* 2011, cells were treated with equal concentration of cysteine and AgNPs [27]. The experiment was set up as described above, where the effect of the AgNP treatment in the presence and absence of cysteine was observed after cells received treatment with 100 $\mu$M of AgNPs for 24 hours (results would correlate to passage 2 in chronic toxicity studies). The results are expressed as percentage viability ($\%$ viability) and total cell count with standard error bars.
4.2.6. Reactive oxygen species (ROS) generation

In order to analyse ROS production in the cells, cells were grown in 25 cm² flasks and treated with 100 µM of AgNPs. The experiment was conducted up to three passages similar to the chronic toxicity assay mentioned above, where cells were reseeded into the flask at the same seeding concentration. After each passage, cells were detached from the plate using TryplE express, counted and brought to 1x10⁵ cells/mL for ROS analysis. Cells were washed once in PBS, by centrifugation at 200x g for 5 minutes and stained with 10 µM carboxy-H₂DFFDA in PBS, for 30 minutes at 37 °C and in the dark. After three washes with PBS, fluorescence was read on a Horiba fluorimeter by exciting at 495 nm. All samples were run in triplicates and three independent experiments were performed. Results are expressed relative to the untreated cells as relative fluorescence with standard error bars, taking an average of all three experiments.

4.2.7. Mitochondria membrane potential (MMP)

In order to analyse MMP, cells were grown in 25 cm² flasks and treated with 100 µM of AgNPs. The experiment was conducted up to three passages similar to the chronic toxicity assay mentioned above, where cells were reseeded into the flask at the same seeding concentration. In order to minimise variability, after each passage, cells were detached from the plate using TryplE express, counted and brought to 1x10⁵ cells/mL for MMP analysis. Cells were washed once in PBS, by centrifugation at 200x g for 5 minutes. Cells were stained with 10 µg/mL of Rhodamine 123, for 30 minutes at room temperature and in the dark. Cells were washed thrice with PBS by centrifugation at 200x g for 5 minutes. Fluorescence was read on Horiba fluorimeter by exciting at 480 nm. All samples were run in triplicates and three independent experiments were performed. Results are expressed relative to the untreated cells as relative fluorescence with standard error bars, taking an average of all three experiments.

4.2.8. Cell cycle analysis

Propidium iodide (PI) is a dye capable of staining DNA. Since the quantity of DNA in the cell varies based on the cell cycle stages, PI staining can be used to determine the relative DNA content in the cells and hence the stage of cell cycle [28]. Cells were treated with the AgNPs for passages P1 and P2 as described above. After each passage, 1x10⁶ cells were washed in PBS and added drop wise to cold 70% ethanol, while vortexing. Samples were stored at -20 °C. When ready for use, samples were thawed and centrifuged
at 1000x g for 10 minutes and washed in PBS. Samples were stained in 400 µL staining solution for 15 minutes are 37 °C. Staining solution consisted of 0.1% (v/v) Triton-X 100 solution, 20 µg/mL PI (from 1 mg/mL stock solution stored at 4 °C) and 0.2 µg/mL DNAse free Ribonuclease A and was prepared fresh each time and was used to prevent PI from staining RNA [28]. Samples were stored on ice and protected from light. Flow cytometry analysis was performed on a BD Accuri flow cytometer and the data analysed using FlowJo software.

4.2.9. Gene expression of proliferating cell nuclear antigen (PCNA) and cyclin D1

Proliferating cell nuclear antigen (PCNA) was first discovered by Miyachi, K., et al. 1978 as an antigen in the serum of patients with systemic lupus erythematosus [29]. Two years later, another group identified a 36 kDa protein differentially expressed during cell cycle and they named it ‘cyclin’ [30]. Mathews, M. B., et al. 1984 identified that PCNA and ‘cyclin’ were one and the same protein and now the term cyclin is used to refer to a family of cell cycle regulating proteins [32]. PCNA has also been referred to as DNA polymerase δ auxiliary factor [33]. It forms a clamp around the DNA, binds to DNA polymerase and initiates DNA synthesis, hence it is imperative for DNA replication and repair [32].

The cycle of duplication and division of cells is essential for normal functioning of the body. Figure 4.2 depicts the various phases of cell cycle. It consists of mitosis and cytokinesis, and together they are referred to as the M phase. During the S phase, DNA replication occurs and the interval or gap periods before and after the S phase are referred to as the G1 and G2 phases, respectively. The internal and external cellular conditions during the gap phases should be suitable for the cell to progress through cell cycle [34]. If these conditions are not met, the cell fails to proliferate and can sometimes result in cell death. With regards to expression during the cell cycle, PCNA levels are lowest in G0-G1 phase, starts to rise during late G1 phase or early S phase, reaches a maximum during late S phase and decreases during G2-M phase, but not as low as that of the G0-G1 phase [32, 33]. The changes in the protein levels during cell cycle are also reflected in the mRNA levels as observed by Morris, G. F., et al. 1989. Hence, monitoring PCNA expression is a good indication of cell cycle progression. While PCNA controls cell cycle progression, cyclin D1 controls PCNA and thereby, is also responsible for the regulation of cell cycle.
Studies suggest that cyclin D1 sequesters PCNA in the cytoplasm, preventing premature translocation into the nucleus, hence preventing DNA replication before it is necessary [32, 35]. Cyclin D1 expression is highest during G₁ and G₂ phase of cell cycle and lowest during the S phase, since nuclear translocation of PCNA is necessary for DNA replication during this phase [35]. Another scenario when cyclin D1 expression decreases is when DNA damage occurs, since PCNA is needed in the nucleus for DNA repair [32]. The presence of cyclin D1 in the nucleus during G₁ phase is considered necessary for the phosphorylation of negative growth regulators and is therefore required for the progression of cells from the G₁ to the S phase [31, 36].

Hence, qRT-PCR was performed for PCNA and cyclin D1 expression in the cells, after passages P1 and P2 in the chronic toxicity treatment mentioned above. This was brought about by extracting ribonucleic acid (RNA) from the cells after each passage, converting the RNA to complementary DNA (cDNA), followed by qRT-PCR to observe the changes in the expression levels.
4.2.9.1. Ribonucleic acid (RNA) extraction by trizol method

Cells were treated up to three passages as described above. After each passage cells were counted on a haemocytometer by staining with trypan blue and 500 μL of trizol reagent was added to 1x10^6 cells. Samples were stored at -80 °C. RNA extraction was performed as per manufacturer’s instructions. Briefly, samples were thawed at room temperature to which 100 μL of chloroform, followed by vigorous shaking and incubation for 5 minutes at room temperature. The chloroform causes the solution to separate into three phases – the lower phase contained proteins, interphase DNA and supernatant contained RNA. Samples were centrifuged for 10 minutes at 12000x g at 4 °C. The supernatant was removed into a new tube and 250 μL of isopropanol was added. It was mixed well and allowed to incubate for 10 minutes. Samples were centrifuged for 10 minutes at 12000x g at 4 °C. The isopropanol was removed and the pellet was washed with 75% ethanol, followed by centrifugation at 12000x g for 10 minutes. The ethanol was removed and pellet allowed to dry for 10 minutes. The pellet was dissolved in RNAse free water and boiled at 65 °C for 10 minutes. RNA quantification was performed on a NanoQ. Quality was based on the A230/A250 ratio. Sample with ratio less than 1.8 were discarded.

4.2.9.2. Complementary deoxyribonucleic acid (cDNA) conversion

Conversion of RNA to cDNA was performed using the High-Capacity cDNA Reverse Transcription Kits. 1 µg of RNA extracted as described above, was dissolved in 10 μL nuclease free water for cDNA conversion. To this, 10 µL of 2X RT master mix was added, which was prepared as shown in Table 3.1. Thermal cycling was performed on a BioRad T-100 thermal cycler and the running conditions are showed in Table 3.2. cDNA samples were stored at -20 °C.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X reverse transcription (RT) Buffer</td>
<td>2.0</td>
</tr>
<tr>
<td>25X deoxynucleotide triphosphate (dNTP) Mix</td>
<td>0.8</td>
</tr>
<tr>
<td>10X RT Random Primers</td>
<td>2.0</td>
</tr>
<tr>
<td>MultiScibe™ Reverse Transcriptase</td>
<td>1.0</td>
</tr>
<tr>
<td>Nuclease-free water</td>
<td>3.2</td>
</tr>
<tr>
<td>1 µg of RNA dissolved in nuclease-free water</td>
<td>10.0</td>
</tr>
</tbody>
</table>
Table 4.2: Thermal cycle conditions optimized for High-Capacity cDNA Reverse Transcription Kits.

<table>
<thead>
<tr>
<th>Step 1</th>
<th>Step 2</th>
<th>Step 3</th>
<th>Step 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature (°C)</td>
<td>25</td>
<td>37</td>
<td>85</td>
</tr>
<tr>
<td>Time (minutes)</td>
<td>10</td>
<td>120</td>
<td>5</td>
</tr>
</tbody>
</table>

4.2.9.3. Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR) was performed using TaqMan Gene Expression Assay using FAM-MGB dye-labelled probes, on a 7500 Fast Applied Biosystems instrument. The experiment was set up as per manufacturer’s instructions. Samples were prepared as shown in Table 3.3. 10 µL of the reaction mix was added to each well of a 96 well reaction plate, sealed and centrifuged briefly before loading into the instrument. Samples were run with two holding stages at 50 °C for 2 minutes, 95 °C for 10 minutes followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. Samples were in triplicates with three independent experiments performed. Gene expression of PCNA and cyclin D1 is expressed as fold change or delta delta threshold cycle (ΔΔCT), using 18s rRNA as the endogenous control.

Table 4.3: Sample preparation for qRT-PCR per 10 µL reaction.

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2x TaqMan gene expression master mix</td>
<td>5.0</td>
</tr>
<tr>
<td>20x TaqMan gene expression assay mix</td>
<td>0.5</td>
</tr>
<tr>
<td>cDNA template (25ng)</td>
<td>2.0</td>
</tr>
<tr>
<td>Ultrapure RNase, DNase free water</td>
<td>2.5</td>
</tr>
</tbody>
</table>

4.2.10. Statistical analysis

The data was presented as mean ± standard error (SE) of three independent experiments. Significant differences were determined by ANOVA on Microsoft Excel. The level of statistical significance was *p < 0.05 and **p < 0.001.
4.3. Results and discussion

4.3.1. Acute toxicity

PC-3 cells were exposed to free phytochemicals and silver ions to assess the toxicity after 24 hours of exposure. Acute cytotoxicity with the phytochemicals alone (Figures 4.3) indicates that curcumin and EGCG showed dose dependent increase in toxicity, which is in agreement with similar toxicity studies conducted with curcumin and EGCG on prostate cancer cells [8, 37]. Curcumin showed the most effect on the cells with less than 20% viability at 250 µM (Figure 4.3 A), while tyrosine showed no effect on cell viability. Since curcumin stock solution was prepared in DMSO, vehicle control employed gave 97% viability indicating that the DMSO used to make the stock solution did not contribute to the cytotoxic effect shown by the curcumin. Since the nanoparticle treatment was based on silver concentration, cytotoxicity of free silver ions was examined. The silver ions showed complete cell death at 60 µM (Figure 4.3) which is in agreement with the findings of others [38].

![Figure 4.3](image)

Figure 4.3: Effect of the free phytochemicals and silver ions to (A) viability and (B) total cell count after 24 hours of treatment of PC3 cells. *p < 0.05 and **p < 0.001 when compared with the corresponding untreated cells.

The AgNPs, on the other hand, showed no toxicity after 24 hours (Figure 4.4) and the concentrations used were higher than 60 µM of the equivalent silver ions (up to 750 µM), thus the AgNPs were less toxic than free Ag ions. Similar acute toxicity results have shown that curcumin coated gold NPs (CurAuNPs) and EGCG coated gold NPs (EGCGAuNPs) do not exhibit toxicity to mammalian cells after 24 hours of exposure [11, 39]. Commercially available AgNPs, on the other hand, exhibit high acute toxicity to
mammalian cells [2-4] and this could indicate that polyphenol coated NPs might be better suited for future commercial products.

Figure 4.4: (A) Cell viability and (B) total cell count after 24 hours treatment with AgNPs. *p < 0.05 and **p < 0.001 when compared with the corresponding untreated cells.

While the AgNPs showed no effect on cell viability, as shown in Figure 4.4 A, EGCGAgNPs showed a slight decrease in cell number with increasing concentration and TyrAgNPs and CurAgNPs showed evidence of decrease in cell number only at the highest concentration (750 µM) (Figure 4.4 B). This effect on cell number has not been reported before, since most viability studies are conducted using MTT assay [3, 8, 9, 14, 40] which is a colorimetric assay and does not take the number of cells into consideration.

4.3.2. Method design for chronic toxicity testing

At present, MTT assay or similar colorimetric assays have a 72 hour limit for toxicity testing [27, 41]. For longer exposure periods, colonogenic assay is used, however, the cell seeding density can be upto 200x less (50 cells per well in a 6 well plate) than what is used for short term assays (1x10^4 cells per well in a 96 well plate) [42]. Seeding density is defined as the number of cells seeded per unit surface area of growth [43]. Hence, to further prove the importance of appropriate seeding densities, 5x10^4 cells were seeded per well in a 24 well plate (1.9 cm^2) and the same number of cells were seeded into a 6 well plate (9.5 cm^2) and allowed to grow for 48 hours, under the conditions described in Section 4.2.2. Phase contrast images were obtained on a Nikon Eclipse TS 100 and cell count and viability were obtained using a haemocytometer as described in Section 4.2.3
As observed in Figure 4.5 A, cells seeded into the 24 well plate show normal morphology and have reached ~95% confluency in the 48 hours. However, cells in the 6 well plate (Figure 4.5 B) show change in morphology and were less than 50% confluent. Cell counts revealed that cells seeded into the 24 well plate had increased to $6.13 \times 10^5$ cells per well, with 98% viability, while those from the 6 well plate only increase to $1.25 \times 10^5$ cells per well, with 92% viability. Since epithelial cells require cell-cell adhesion, studies indicate that low seeding density can significantly affect cell viability and hinder vital cellular functions due to insufficient cell-cell contact [44]. Hence the colonogenic assay or similar assays that use low seeding densities might not a suitable method for observing long term toxicity [45].

Figure 4.5: Phase contract images depicting the level of confluency and morphology of cells seeded at a density of $5 \times 10^4$ cells per well in (A) 24 well plate and (B) 6 well plate. Scale bars correspond to 50 μm.

To minimise experimental variation and to maintain the same cell seeding density to AgNP treatment ratio as that of the acute toxicity, the chronic toxicity experiment was designed such that the same seeding density was used as acute toxicity. This was achieved by passaging cells every 48 hours, for a maximum of four passages, with the same seeding density each time. This enabled a better understanding of the toxicity profiles of the AgNPs in a realistic scenario, where the number of cells in an organ would remain the same irrespective of the length of nanoparticle exposure. This assay was restricted to four passages, since the number of cells continuously decreased in treatment (Figure 4.6 D – F), preventing further passages at the same seeding density.
4.3.3. Chronic toxicity

4.3.3.1. Treatment of cells with the silver nanoparticles (AgNPs)

Cell viability and total count for cells that continuously received treatment are shown in Figure 4.6. All three AgNPs show a decrease in viability and cell number with increasing concentrations. TyrAgNPs showed the greatest effect when compared to the other two AgNPs, with complete cell death by passage 2 (P2) when treated with 500 μM. CurAgNPs showed the least toxicity when compared to the other two AgNPs, as seen in the higher viabilities and cell numbers at each passage and at all concentrations tested.

![Figure 4.6: Cell viability (A, B and C) and total cell count (D, E and F) of successive passages of cells that were continuously in treatment with 100 μM, 250 μM and 500 μM of AgNPs, respectively. Data is shown as the mean ± SE of three independent experiments. *p < 0.05 and **p < 0.001 when compared with the corresponding untreated cells.](image)

The results for cells that were treated after 24 hours of recovery are shown in Figure 4.7. The results indicate that these cells fare better than the cells that were continuously treated; evident by higher cell numbers and better viability than cell in continuous treatment. However, the toxicity trend is similar to that of the continuous treatment and once again, TyrAgNPs showed the most toxicity and CurAgNPs were the least toxic. The decrease in toxic effects on the cells could be attributed to the fact that the cells were allowed to recover for 24 hours before receiving treatment. It could indicate that perhaps the cell adhesion
was affected by continuous treatment with the AgNPs through a process known as *anoikis*, which is cell death induced by detachment of the cells from the extracellular matrix [36]. This is further investigated in Chapter 5.

![Figure 4.7](image)

**Figure 4.7**: Cell viability (A, B and C) and total cell count (D, E and F) of successive passages of cells that received treatment after 24 hours of recovery with 100 µM, 250 µM and 500 µM of AgNPs, respectively. Data is shown as the mean ± SE of three independent experiments. *p < 0.05 and **p < 0.001 when compared with the corresponding untreated cells.

Overall, the results suggest that, although 24 hour acute toxicity shows no effect to the cells, continued exposure to all three AgNP treatments show significant effect to cell viability and cell number, in a dose dependent manner. Similar results were observed by Nowrouzi, A., *et al.* 2010, Comfort, K. K., *et al.* 2014, in that, commercially available AgNPs showed significant chronic toxicity with concentrations that were sublethal at 24 hours of treatment. In this experiment, the cells showed a steady decline in viability and total cell number, with TyrAgNPs being the most toxic, followed by EGCGAgNPs and CurAgNPs were the least toxic. These results suggest that the coating on the AgNP could play a role in the toxicity profiles, since the silver core and nanoparticle size is similar in all three cases.
4.3.3.2. Recovery of cells after treatment with the AgNPs

To investigate if the AgNPs have any lingering effects on the cells after treatment has ceased, cells were allowed to recover for three passages (6 days) after 24 hours of treatment with the AgNPs. The results (Figure 4.8) indicate that in spite of the fact that cells showed good viability and cell number after 24 hours of treatment, the cell number is significantly decreased with all three AgNPs, even when the cells were not receiving any treatment. The cell number of the untreated cells is not affected, which indicates that the effect seen is not due to the methodology used, but an effect of the AgNPs. The number of passages it takes for the cell number to recover varies depending on the nanoparticle. TyrAgNPs showed the least recovery, since the total count of cells treated with 500 µM of TyrAgNP continued to decline with no signs of recovery (Figure 4.8 C and F). The lower concentrations of TyrAgNPs also took more number of passages to recover than the other two AgNPs. Cells treated with CurAgNPs, however, showed the most recovery, since cells recovered by passage P4, even at the highest concentration tested (500 µM) (Figure 4.8 D, E and F). Yet again, TyrAgNPs were the most toxic, followed by EGCGAgNPs and CurAgNPs were the least toxic.

Figure 4.8: Cell viability (A, B and C) and total cell count (D, E and F) of successive passages of cells that were allowed to recover after 24 hour treatment with 100 µM, 250 µM and 500 µM of AgNPs, respectively. Data is shown as the mean ± SE of three independent experiments. *p < 0.05 and **p < 0.001 when compared with the corresponding untreated cells.
Since the cells took at least 4 days (P2 and P3) to recover to the same cell number as the untreated, it signifies that all three AgNPs had a significant effect on cellular functioning even after a short exposure for 24 hours. These results on recovery of cells are important since there are no known reports that consider this aspect of toxicity of AgNPs. This information can be used to determine better Occupational Health and Safety (OH&S) guidelines for nanomaterials, since current toxicity testing only consider the effect of NPs during treatment and neglect the after effects that NPs might have on the human body.

4.3.4. Effect of silver ions on toxicity

Silver ions are highly toxic to mammalian cells [21, 48, 49]. To determine whether silver ions generated from the AgNPs during potential dissolution under tested conditions might play a role in toxicity, the effect of the AgNPs was studied in the presence of cysteine, since the thiol group in cysteine is capable of scavenging silver ions [27, 40]. The results (Figure 4.9) indicate that the total cell number increased in the presence of cysteine, irrespective of the AgNP treatment, which suggests that silver ions are generated from the AgNPs and they contribute to the toxic effects observed. Cells that were allowed to recover in the presence of cysteine (Figure 4.9 D, E and F) showed a significant increase in cell number equivalent to the untreated cells, which suggests that silver ions alone are the source of the toxic effects. This indicates that the AgNPs remain within the cell and are possibly passed on to daughter cells during cell division, where they continue to generate silver ions leading to the toxic effects seen in Figure 4.8. Hence the cells take a few days to recover to the same level as the untreated cells. Cells treated with TyrAgNPs (24 hours and T) and cysteine also showed an increase in cell number equivalent to the untreated cells (Figure 4.8 D), which indicates that the toxicity seen with TyrAgNPs stems from silver ions, which could also account for the higher toxicity observed with these AgNPs. The fact that treatment with CurAgNPs and EGCGAgNPs (Figure 4.9 E and F) in the presence of cysteine does not show a significant increase in cell number, indicates that the toxicity is not solely from the silver ions. Similar results have been obtained by Verano-Braga, T., et al. 2014, who found that silver ions are not always the sole cause of toxicity in AgNPs [50]. These results could also indicate that the stability of these two AgNPs is better than the TyrAgNPs and they do not generate as many silver ions [21]. The findings of Selvakannan, P., et al. 2013 and the FTIR data from Chapter 2, suggests that the phenolic groups of tyrosine, curcumin and EGCG are utilised in the synthesis of the AgNPs [51]. Since the curcumin and EGCG molecules have more number of phenolic
groups than tyrosine, it is possible that curcumin and EGCG provide better coverage to the surface of the AgNPs than tyrosine, thereby preventing the generation of ions. Moreover, it is known that polyphenols may act as chelators of metal ions, thereby providing an opportunity to sequester silver ions before their release into the surrounding environment [52].

**Figure 4.9:** Cell viability (A, B and C) and total cell count (D, E and F) of cells treated with AgNPs in the presence and absence of cysteine. Data is shown as the mean ± SE of three independent experiments. *p < 0.05 and **p < 0.001 when compared with the corresponding untreated cells. +p < 0.05 and ++p < 0.001 when compared with the corresponding treatment in the absence of cysteine.

### 4.3.5. Effect of the AgNPs on oxidative stress

ROS are oxygen molecules that contain unpaired electrons and are naturally generated within cells during various metabolic processes [37, 38, 53]. The role of these free radicals in mammalian cells appears to be a necessary evil, with ROS functioning as a signalling system for various cellular pathways responsible for growth regulation [53]. However, a balance in ROS is imperative, since overproduction can cause damage to DNA, proteins and lipids, leading to cell death [37]. ROS can be generated through endogenous and exogenous sources and the main endogenous source is the mitochondria. In order to maintain the ROS balance in the cell, the mitochondria produce numerous antioxidants and enzymes such as glutathione (GSH), superoxide dismutase (SOD) and glutathione peroxidase (GPx) [53]. Hence, increase in ROS production has been seen to disrupt the mitochondrial membrane potential (MMP) and is an early indicator of apoptosis [54]. Another reason for observing ROS and MMP was to assess antioxidant effect of the
AgNPs, especially since the phytochemicals on the surface of the NPs are well known for their antioxidant activity [7, 15].

Figure 4.10 shows the results of ROS generation and MMP in cells receiving continuous treatment and recovering cells for three passages. Cells treated with TyrAgNPs and EGCGAgNPs showed a decrease in ROS with an increase in MMP, irrespective of whether they received continuous treatment or were recovering. This antioxidant activity from the TyrAgNPs is likely to be due to the conversion of the phenolic group of tyrosine to a quinone [53], since quinones exhibit antioxidant activity [19, 55]. Similarly, the antioxidant activity with EGCGAgNPs appears to arise from the phenolic compounds coating the NPs [17]. This is further supported by the fact that AgNPs synthesised without phenolic compounds, such as citrate–coated, sodium borohydride coated, starch-coated, commercial AgNPs and silver ions act as prooxidants by increasing ROS levels [2-4, 50, 56-58], which leads to strong indications that the coating on the nanoparticle does play a role in the toxic effects on the cells. It also suggests that the toxicity occurs through a synergistic effect between the surface coating and the silver core of the AgNPs.

Cells treated with CurAgNPs, on the other hand, showed no effect with continuous treatment, however, there was an increase in ROS production and a decrease in MMP in cells recovering after treatment with CurAgNPs (Figure 4.10 C and D). This activity of CurAgNPs could result from the fact that pure curcumin in low concentrations has been shown to exhibit pro-oxidant activity, while at high concentrations it shows antioxidant activity [18, 59]. It is possible that the curcumin from the surface of the AgNPs could have this affect on recovering cells. If these results were correlated to the toxicity results (Figure 4.8), where cells treated with CurAgNPs recovered faster than the other two AgNPs, it could be attributed to the increase in ROS production. This is because evidence suggests that continuous oxidative stress can promote cell proliferation rather than apoptosis [60], since cancer cells require higher amounts of ROS for maintaining their cancerous properties [40]. Highly aggressive, metastatic prostate cancer cells lines, such as PC-3 cells, have been shown to require a higher amount of ROS for survival, than less aggressive, non metastatic cell lines [61]. Low concentrations of curcumin has also been shown to increase cell proliferation [62, 63] and perhaps the concentration of curcumin on the AgNPs falls in this range.
Figure 4.10: (A) ROS generation and (B) MMP in cells that continuously received treatment for three passages. (C) ROS generation and (D) MMP in cells that were allowed to recover after 24 hours of treatment with 100 μM of AgNPs.

4.3.6. Effect of the AgNPs on cell cycle

4.3.6.1. Cell cycle analysis

One of the hallmarks of cancer is the increase in cell proliferation due to alterations in this cell cycle machinery and bringing about cell cycle arrest in cancer cells is a good method to eradicate the disease [64, 65]. Hence, the effect of these AgNPs on cell cycle regulation was studied and results are displayed in Figure 4.11.
The results indicate that after 24 hours of treatment with CurAgNPs and EGCGAgNPs, there is an increase in cells in the S-phase, while cells treated with TyrAgNPs show an increase in G2/M phase (Figure 4.11 A and C). However, with continuous treatment there is an increase in G2/M phase in passage P2 with both TyrAgNPs and EGCGAgNPs, while CurAgNPs continue to show an increase in S phase (Figure 4.11 B). EGCG gold NPs similarly showed an S phase arrest after 24 hours of treatment [66] and this could possibly be attributed to the EGCG coating, since the core in both cases are different. Citrate–capped, starch-coated AgNPs and commercial AgNPs showed G2/M phase arrest [1, 57, 58, 67], while EGCG alone shows G0/G1 phase arrest [68] and curcumin shows G2/M phase arrest in PC-3 cells [69, 70]. Hence the effect of the AgNPs on cell cycle in this case is possibly a result of the AgNPs as a whole, rather than the effect of the surface coating alone. Cells that were allowed to recover also show an increase in S phase with CurAgNPs, while there is an increase in G2/M phase with EGCGAgNPs (Figure 4.11 C).

4.3.6.2. Proliferating cell nuclear antigen (PCNA) and cycle D1

During G0-G1 phase, cyclin D levels are high to keep PCNA in the cytoplasm, thereby preventing premature DNA replication during G0-G1 phase of cell cycle. It is also necessary for the progress of cell cycle from G1 to S phase. Once the cell moves into the S phase, cyclin D expression decreases, releasing PCNA to enable it to move into the nucleus to bring about DNA replication [31-33, 35, 36]. This also occurs during DNA damage, since PCNA is necessary for DNA repair [32]. Since they play a major role in the regulation of cell cycle, the gene expressions of PCNA and cyclin D1 was studied in conjunction with cell cycle analysis to determine the effect of the AgNPs.
The real time RT-PCR results for PCNA and cyclin D1 are displayed in Figure 4.12. The results indicate the CurAgNPs show a significant increase in PCNA and cyclin D1 expressions after 24 hours treatment and in cells in passage P2. The increase in PCNA corresponds to the increase in number of cells in S phase seen in the flow cytometry data (Figure 4.11) [31-33, 35, 36]. Evidence suggests that an increase in cyclin D1 causes an increase in cells transitioning from G_{1} to S phase, thereby shortening the G_{1} phase [71]. This, taken in conjunction with the increase in cells in S phase and the prooxidant effect (Figure 4.10 C and D) could indicate that the CurAgNPs are encouraging the cells to grow, rather than exhibiting toxicity. Cells treated with TyrAgNPs and EGCGAgNPs show a decrease in cyclin D1 after 24 hours and when continuously treated in passage P2, there is also a decrease in PCNA. This corresponds to the G_{2}/M phase arrest seen in Figure 4.11 A [58, 67].

![Figure 4.12: PCNA and cyclin D1 gene expression in cells at (A) passage 1 (P1), (B) continuous treatment in passage 2 (P2) and (C) recovery of cells at passage 2 (P2).](image)

### 4.4. Conclusions

In order for AgNPs to be successfully incorporated into emerging areas of medicine, it is imperative to understand the long term effects these NPs have on mammalian cells and also the extent to which the surface coating plays a role in the cellular responses elicited. The results of this Chapter have shown that while 24 hour exposure to AgNPs might not show any effect on cell viability, continuous exposure does have a significant effect on mammalian cells. This is seen in the inhibition of cell growth, disruption of ROS balance and mitochondrial membrane potential, and deregulation of cell cycle. On the other hand, while acute toxicity studies might seem harmless, the consequences of a short 24 hour treatment with AgNPs are evident in subsequent generations of cells, lasting up to at least three passages after exposure.
This information proves that the after effects of NP exposure need to be considered when determining biocompatibility and when setting regulations for safe exposure levels to nanomaterials.

This Chapter also dealt with the use of a chronic toxicity testing method that allows the testing of NPs on mammalian cells for longer than 72 hours, without compromising normal cellular functioning. This method allowed the observation of the recovery of cells, which can provide a better understanding of the toxicity profiles of nanomaterials. It can also be used with any cell line to test any substance, without the use of expensive chemicals and equipment.

With regards to toxicity, the results indicate that silver ions play an important role in toxicity. This was evident with the TyrAgNPs, where the use of cysteine indicated that silver ions released from the TyrAgNPs were the cause for the decrease in cell viability. This effect could be due to the amount of coverage that the coating provides to the NP surface. Greater amount of coverage from a larger molecule, such as curcumin and EGCG could protect against silver ion generation better than a smaller molecule like tyrosine. The release of larger amounts of silver ions from the TyrAgNPs (Figure 4.6 A and D) could also account for the higher toxicity observed with the TyrAgNPs, but not with the free tyrosine (Figure 4.3), suggesting that the silver ions are the cause of toxicity with the TyrAgNPs rather than the Tyr coating.

With the phytochemicals coated AgNPs, on the other hand, the change in structure of the phytochemicals on the AgNPs, observed in the FTIR data (Chapter 2, Figure 2.14), and possibly the lower concentrations of the phytochemicals on the surface of the AgNPs could account for the decrease in toxicity observed with the phytochemical coated AgNPs in comparison to the free phytochemicals.

The antioxidant effect observed appeared to be caused by the tyrosine coating on the AgNPs, since the phenolic group on the tyrosine molecule is converted to a quinone during the NP synthesis. This antioxidant effect, coupled with the silver ion generation appeared to work synergistically, resulting in TyrAgNPs showing the most toxicity. Similarly, with EGCGAgNPs, the antioxidant effect appears to be due to the EGCG coating, while the G2/M phase arrest in cell cycle is possibly due to the NP itself, rather than the coating.

The role of the surface coating as a major contributor to the cellular effects elicited by these NPs is further demonstrated by the CurAgNPs, where the curcumin coating appears to have a protective effect against
cytotoxic effects, possibly leading to the better recovery of the cells in comparison to the other two AgNPs. It can also be hypothesised that the increase in cells in S phase and the increase in PCNA and cyclin D1 by the CurAgNPs are an indication that the CurAgNPs appear to promote cell growth.

With regards to anticancer properties, the EGCGAgNPs are possibly the most promising, since there was minimal toxicity arising from silver ion generation, which would be safer to the normal cells in the body. The cause of toxicity is possibly due to the antioxidant effect, from the EGCG coating and a G2/M phase arrest in cell cycle. Cells treated with these AgNPs showed signs of recovery, even when treated with 500 μM, unlike with the TyrAgNPs, which showed no recovery. This lends further support to the potential use of these AgNPs as anticancer agents. Overall, the results of this Chapter suggest that altering the coating on the NPs can alter the function and application of these NPs in a biological scenario.

4.5. References


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Chapter 5: Effect of phytochemical-stabilised silver nanoparticles on cell adhesion and migration

5.1. Introduction

Cell interaction with the extracellular matrix (ECM) is fundamental for normal functioning of cells. The ECM occupies the extracellular space in tissues and consists of a network of protein molecules [1, 2]. These molecules are produced by the cells in the vicinity and are made up of polysaccharides and fibrous proteins such as collagens, fibronectin and laminin [1, 2]. While different cells can interact with the ECM differently, integrins are responsible for connecting the cell surface with the ECM in all cases [3]. This occurs through the formation of focal adhesions (FAs). More than sixty different focal adhesion proteins have been identified and vinculin is a key protein involved in FAs formation [4]. Vinculin interacts with talin, which helps bind it to the integrins on the extracellular surface, while on the intracellular side of the cell, vinculin anchors the actin cytoskeleton stress fibres in place, thus forming a FA [4-6]. The actin cytoskeleton, in turn, is responsible for signalling various cellular functions [6, 7], hence cell – ECM adhesion is imperative to cell survival. Loss of cell adhesion, on the other hand, can lead to apoptosis and is called anoikis [8].

Cell migration is another important cellular process. In an embryo, it occurs during tissue and organ formation, while in an adult form, it occurs during tissue renewal, wound healing and angiogenesis. In the case of cancer cells, migration occurs during metastasis [9, 10]. A schematic representation of cell migration is depicted in Figure 5.1. For migration to occur, whether it be a normal or a cancer cell, the edge of the cell produces a protrusion, known as a lamellipodium, which adheres to the ECM by FAs [7]. The body of the cell then contracts and the rear end of the cell detaches from the matrix, moving the cell forward. This involves the formation and disassembly of the FAs at the leading edge and rear of the cell, respectively [6, 7]. Whether it is single cell cancer migration or collective cell migration seen in endothelial cells and carcinomas, the cells need to maintain strong cell – cell and cell – ECM adhesion [10].
When considering the effects that nanoparticles (NPs) can have on cells, most reports focus mainly on cell viability and apoptosis [11-20]. Studies indicate that various NPs are capable of inhibiting cell adhesion and migration [21-26] and while this might be beneficial in the case of cancer cells, it is not a desirable effect for healthy cells in the body. With the increasing use of AgNPs in commercial products, it is imperative to know the extent to which various cellular aspects might be affected from use of these products. This Chapter aims to study the effects that silver nanoparticles (AgNPs) might have on cell adhesion and migration, using tyrosine-capped (TyrAgNPs), curcumin-capped (CurAgNPs) and epigallocatechin-3-gallate-capped silver nanoparticles (EGCGAgNPs). Since actin and vinculin play integral roles in cell adhesion and migration, their gene and protein expression levels were assessed. Cell adhesion assay, wound healing assay, real time quantitative reverse transcription polymerase chain
reaction (qRT-PCR), and confocal microscopy were used to observe and analyse the effect of the AgNPs on mammalian cells.

5.2. Materials and methods

5.2.1. Chemicals and materials

Medium 200, low serum growth supplement (LSGS), crystal violet, Alexa Fluor® 488 phalloidin, mouse anti human vinculin monoclonal antibody, goat anti mouse polyclonal antibody tagged with Alexa Fluor® 594, Hoechst 33342 and geltrex were purchased from Life Technologies, Mulgrave, Victoria, Australia. Coverslips and glass microscopy slides were purchased from Electron Microscopy Sciences, The Patch, Victoria, Australia. Magnesium chloride (MgCl₂), calcium chloride (CaCl₂), methanol, laminin, collagen and fibronectin were purchased from Sigma-Aldrich, Castle Hill, New South Wales, Australia.

5.2.2. Cell lines

Prostate cancer (PC-3) cells were maintained as described in Chapter 4, Section 4.2.2. Human umbilical vein endothelial (HUVEC) cells were acquired from American Type Cell Collection (ATCC), Manassa, Virginia, USA and maintained in M200 media supplemented with LSGS, at 37 °C in the presence of 5% CO₂ and 95% humidified air.

5.2.3. Cell adhesion assay

Cells were treated with increasing concentrations of AgNPs for 24 hours as described in Chapter 4, Section 4.2.3. Substrate coatings with 5 µg/mL solutions of laminin, collagen and fibronectin were performed in 96 well plates, for 2 hours at room temperature. The wells were washed twice with PBS and blocked with cell culture media containing 10% FBS for 30 minutes at 37 °C. Wells were washed once with PBS and 2 x 10⁵ cells were added per well in 100 µL of media. Cells were allowed to attach for 30 minutes at 37 °C. Non-adherent cells were removed and adherent cells were washed twice in PBS containing 1 mM MgCl₂ and 1 mM CaCl₂. Cells were fixed in 100% cold methanol for 10 minutes, washed twice in PBS and stained with 0.5% crystal violet in 20% ethanol for 10 minutes on a shaker. Excess crystal violet was washed by dipping the plate into a beaker of distilled water. Crystal violet from stained cells was extracted in 100% methanol for 15 minutes on a shaker and 100 µL of the methanol was removed into a fresh 96 well plate. Absorbance was read at 590 nm on a SpectraMax Paradigm Multi – Mode Microplate Reader, Molecular
Devices. Results are expressed as percentage of cell adhesion in comparison to the untreated cells with standard error (SE) bars, taking an average of the replicates.

5.2.4. Wound healing assay

To determine the effect of the AgNPs on cell motility and migration, wound healing assay was performed by growing $1 \times 10^6$ cells per well in a 6 well plate, on glass cover slips for 24 hours. Since the effect of the AgNPs on PC-3 cells was to be assessed for longer than 24 hours, cells were removed from half the cover slip. In the case of HUVEC cells, however, a wound was made in the cells using a 200 µL sterile pipette tip. Cells were washed thrice with PBS and treated with AgNPs in cell culture media with 10% FBS for 24 hours. Phase contrast images were taken to observe the migration of the cells into the wounded area using a Nikon Eclipse TS100 phase contrast microscope. The number of cells that migrate into the wound were counted using NIS Elements D 4.1 software and compared against the controls. In order to observe the recovery of the PC-3 cells, the media in the wells was replaced with treatment free complete media and observed 48 hours later. The number of migrated cells was counted as above and percentage of cell migration was calculated in relation to the untreated cells. All samples were done in triplicates and three independent experiments were performed. Results are expressed as percentage of migrated cells with standard error (SE) bars, taking an average of all three experiments.

5.2.5. Confocal staining on wounds for actin and vinculin

After the wound healing assay, fluorescent staining for actin and vinculin was performed on the cover slips. The cells were fixed in 4% paraformaldehyde solution for 10 minutes, after which, cells were washed thrice in PBS and stored in PBS at 4 °C until staining could be performed. For the staining, the PBS was removed and cells were permeabilised with 0.02% Triton X-100 in PBS for 90 seconds. Cells were washed once with PBS, followed by blocking with 2% bovine serum albumin (BSA) in PBS for 30 minutes. Cells were then incubated with 1:50 dilution of mouse anti human vinculin antibody in blocking buffer, for 1 hour. After washing thrice with PBS, cells were incubated with 1:500 dilution of goat anti mouse secondary antibody tagged with Alexa Fluor® 594 in blocking buffer, for 30 minutes, in the dark. Cells were washed 5 times, followed by incubation with 1:40 dilution of Alexa Fluor® 488 phalloidin in PBS, for 20 minutes, in the dark. Three washes in PBS were performed, followed by staining with Hoechst 33342 (2µg/mL in PBS) for 10 minutes. After the final three PBS washes, cells were mounted on to glass slides.
using antifade mounting medium and imaged on a N–STORM SuperResolution/Confocal microscope, Nikon. Images were obtained using the NIS Elements 4.0 software and fluorescence intensity analysis was performed using ImageJ software.

5.2.6. Gene expression of actin and vinculin

Cells were treated with the AgNPs as described in Chapter 4, Section 4.2.4 for three passages (P1, P2 and P3). The RNA extraction, cDNA conversion and real time quantitative reverse transcription polymerase chain reaction (qRT-PCR) were performed as described in Chapter 4, Section 4.2.9.

5.3. Results and discussion

5.3.1. Effects of the silver nanoparticles (AgNPs) on cell adhesion

Cell adhesion or the ability of a cell to stick to the ECM is imperative, since adhesion is required for vital cell signalling, regulation of cell cycle, cell migration and survival [27]. Attachment to the various components of the ECM is brought about by integrins or through specific cell surface receptors. These integrins link the ECM and the cytoskeleton of the cells through the formation of FAs, which consist of vinculin, talin and actin stress fibres [2]. Since cell adhesion is imperative to normal cellular functioning, the effect the AgNPs on adhesion was studied. The cell adhesion assay was performed using collagen, fibronectin and laminin, components that commonly constitute the ECM [1, 2, 27]. Collagens are fibrous proteins constituting a major component of skin and bones [1]. Fibronectin is known as “biological glue” since it not only has specific domains for binding to other proteins such as collagens and heparin but also for binding to surface receptors on cells [1, 2]. Laminin is a large, flexible protein and also binds to specific receptors on the surface of cells [1]. It can self-assemble into a web or form a network with collagens in basement membranes of tissues [2].

Cell adhesion with untreated cells was first observed and the results are displayed in Figure 5.2. The data indicates that there was an increase in cell adhesion with all three substrates tested, however fibronectin showed the most cell adhesion. This could be due to the fact that fibronectin causes the stimulation of survivin in PC-3 cells, which protects the cells from apoptosis by tumour necrosis factor – α (TNF – α) [28]. This could also possibly account for the lesser toxicity observed with CurAgNPs in Chapter 4, Section 4.3.3 since CurAgNPs showed increase in cell adhesion (Figure 5.3 B).
Figure 5.2: Adhesion of untreated cell on different substrates. UC – uncoated, Col – collagen, Fib – fibronectin and Lam – laminin. Data is shown as the mean ± SE.

Cells were treated for 24 hours with increasing concentrations of the AgNPs, and cell adhesion on the various substrates was tested. Since the cells showed an increase in adhesion in the presence of the substrates, percentage of cell adhesion was calculated in comparison the untreated cells on the respective substrates. The results indicate that cells treated with TyrAgNPs and EGCGAgNPs (Figure 5.3 A and C) show concentration dependent decrease in cell adhesion in uncoated well. Cells treated with the CurAgNPs (Figure 5.3 B), on the other hand, showed concentration dependent increase in cell adhesion, both in the uncoated wells as well as in the presence of the substrates. This indicates that CurAgNPs cause an increase in cell adhesion mechanisms. If these results were to be correlated to the cytotoxicity results in Chapter 4, Section 4.3.3, the decrease in cell adhesion with TyrAgNPs and EGCGAgNPs could play a role in the cell death and decrease in cell number observed with these AgNPs, since cell adhesion is imperative to cell survival [6, 7]. This could also explain the lesser degree of toxic effects observed with the CurAgNPs on the cells.

In the presence of substrates, cells treated with TyrAgNPs (Figure 5.3 A) showed a significant increase in the presence of all three substrates in comparison to the respective treatments in the uncoated well. This could further indicate that the loss of adhesion is possibly responsible for the decrease in cell viability and cell count observed in Chapter 4, Section 4.3.3. Cells treated with EGCGAgNP (Figure 5.3 C) similarly
showed a significant increase in cell adhesion in comparison to the respective treated cells in uncoated wells. While there was evidence of an increase in cell adhesion at lower concentrations (0.13 μM and 0.25 μM) with collagen and fibronectin, as concentration increase, there is a decrease in adhesion. This could suggest that the cell adhesion mechanisms have been compromised in cells treated with TyrAgNPs and EGCGAgNPs. Hence, when substrates were provided to aid in cell adhesion, an improvement is evident when compared to the untreated cells.

At higher concentrations of EGCGAgNPs, however, the concentration dependent effect on adhesion is still evident. Shukla, R., et al. 2012 have shown that EGCG – capped gold NPs (EGCG-AuNPs) gain entry into PC-3 cells through the 67 kD laminin receptor (67LR) [29]. Ren, X., et al. 2014 have also shown that EGCG alone is capable of causing the internalisation of the 67LR, causing a decrease in cell adhesion and migration [30]. If EGCGAgNPs were similarly engaging some of the 67LR in the PC-3 cells, it would account for the decrease in cell adhesion both in the uncoated wells and when laminin substrate was provided since all the receptors are not available to attach to the substrate. This could also account for the decrease in cell migration observed in Figures 5.3 and 5.4.

![Figure 5.3: Adhesion of cells on collagen, fibronectin and laminin substrates, after 24 hour treatment with increasing concentrations of (A) TyrAgNPs, (B) CurAgNPs and (C) EGCGAgNPs. Data is shown as the mean ± SE. *p < 0.05 and **p < 0.001 when compared with the corresponding cell treatment in uncoated wells.](image)

5.3.2. Effects of the AgNPs on cell migration

When a cell is about to migrate, it becomes polarised, which means certain structures form in the front or the leading edge of the cell, while others form at the rear of the cell. As depicted in Figure 5.1, the cell membrane at the leading edge forms a protrusion called a lamellipodium, which attaches to the substrate.
with the help of the FAs [4, 6, 31]. This lamellipodium fills with cytosol, followed by contraction of the cell, causing the FAs at the rear of the cell to detach from the substrate and move forward. This can sometimes result in FAs being left behind as the cell moves forward [6, 31]. Cell migration occurs during tissue repair and angiogenesis [9, 10], hence plays a vital role in the body.

In order to investigate the effect of the AgNPs on cell migration, wound healing assay was performed. Cells were exposed to 100 µM of AgNPs for 24 and 48 hours. The phase contract images are displayed in Figure 5.3. From these images, migrated cells were counted and percentage of migration was calculated in comparison to the untreated cells. The results are displayed in Figure 5.4.

![Phase contrast images of wound healing assay of PC-3 cells after 24 and 48 hours of treatment with the AgNPs. Cells were also allowed to recover for 48 hours after 24 hours treatment with the AgNPs. Scale bars corresponds to 200 μm.](image)

**Figure 5.4:** Phase contrast images of wound healing assay of PC-3 cells after 24 and 48 hours of treatment with the AgNPs. Cells were also allowed to recover for 48 hours after 24 hours treatment with the AgNPs. Scale bars corresponds to 200 μm.
The results indicate that all three AgNPs showed a decrease in cell migration after 24 hours of exposure to the AgNPs (Figure 5.5 A). This also indicates that while acute toxicity observed in Chapter 4, Section 4.3.1 showed no effect on cell viability, the cell motility and migration is significantly affected when compared to the untreated cells. Further, when cells were allowed to recover for 48 hours without treatment (Figure 5.5 B), only cells treated with CurAgNPs showed significant increase in migration in comparison to the other two AgNPs. If these results were correlated with the ROS generation data obtained in Chapter 4, Section 4.3.5, Figure 4.9, it is possible that the increase ROS levels observed with the CurAgNPs could account for the increase in migration observed. These results are in agreement with previous reports which indicate that increased ROS results in an increase in Nox1 protein levels, which is responsible for increased in migration of cells [32, 33].

Cells in continuous treatment with all three AgNPs showed a significant decrease in migration when compared to the untreated cells. Interestingly, cells that were allowed to recover after treatment with TyrAgNPs and EGCGAgNPs showed approximately the same level of migration as those cells continuously in treatment. This could be attributed to the decrease in cell adhesion in the presence of these two AgNPs (Figure 5.3 A and C), since cell adhesion is imperative to migration [10].

Figure 5.5: Cell migration of PC-3 cells in the presence of 100 μM of AgNPs after (A) 24 hours of exposure to the AgNPs and (B) 48 hours of exposure. Data is shown as the mean ± SE. *p < 0.05 and **p < 0.001 when compared with the untreated cells. + p < 0.05 and ++ p < 0.001 when compared to cells treated with TyrAgNPs. # p < 0.05 and ## p < 0.001 when compared to cells treated with EGCGAgNPs.
5.3.3. Effects of the AgNPs on actin and vinculin

A schematic diagram of the proteins involved in the formation of FAs is shown in Figure 5.6. Actin exists as globular monomers known as G–actin or as filamentous, linear chains of G–actin, known as F–actin. These actin filaments are commonly arranged as bundle or networks, which together form the framework supporting the plasma membrane of the cell. The networks are also responsible for the gel–like appearance of the cytosol [34]. Actin stress fibres are also found emanating from the FAs which are responsible for cell adhesion and migration [6, 34].

![Schematic diagram of proteins involved in focal adhesion formation](image)

**Figure 5.6: Schematic representation of the proteins involved in focal adhesion formation in cell-matrix adhesion and migration.**

The polymerisation of G–actin to F–actin is responsible for the dynamic shape of the cell and toxins such as phalloidin from the “Angel–of–death” mushroom (*Amanita phalloides*) can prevent the depolymerisation of F–actin. Hence fluorescently – labelled phalloidin is used to stain F–actin for microscopy [34]. In this case, phalloidin tagged with Alexa Fluor 488 was used to observe actin in migrated cells after 24 hours of treatment with the AgNPs.

Vinculin is a 116 kD, F–actin binding protein, which is necessary for the formation of FAs for cell adhesion and migration [35, 36]. It consists of an N–terminal head, a flexible, proline rich neck and a C–terminal tail. In the inactive form, it exists in the cytoplasm with the head and tail interacting with each other [35, 37]. When activated, vinculin unfolds, talin binds to its head while F–actin binds to the tail, thereby forming a FA [35-37]. The FA is destabilised by retrograde movement of F–actin, causing it to disengage from vinculin, which then reverts to the inactive form [4, 37]. Since actin and vinculin work
together in FA formation, vinculin was also observed in migrating cells after 24 hours of treatment with the AgNPs.

Fluorescent images from confocal microscopy are displayed in Figure 5.7. The images indicate that the cells treated with CurAgNPs have numerous FAs and lamellipodia, indicating the cells are actively migrating (Figure 5.7 C). On the other hand, cells treated with TyrAgNPs (Figure 5.7 B) and EGCGAgNPs (Figure 5.7 D) show evidence of change in morphology when compared to the untreated cells (Figure 5.7 A). Fluorescence intensity of actin was analysed and the results are displayed in Figure 5.8. The relative fluorescence intensity shows an increase in the presence of CurAgNPs in comparison to the untreated cells, while there is a decrease in intensity with TyrAgNPs and EGCGAgNPs. This indicates an increase in F-actin protein levels in cells treated with CurAgNPs while there is a decrease with the other two AgNPs.

Figure 5.7: Confocal microscopy images depicting actin and vinculin in (A) untreated cells and cells treated with (B) TyrAgNPs, (C) CurAgNPs and (D) EGCGAgNPs after 24 hours of treatment. (E) Untreated cells and (F) recovery of cells after treatment with CurAgNPs. Scale bars = 20 µm. Red arrows indicate vinculin in focal adhesions and white arrows indicate the lamellipodia.
Figure 5.7 E and F are images of untreated cells and cells that were allowed to recover after treatment with CurAgNPs, respectively. The recovering cells also showed an increase in the number of FAs and lamellipodia after treatment with CurAgNPs. This could account for the better migration of the cell treated with CurAgNPs after 48 hours in comparison to the other two AgNPs (Figures 5.4 and 5.5 B). This data is further supported by the gene expression levels of actin and vinculin (Figure 5.9). Cells that were in continuous treatment with the AgNPs and cells recovering after treatment with TyrAgNPs and EGCGAgNPs showed decrease in cell adhesion and staining was not possible due to washing away of cells during the numerous washing steps involved in the staining procedure.

![Relative fluorescence intensity obtained using Image J from the confocal images after 24 hours of treatment with the AgNPs.](image)

Gene expressions of actin and vinculin are shown in Figure 5.9. The results indicate that cells treated with CurAgNPs show significantly higher levels of both actin and vinculin in comparison to the untreated cells (Figure 5.9). The expressions increased with successive passages of continuous treatment with the CurAgNPs (Figure 5.9 C and D). Cell treated with TyrAgNPs and EGCGAgNPs, however, showed a reduction in gene expression of both genes after 24 hours of treatment, which could be attributed to the decrease in cell adhesion (Figure 5.3 A and C) and cell migration (Figures 5.4 and 5.5). The expression levels in cells treated with both these AgNPs continue to decrease with continuous treatment. However,
there is evidence of increase in expression levels in cells that were allowed to recover after 24 hours of exposure to these AgNPs. The increase in expression levels could be attributed to the increase in cell viability in recovering cells by P3, observed in Chapter 4, Section 4.3.3.2.

Figure 5.9: Gene expression of (A) actin and (B) vinculin in cells allowed to recover after 24 hours of treatment and gene expression of (C) actin and (D) vinculin in cells continuously in treatment with the AgNPs. Error bars correspond to standard error of mean.

Holy, J. 2004 has shown that curcumin is capable of increasing the appearance of F–actin in PC-3 cells [38] and studies have shown that low doses of curcumin are capable of increasing cell migration [39, 40]. On the other hand, EGCG has been shown to suppress the migration of cells [41, 42]. Evidence also suggests that vinculin is capable of controlling cell proliferation by controlling the paxillin – focal adhesion kinase (FAK) interaction, which in turn upregulates the extracellular signal-regulated kinase (ERK) activity, thereby suppressing apoptosis [36]. Actin filaments have also been shown to regulate ERK activity [43, 44].
Hence, the increase in actin and vinculin in cells treated with CurAgNPs could also contribute to the quicker recovery of the cells observed in Chapter 4, Section 4.3.3.2. This could also account for the increase in cell death observed with continuous treatment with TyrAgNPs and EGCGAgNPs in Chapter 4, Section 4.3.3.2. Since CurAgNPs showed an increase in actin and vinculin with the potential to increase cell migration, the effect of these AgNPs was tested on migration on endothelial cells (Figures 5.9 and 5.10).

5.3.4. **Effect of curcumin-capped silver nanoparticles (CurAgNPs) on endothelial cell migration**

Endothelial cells constitute the lining of blood vessels throughout the body and play a vital role in various bodily functions, such as fluid filtration in the kidneys, maintenance of blood pressure, functions as a selective barrier for the movement of white blood cells in and out of the blood stream and regulation of the flow of nutrients and metabolites [1, 45]. With regards to wound healing, endothelial cells are important for the occurrence of hemostasis and the regulation of coagulation and anticoagulation proteins in the blood vessels [1, 45, 46].

When vascular injury or an open wound occurs in the body, the process of hemostasis is initiated. Hemostasis is the formation of a blood clot and subsequent repair of the injured tissue [47]. The first step is constriction of the blood vessels, limiting the flow of blood to the site of injury. This is followed by activation of thrombin and aggregation of platelets at the injury, forming a platelet plug. The aggregation of platelets is stimulated by platelet-activating factor (PAF) and the von-Willebrand factor (vWF), both of which are synthesised by endothelial cells [46, 47]. Thrombin then brings about the conversion of fibrinogen to fibrin, which helps to increase the stability of the platelet plug, forming a clot [48]. The activity of thrombin in circulating blood is kept in check by antithrombin and by the binding of heparin to antithrombin, which has a higher affinity for thrombin than antithrombin alone [46]. Endothelial cells are responsible for regulating the activity of thrombin through the protein C/protein S pathway. These cells also bear heparin-like glycosaminoglycans on their surface, which helps inactivate thrombin [46, 47]. Once the site of injury has been repaired, endothelial cells participate in the degradation of the clot by enabling the formation of plasmin [46, 47]. Thus, the endothelial cells play a pivotal role in wound healing. If the endothelial layer is damaged during the injury, cells surrounding the wound migrate to the wound area to
bring about hemostasis [49], hence the migration of endothelial cells are necessary for efficient wound healing.

Since the CurAgNPs showed increase in cell migration of epithelial cells, the effect was studied on endothelial cell migration. The concentration of CurAgNPs used was 25 μM, which is lower than the lowest concentration (50 μM) used for acute toxicity studies on the PC-3 cells. At the lowest concentration, there was no effect to cell viability after 24 hours of exposure (Chapter 4, Section 4.3.1). To establish if the effect on migration was due to low concentrations of curcumin that might be on the surface of the AgNPs, the effect of 0.01 μM and 0.1 μM of curcumin was also assessed.

Wound healing assay and gene expression of actin and vinculin were performed. Phase contrast images of the wound healing assay are displayed in Figure 5.10. From these images, migrated cells were counted and percentage of migration was calculated in comparison to the untreated cells. The results are displayed in Figure 5.11 A. Gene expression results are displayed in Figure 5.11 B.

![Figure 5.10: Phase contrast images of wound healing assay of HUVEC cells after 24 hours of treatment with curcumin and CurAgNPs. Scale bars corresponds to 200 μm.](image)

The results indicate that 0.1 μM of curcumin showed 20% greater migration than the untreated cells, which indicates that low concentrations of curcumin can increase migration of cells. Similar results were obtained by recent reports, using the same concentration of curcumin on Schwann cells [40]. While the CurAgNPs did not show as much migration as free curcumin, the gene expression of actin and vinculin show an increase in both, in the presence of CurAgNPs, while free curcumin showed a decrease in vinculin. Since a decrease in vinculin can be detrimental to cell proliferation [36] and since curcumin has been shown to
deteriorate under biological conditions [50], the CurAgNPs could provide a better solution in wound healing applications and will have to be explored further at different concentrations and for longer exposure periods.

Figure 5.11: (A) Cell migration and (B) gene expression of actin and vinculin in HUVEC cells when treated with 25 μM of CurAgNPs, 25 μM, 0.01 μM and 0.1 μM of curcumin. Data is shown as the mean ± SE. *p < 0.05 and **p < 0.001 when compared with the untreated cells.

5.4. Conclusions

With the increasing use of AgNPs in commercial and medical products, it is important to understand what other effects the NPs can have on cells, besides affecting cell viability. The results of this Chapter have shown that AgNPs are capable of affecting cell adhesion and migration and whether this effect is beneficial or detrimental to the cells can be determined by the surface coating on the AgNPs. These results also further support the observations of Chapter 4, which suggested that the AgNPs can have an effect on the cells even after a short 24 hour treatment, with the effects evident in subsequent generations of cells. This also stresses the importance of testing long term effects of AgNPs on various aspects of cellular functioning.

The increase in cell adhesion and migration observed with the CurAgNPs and increase in actin and vinculin gene expressions could indicate that these AgNPs might prove to be useful in wound healing applications. The increase in cell adhesion and migration appears to be due to the increase in actin and vinculin levels in the cells. This could also account for the lesser effect on cell viability observed with
these AgNPs, since adhesion and migration play a part in the prevention of apoptosis. Low doses of curcumin have shown wound healing capabilities, though the inhibition of inflammatory markers such as TNF-α [51], NF-κB [51] and IL-1β [52]. It has also exhibited the ability to increase the migration of cells [39, 40], which is an essential process in wound healing. Hence, the increase in cell adhesion and migration observed with the CurAgNPs and increase in actin and vinculin gene expressions could be attributed to the curcumin coating the surface of the AgNPs. However, further in depth studies on wound healing applications of the CurAgNPs are warranted.

Once again, the EGCGAgNPs appear to be a promising anticancer agent since it caused the decrease of cell adhesion and migration of cancer cells. This could prove useful especially with metastatic cancers. Similar to the CurAgNPs, the EGCG coating appears to be responsible for the effects on the cells. Overall, the results of this Chapter suggest that the surface coating on the AgNPs plays a major role in the cellular effects elicited by the AgNPs.

5.5. References


Chapter 6: Conclusions and future directions

6.1. Summary

Silver nanoparticles (AgNPs) are currently used in numerous commercial products [1-3]. However, there is a lack of information of the full extent of the toxicity that these AgNPs may exert on mammalian cells, specifically long term toxicity [4]. The influence of the surface coating on toxicity and interaction with proteins in an in vivo scenario has not been well understood [5, 6], such as, the role that the surface coating plays in the interaction with proteins in the blood stream. If humans are to come into contact with these AgNPs on a regular basis in everyday life, the full effect that these AgNPs may have on mammalian cells needs to understood in its entirety. To provide detailed insights on these aspects, AgNPs were synthesised with mono, di and polyphenolic compounds, namely tyrosine, curcumin and epigallocatechin-3-gallate (EGCG). These AgNPs were used to study the role of the surface coating in interaction with proteins in the body, effect on long term toxicity and on normal cellular functioning of mammalian cells.

To better understand the behaviour of these AgNPs in a biological scenario, the interaction of the AgNPs with human serum was examined. The studies were conducted with human serum albumin (HSA), and the results revealed that interaction with serum proteins occurs in a concentration dependent manner. Apart from concentration, the surface coating on the AgNPs, pH and temperature of the medium may also influence the interaction. The surface coating may determine the type of interaction that occurs with the proteins with phytochemical coated AgNPs interacting through van der Walls forces and hydrogen bonding, while citrate-capped silver nanoparticles (CtNPs) showed stable electrostatic interactions. The thermodynamic and temperature dependent studies revealed that the phytochemical coated AgNPs formed less stable complexes with serum proteins, as opposed to CtNPs with fewer changes to the secondary structure of the protein. This instability of AgNP-HSA complex could indicate that phytochemical coated AgNPs might be better suited for targeted nanomedicine.

With regards to toxicity testing of AgNPs, colimetric assay such as MTT [3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide] and lactate dehydrogenase (LDH) assays are currently utilised. However, nanomaterial can interact with the MTT solution or LDH molecules, thereby producing false results, hence these assay are not reliable for the in vitro testing of nanomaterials [7]. These assay also limit the testing
period to a maximum of 72 hours, as further testing can compromise normal cellular functioning due to low seeding density of the cells [8]. This thesis dealt with the development of a long term *in vitro* toxicity assay, which allowed testing for up to four generations of cells. It is possible to use this method for longer time periods, provided the viability and cell numbers allow it. The advantage of this assay is that the cell seeding density remains the same as acute toxicity testing methods, thereby allowing a better comparison between short and long term results. This method enabled testing cells in a realistic scenario, where the same dose of treatment would be received every day. It also allowed a study on the recovery of cells after a short 24 hour period, to examine whether the AgNPs left any residual effects on the cells after treatment had stopped. This is beneficial, since this aspect is often neglected when testing the biocompatibility of AgNPs. This method can be potentially utilised for testing any nanomaterial or drug to determine the long term effect on mammalian cells. This can also help determine the Occupation Health and Safety exposure limits of nanomaterials, since currently there is very limited information available to determine the acceptable exposure concentrations of nanomaterials.

The results of toxicity studies indicated that while acute toxicity suggested no effect on the AgNPs, the long term exposure studies showed significant effect. This was evident in the decrease in cell number and viability, in a dose-dependent manner, along with evidence of oxidative stress, disruption of cell cycle and inhibition of cell adhesion and migration. The results also showed that AgNPs can continue to have an effect on the cells for successive generations even after a mere 24 hours of exposure to the AgNPs. This raises questions regarding reports that indicate nanomaterials are biocompatible after 24 hours of treatment.

There has been much speculation on the effect that surface coatings on AgNPs might have on their activity [6]. The results of this thesis indicate that the surface coating does in fact play a big role in the interaction of AgNPs with biological components. Beginning with introduction of the AgNPs into the blood stream; the surface coating may dictate the extent to which NPs will interact with serum proteins, and altering the coating may either increase or decrease this interaction, as per the requirement of the application.

With regards to interaction with cells, the surface coating may dictate the amount of silver ions that are released from the surface of the AgNPs. Coating of AgNPs with polyphenolic compounds such as EGCG could result in less ions being generated from the surface, while molecules such as tyrosine may allow
greater amount of silver ion generation. The coating may also work synergistically with the silver ions being generated, to produce greater toxic effects on the cells. The concentration of the surface coating may also have an effect on mammalian cells, as evident from the CurAgNPs, where the low concentration of curcumin coating the AgNPs resulted in less toxicity in comparison to the other two AgNPs and an increase in cell adhesion and migration.

These results further indicate the versatility of phytochemical-coated AgNPs. Phytochemicals not only provide an environmentally friendly route for synthesising AgNPs [9], they confer added benefits in the form of antioxidant and anticancer activity or wound healing capability, resulting from the phytochemical coating on the AgNPs. The results of this thesis also suggest that simply changing the phytochemical coating on the AgNPs may change the applications for which the AgNPs can be used, such as EGCGAgNPs which may be tested for anticancer properties, while CurAgNPs shows promising wound healing properties. This thesis has also provided a method for improved testing of long term toxicity of nanomaterials before use in commercial or medical products.

6.2. Future directions

The results of this thesis provided new information in understanding the full potential of AgNPs, which will facilitate safer and wider usage of these NPs; however, there are many areas still lacking in information and further research is required. The long term toxicity testing method may be utilised to test these AgNPs on other mammalian cells, to obtain a better understanding of their effect on other organs in the body. It may also be used to identify if specific proteins interact with the surface coating on the AgNPs. This may enable the identification of unique biomarkers specific to the phytochemicals coating on the AgNPs. This method may also be used to test long term toxicity of other nanoparticles.

Since the CurAgNPs showed promising wound healing activity with endothelial cells, further investigation in required into the optimum concentration of the CurAgNPs for maximum wound healing. The effect of low concentrations of curcumin will also need to be investigated since this appears to be the reason behind the wound healing capabilities of the CurAgNPs. Following this, in vivo testing of these NPs is recommended to fully understand their wound healing potential.

The EGCGAgNPs showed promising anticancer properties, evident in the dose-dependent toxicity, G2/M phase cell cycle arrest, decrease in cell adhesion and migration of prostate cancer cells. Evidence suggests
that pure EGCG and EGCG gold nanoparticles (EGCGAuNPs) utilise the laminin 67 kD receptors to gain entry into cells [10, 11]. The EGCGAgNPs also appeared to be engaging these receptors on the PC-3 cells and this could provide a mechanism for specifically targeting these AgNPs to cells that possess these receptors. Hence, the role of this receptor in uptake of these NPs can be further investigated and the effect of these NPs on other cells that possess the laminin 67 kD receptor should be investigated. In vivo testing of these NPs is also recommended to fully investigate their anticancer properties.

The increasing usage of AgNPs in commercial products would suggest a greater elimination of AgNPs as domestic and commercial waste, which will lead to greater deposition of AgNPs in the environment. This would suggest that a more comprehensive understanding of the effects these NPs may have on the environment is necessary [12]. The fact that the AgNPs were capable of having adverse effects on mammalian cells after long term exposure would suggest that the AgNPs are possibly accumulating in the cells. This could indicate that the AgNPs could remain within an organism, which can lead to bioaccumulation and increased toxicity at the various trophic levels in an ecosystem. Detailed studies on the accumulation of phytochemical coated-AgNPs in the different trophic levels in the ecosystem are warranted to understand further the long term effects of these AgNPs.

6.3. References


Appendix: Publications

Articles under preparation

Chapter 3

“The effect of surface corona on nanoparticle-protein interactions”
Amanda Nicole Abraham, Tarun K. Sharma, Vipul Bansal, Ravi Shukla.
Communicated to Langmuir.

Chapter 4

“Toxicity of phytochemical-stabilised silver nanoparticles on successive generations of mammalian cells”
Amanda Nicole Abraham, Vishal Mistry, Vipul Bansal, Ravi Shukla.
Communicated to Journal of Nanobiotechnology.

Chapter 5

“Curcumin-reduced biocompatible silver nanoparticles induce cellular migration”
Amanda Nicole Abraham, Rajesh Ramanathan, Vipul Bansal, Ravi Shukla.
Communicated to Nanoscale.

Journal publications