8-2013

Functionalized nanomaterials and their biological applications

Pallavi Vedantam
Clemson University, pvedant@clemson.edu

FUNCATIONALIZED NANOMATERIALS AND THEIR BIOLOGICAL APPLICATIONS

A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Microbiology

---

by
Pallavi Vedantam
August 2013

Accepted by:
Dr Tzuen-Rong J. Tzeng, Committee Chair
Dr Pu-Chun Ke
Dr Lesly Temesvari
Dr Ramakrishna Podila

ABSTRACT

Bionanomaterials have been used in drug delivery, cancer therapy and biodiagnosis of pathogens based on their size and surface functionalization. In this present work, different kinds of nanoparticles (NPs), their cellular interactions, cytotoxicity profiles, and finally role of gold nanoparticles (GNPs) in biodetection of *E. coli* was investigated.

Firstly, cytotoxicity profiles of commercial, laser ablation, and green synthetic NPs were studied. Induction of apoptosis in cancer cells was found to be size dependent. The plain 80 nm GNPs and AgNPs enhanced toxic effects in cancer cells when compared to 20 nm ones. Apoptotic profiles of ALB- or FBS-coated NPs were significantly low in cancer cells when compared to plain NPs. The FBS-coated NPs were relatively bigger. Whereas, green synthetic NPs prepared from floral extracts of *Tacoma stans* and *Tagetus erecta* caused significant increase in cell membrane damage in cancer cells when compared to commercial GNPs. The phytochemicals in the extract were found to have a synergistic effect on pathogens like *E. coli*, *Staphylococcus aureus* and *Enterococcus faecalis* when used with antibiotics like tetracycline, ampicillin and vancomycin. This effect can be capitalized in developing NPs as effective drug carriers.

Next, use of commercial GNPs as diagnostic tools to detect competitive binding in DU-145 in the presence of UTI-causing *E. coli* ORN178 was studied. GNPs functionalized with D-mannose (Mn) showed competitive binding between Mn-GNPs and *E. coli* ORN178 when presented together with DU-145 cells. Cytotoxicity assays of plain and Mn-GNPs showed significant decrease in viability of DU-145 cells. The

plain/Mn 20 nm GNPs were taken up more by the cell when compared to the 200 nm ones. The protein-coated GNPs were found to be stable in culture medium. This competitive binding can be further developed to prevent/detect recurrent UTI in DU-45 cells.

Lastly, primary and fine sugar specificity of fimbrial lectins of *E.coli* ORN178 and *E. coli* 13762 to D-mannose and Neuαc(α2-3)-Gal-(β1-4)Glc–Paa functionalized GNPs showed that *E. coli* ORN178 binds specifically only to Mn-GNPs and *E. coli* 13762 to the latter. Hence, adhesin-specific adhesion shows great potential for designing NPs to specifically bind to microorganisms as biodiagnostic tools.

DEDICATION

It is true that not even a blade of grass moves without His Will. This task would not be possible without the Divine blessings, guidance and grace of my most beloved *guru and sakha*, **Bhagavan Sri Sathya Sai Baba**. His Divine love helped me overcome every obstacle in this endeavor. I humbly dedicate this dissertation to Him and thank Him for everything.

ACKNOWLEDGMENTS

First and foremost, I would like to immensely thank Dr. Jeremy Tzeng for giving me an opportunity in pursuing PhD. But for his constant guidance and support I could not have completed this task. His continuous encouragement and sense of humor has definitely been instrumental in completing this project. More than anything his patience and outlook towards research has taught me a lot and shaped me as a better person.

I would like to thank my committee members, Dr. Robert Latour for constructive feedback, Dr. Pu-Chun Ke for his invaluable suggestions, Dr. Lesly Temesvari for her inputs and Dr. Ramakrishna Podila for readily agreeing to be on my committee and for his stimulating scientific ideas.

I would like to thank Dr. Wheeler for all the help he has extended during my education in the past six years. I am also thankful to Dr. Charlie Rice who had given me an opportunity to work in his lab. I am thankful to the staff of the Department of Biological Sciences for all their services.

I am grateful to Dr. Thompson Mefford, Department of Material Sciences, Clemson University for help with instrumentation.

I would like to extend my gratitude to Dr. R Sai Sathish, Sri Sathya Sai University for providing me the samples for research.

I would like to express my gratitude to Dr. Guohui Huang who not only trained me in cell culture but also helped me design experiments and prepare the cells from time to time.

I would like to most importantly thank John Abercrombie who has been very patient, cooperative and resourceful in helping me overcome unforeseen obstacles in my research and teaching.

I am grateful to all the past and present lab mates and undergraduate students who have helped me immensely.

I am extremely thankful to almighty for gifting me friends like Dr. Pooja Puneet and Dr. Sally Wahba for being a part of my life from the beginning to the end of this educational pursuit. Their friendship has kindled optimism and enthusiasm in me to reach the finish line!

I would like to express my heartfelt gratitude to my beloved sister, Indira Vedantam, for all her support and endless love.

Lastly, I would like to thank my mother who has been the backbone of my life and co-advisor in checking the progress of my research from time to time! I am forever indebted to her for all her prayers, patience, compassion, blessings, sacrifices and love.

LIST OF PUBLICATIONS

1. **Vedantam**, **P**., George Huang, and T. R. Jeremy Tzeng. “Size-Dependent Cellular Toxicity and Uptake of Commercial Colloidal Gold Nanoparticles in Du-145 Cells”. Cancer Nanotechnology, (2013): 4(1-3): 13-20.

2. **Vedantam P**., T. R. J. Tzeng, A. K. Brown, R. Podila, A. Rao, and K. Staley. “Binding of Escherichia coli to Functionalized Gold Nanoparticles”. Plasmonics 7, no. 2 (2012): 301-308.

3. J. Zeng, Y. Denga, P Vedantam, T. Tzeng, X. Xuan. “Magnetic separation of particles and cells in ferrofluid flow through a straight microchannel using two offset magnets”. Journal of Magnetism and Magnetic materials. 2013. dx.doi.org/10.1016/j.jmmm.2013.07.021

4. Podila, R., **P. Vedantam**, P. C. Ke, J. M. Brown, and A. M. Rao. "Evidence for Charge-Transfer-Induced Conformational Changes in Carbon Nanostructure–Protein Corona." The Journal of Physical Chemistry C 116, no. 41 (2012): 22098-22103.

5. Zeng, J., Chen, C., **P. Vedantam**., Tzeng, Tzuen-Rong, Xuan, Xiangchun. “Magnetic concentration of particles and cells in ferrofluid flow through a straight microchannel using attracting magnets”. Microfluidics and Nanofluidics, 2012: p. 1-7.

6. Patel, S., Daniel S., **P. Vedantam**, Tzuen-Rong T., Shizhi Q., and Xiangchun X. “Microfluidic Separation of Live and Dead Yeast Cells Using Reservoir-Based Dielectrophoresis.” Biomicrofluidics 6, no. 3 (2012): 034102-12.

7. Zeng, Jian, Chen Chen, **P. Vedantam**, Vincent Brown, Tzuen-Rong J Tzeng, and Xiangchun Xuan. “Three-Dimensional Magnetic Focusing of Particles and Cells in Ferrofluid Flow through a Straight Microchannel.” Journal of Micromechanics and Microengineering 22, no. 10 (2012): 105018.

8. Zhu, J., Robert C., Gyunay K., **P. Vedantam**, Tzuen-Rong J., and Xiangchun X. “Continuous-Flow Particle and Cell Separations in a Serpentine Microchannel Via Curvature-Induced Dielectrophoresis.” Microfluidics and Nanofluidics 11, no. 6 (2011): 743-752.

9. Badal, S., S. A. Williams, G. Huang, S. Francis, **P. Vedantam**, O. Dunbar, H. Jacobs, T. J. Tzeng, J. Gangemi, and R. Delgoda. “Cytochrome P450 1 Enzyme Inhibition and Anticancer Potential of Chromene Amides from Amyris Plumieri.” Fitoterapia 82, no. 2 (2011): 230-236.

TABLE OF CONTENTS

Page

LIST OF TABLES

Table Page

LIST OF FIGURES

List of Figures (Continued)

Figure Page

CHAPTER ONE

LITERATURE REVIEW

Nanotechnology

Nanotechnology is a field that is undergoing explosive development on various fronts. It has found its applications in drinking water purification systems, biological and biomedical research areas. The term 'nano' indicates one billionth or 10^{-9} units (Sergeev 2003; Sergeev and Shabatina 2008). Such nanomaterials act as bridge between technology and innovative scientific advances. Bionanotechnology is another interdisciplinary science that sheds a whole new light of knowledge in understanding the intrinsic cellular mechanisms that has opened the window to plethora of treatment approaches in biomedical and biological applications of nanomaterials.

Synthesis

Once the nanoparticles are synthesized by the desired method of synthesis, they need to be water solubilized, biofuncationalized before use in various biomedical applications. Gold, cobalt, nickel, iron oxide, silica, silver and cadmimum have been reported to be synthesized in aqueous solutions. These particles need to be water-dispersible, the main criteria for their use in biological systems. Size – selective precipitation often is tedious multi-step procedure that is not the most desired procedure for nanoparticle synthesis. The coprecipitation process is often used for iron oxides (Stöber, Fink *et al.* 1968; Rogach 2000; Wang, Sato *et al.* 2003; Podsiadlo, Paternel *et al.*

2005; Bao, Wang *et al.* 2006; Sweeney, Woehrle *et al.* 2006; Gnanaprakash, Mahadevan *et al.* 2007; Lu, Tung *et al.* 2008). Gold and silver nanoparticles are prepared by the reduction method with the use of reducing agents (Daniel and Astruc 2003; Gnanaprakash, Philip *et al.* 2007). Non-polar solvents also have been used to synthesize nanoparticles with the help of organic solvents that help grow the crystal structure and regulate the size of the nanoparticles (Brust, Walker *et al.* 1994; Sun, Murray *et al.* 2000; Gittins and Caruso 2001; Gnanaprakash, Mahadevan *et al.* 2007; Lu, Tung *et al.* 2008). Spherical nanoparticles are generally produced via the simple reduction of metal salts by the use of reducing agents. Since spherical nanoparticles exist in low-energy state, they have been frequently prepared by the Turkevich method, Frens method, microemulsion method, and the Brust method (Murphy, Sau *et al.* 2005).

Lately laser ablation synthesis in liquid solution has gained increased attention. Laser ablation process involves synthesis of nanoparticles in a one-step process. Such particles have extremely high colloidal stability due to their inherent surface charge, resulting in stable free NPs. The surface chemistry of laser-ablated metal nanoparticles is reported to have negative charge which makes it electrostatically stable (Muto, Miyajima *et al.* 2008). NPs prepared by this method allow for control of nanoparticle size distribution and produce mono-disperse particles (Menéndez-Manjón, Chichkov *et al.* 2010). In this method nanomaterials are produced by focusing laser pulses on a target (single piece or pressed powder) immersed in a liquid solution. The material and laser parameters result in nanoparticles that are stable colloids (Amendola and Meneghetti 2007; Bueno-Alejo, D'Alfonso *et al.* 2012). It is a sustainable technology in which

various aqueous media can be used (Murphy 2008). Apart from being a low-cost method, it is one of the fastest and cheapest ways to produce colloidal nanoparticles (Amendola, Meneghetti *et al.* 2011). This process allows for significant control of particle size, concentration, and aggregation. These parameters allow for easy functionalization by various ligands (Amendola, Polizzi *et al.* 2006).

Synthesis and characterization of silver and gold nanomaterials can be done by chemical and physical methods as discussed above.

The biological method of synthesis of nanoparticles is also called as the 'green approach'. Polymers, surfactants, co-polymers, dendrimers, starch, and lipids are necessary to achieve fine and stable catalysis of nanoparticles in the chemical and physical methods. Some of the compounds that are used as stabilizing and/or capping agents in chemical synthesis of nanoparticles could be toxic. Whereas the green approach is economical, environment friendly, and can be easily adapted for large scale production (Buffat and Borel 1976; Jain, Varshney *et al.* 1989; Esumi, Hosoya *et al.* 2000; Chen, Herricks *et al.* 2005; Bakshi, Possmayer *et al.* 2008; Agasti, Chompoosor *et al.* 2009; Gupta, Singh *et al.* 2010). It also does not involve high energy, pressure, temperature, and toxic chemicals. Plenty of research has been published where in extracts from algae, bacteria, fungi, leafs, plants, and yeast have been used to synthesize silver and gold nanoparticles. It is quite interesting to note here that these extracts themselves have been shown to act as reducing, capping, and sometimes shape directing agents (Freund and Spiro 1985; Mehra and Winge 1991; Wang and Herron 1991; Grunwaldt, Kiener *et al.* 1999; Gardea-Torresdey, Parsons *et al.* 2002; Sanghi and Verma 2009). Extracts of neem

leaves, geranium, lemongrass, tamarind, and aloe vera plant have been used to synthesize various shaped mono- and bi-metallic gold and silver core shell nanoparticles (Shankar, Ahmad *et al.* 2003; Shankar, Rai *et al.* 2004; Shankar, Rai *et al.* 2004; Ankamwar, Damle *et al.* 2005; Shankar, Rai *et al.* 2005; Chandran, Chaudhary *et al.* 2006). The extracts of fruit, fruit peel, root, callus, and bark also have been used to synthesis nanoparticles (Ahmad, Sharma *et al.* 2010; MubarakAli, Thajuddin *et al.* 2011; Prathna, Chandrasekaran *et al.* 2011; Rai, Yadav *et al.* 2012). Vegetable extracts have also been used for the facile synthesis of silver, anisotropic gold, and bimetallic nanoparticles using cruciferous vegetable extracts (Jacob, Mukherjee *et al.* 2012).

Researchers have also used other leaf extracts of *Crossandra, infundibuliformis*, *Acalypaindica*, *Rizophora mucronata*, *Menthapiperita*, *Azadirachta indica*, *Stevia rebaudiana*, *Chenopodium album*, *Cassia fistula*, and even banana peel extract (Shankar, Rai *et al.* 2004; Rai, Yadav *et al.* 2009; Gnanadesigan, Anand *et al.* 2011; MubarakAli, Thajuddin *et al.* 2011; Yilmaz, Turkdemir *et al.* 2011). Silver nanoparticles synthesized in this manner shown enhanced fluorescence and surface-enhanced Raman scattering thus making it most useful in optical applications (Dastjerdi, Montazer *et al.* 2009; Li, Xie *et al.* 2010). Raju *et al.* used *Annonna squamosa* leaf extract to extract dominant stable spherical silver nanoparticles with an average size ranging from 20-100 nm (Vivek, Thangam *et al.* 2012). Green tea leaves extract has been used to synthesize plant mediated catalysis of 20 nm gold nanoparticles (Zaki, El Kady *et al.* 2011). Fifteen nm silver nanoparticles synthesized using dried medicinal plant of basil have also been reported (Ahmad, Sharma *et al.* 2010). Green production of zero-valent iron

nanoparticles using 26 different tree leaf extracts have been reported by Machado *et al.* (Machado, Pinto *et al.* 2013). Biosynthesis of NPs from plants, such as *Chenopodium album*, *Sorbus aucuparia*, sugar beet pulp, *Terminalia catappa*, *Hisbiscus rosa sinensis*, *Mangifera indica*, *Syzygium aromaticum*, *Anacardium occidentale* and *Murraya koenigii* have been recently demonstrated (Ankamwar 2010; Dwivedi and Gopal 2010; Philip 2010; Philip 2010; Philip, Unni *et al.* 2011; Sheny, Mathew *et al.* 2011).

The synthesis methods using plant extracts involve phytochemicals like terpenoids, flavonoids, phenol derivatives, di-hydric phenols, plant enzymes and hence act as reductants in the presence of metal salts. It is established that based on the type of extract, structure and type of phenolic phytochemicals present in the extract, different shaped and sized nanoparticles are formed (Jha, Prasad *et al.* 2009; Kumar, Yadav *et al.* 2010; Raghunandan, Bedre *et al.* 2010; Thakkar, Mhatre *et al.* 2010; Jacob, Biswas *et al.* 2011; Jagajjanani Rao and Paria 2013). Interestingly, microorganisms also have been used for the biological synthesis of nanoparticles such as *Pseudomonas stutzeri* AG259, fungi, actinomycetes, cyanobacteria, and biomolecules (Gopinath, MubarakAli *et al.* 2012).

Green catalysis aspects obviously require that environmentally friendly catalysts be designed for easy removal from the reaction media and recycling many time with very high efficiency. But most importantly, application of eco-friendly nanoparticles makes this method preferable for large scale synthesis (Khan, Bashir *et al.* 2012). Almost all studies report a general trend of antimicrobial properties more enhanced in green synthesis particles than nanoparticles synthesized by chemical or physical methods.

Ligands

Having briefly discussed the methods of synthesis, the stabilization of the nanoparticles is the next step where one needs to make sure that the nanoparticle colloidal suspensions are physically and chemically stable, do not aggregate, dissociate or do not lose their metallic properties as they age. For this purpose, they are stabilized with hydrophilic ligands. There are different kinds of ligands that can be used. Ionic stabilization has been studied extensively and it has been reported that even though they are charged well, their high charge can sometimes be disadvantageous as they can be readily opsonized by cells easily (Berry 2009). Since it has been established that positively charged nanoparticles have longer circulation in the blood compared to negatively charged nanoparticles, the coulumbic repulsion based on the charge of the nanoparticle plays an important role in medical applications (Rideal 1951; Mandeville, Marota *et al.* 1998; Daou, Begin-Colin *et al.* 2007; Longmire, Choyke *et al.* 2008). Ligands are often used to maintain the stability of the particle, which in turn maintains the size distribution post-synthesis. These ligands are usually surfactants such as fatty acids, thiols or alkene thiols, which usually render the particle hydrophobic and hence, they need to be further stabilized with amphiphilic ligands such as protein, peptides and thermoresponsive polymers (N.T.K. Thanh 2004; Robinson, Alexander *et al.* 2007).

Steric stabilization using polymeric ligands and small-molecule ligands like ethylene glycol, which is commonly used, can be used to stabilize the nanoparticle to provide enhanced chemical functionality or ionic stabilization (Hong, Han *et al.* 2006;

Chompoosor, Han *et al.* 2008; Nativo, Prior *et al.* 2008). The ethylene glycol end can be further modified to attach another amphiphilic molecule for further functionalization (Berry, Wells *et al.* 2003; Park, Im *et al.* 2005; Chairam and Somsook 2008). Oxygen based ligands like polyols, polyehters, carbohydrates, alcohols, carbonyls are neutral ligands that have been used to control the size selective precipitation of the nanoparticles (Chen, Liu *et al.* 2002; Daniel and Astruc 2003; Xu, Xu *et al.* 2004). However, the stability varies for different nanoparticles. Nitrogen- based ligands are also used but these ionically stabilized nanoparticles salt out in the presence of electrolyte concentrations well below physiological conditions (Brust, Walker *et al.* 1994; Gittins and Caruso 2001; Charbonniere, Rehspringer *et al.* 2008). Even though phosphorous and sulfur-based ligands have been used to functionalize nanoparticles, it has been reported that the stability is the main concern of the entire process (Ulman 1996; Letsinger, Elghanian *et al.* 2000; Yonezawa, Yasui *et al.* 2000; Sahoo, Pizem *et al.* 2001; White, Johnson *et al.* 2006; Joumaa, Toussay *et al.* 2008; Polito, Colombo *et al.* 2008). This is determined by the ligand-surface bond, the packing of the ligand chain, length of the ligand tail, and stacking of the ligand (Porter, Bright *et al.* 1987; Tadmor, Rosensweig *et al.* 2000; Parak, Pellegrino *et al.* 2002; Agasti, You *et al.* 2008). Light induced reactions often lead to the desorption of the ligands (Stouwdam, Shan *et al.* 2006).

Effect of ligands

Since the ligands are essential for the nanoparticle surface characterization, it must be noted that they will, most of the time, determine how a nanoparticle will behave

in *in vitro* or *in vivo* conditions. It has been reported that uncoated nanoparticles are cytotoxic (Gupta, Berry *et al.* 2003; Berry 2009; Nune, Gunda *et al.* 2009). There is no defined map that can be laid out and direct the nanoparticle to cause the desired effects in a planned fashion. This aspect of nanoscience in fact has attracted a large number of research groups to study the cytotoxicity of the nanoparticles in order to decipher their behavior in the biological system and make them safer for various applications (Maenosono, Yoshida *et al.* ; Lewinski, Colvin *et al.* 2008; Verma and Stellacci 2010; Zolnik, González-Fernández *et al.* 2010). Since this review mainly pivots around the biological applications of the nanoparticles, it is important to know the different biofunctionalization moieties that are being used.

Surface functionalization

The optical and electronic properties of Au-NPs offer an enormous potential in nanoscience and engineering applications (Bawendi, Steigerwald *et al.* 1990; Wang and Herron 1996; Collier, Vossmeyer *et al.* 1998). Therefore, surface functionalization of metallic nanoparticles will greatly expand the range of applications of these materials. Two basic approaches can be taken toward the creation of functionalized gold nanoparticles. One is performing the surface modification of already available colloidal gold preparations by chemisorbing thiols, and the other implies the synthesis of colloidal metals with an organic monolayer in a one-step procedure. The latter is most commonly based on modifications to the synthetic procedure originally reported by Brust to create metallic nanoparticles (Brust, Walker *et al.* 1994; Brust, Fink *et al.* 1995). Brust's

synthesis consists of reducing a metallic salt (e.g., AuCl4H) in the presence of an alkanethiol and results in nanoparticles protected by an organic monolayer which are called monolayer-protected clusters (MPCs). Such a monolayer confers these systems with new surface properties and also with extraordinary stability such that they can be isolated and manipulated with a variety of chemical and physical procedures to the extent that they can be dried and resuspended in solution again without suffering irreversible aggregation or decomposition (Chen and Kimura 1999; Yee, Scotti *et al.* 1999; Aguila and Murray 2000; Cliffel, Zamborini *et al.* 2000; Templeton, Wuelfing *et al.* 2000; Templeton, Pietron *et al.* 2000; Kometani, Tsubonishi *et al.* 2001; Shon, Mazzitelli *et al.* 2001).

In general, the synthesis of MPCs yields polydisperse size distributions of nanoparticles of sizes under 10 nm. (Bawendi, Steigerwald *et al.* 1990; Chen and Kimura 1999; Yee, Scotti *et al.* 1999; Kometani, Tsubonishi *et al.* 2001). For some applications, it may be desirable to obtain functionalized gold nanoparticles of sizes larger than these MPCs and with monodisperse sizes. For example, quantum dot (QD) superlattices can be better assembled from single-sized nanoparticles or from colloids with narrow size distributions (Bawendi, Steigerwald *et al.* 1990). This and the fact that larger colloids may offer a broader range of properties and applications have motivated us to study the stability of commercial preparations of colloidal gold upon surface modification by chemisorption of alkanethiols. Colloidal gold is commercially available in monodisperse sizes on the order of tens of nanometers, whereas MPCs have typical polydisperse size distributions on the order of 1-10 nm. Also, the synthesis and characterization of MPCs

may become expensive and time consuming. However, colloidal gold can undergo irreversible aggregation upon chemisorption of alkanethiols (Weisbecker, Merritt *et al.* 1996; Mayya, Patil *et al.* 1997; Templeton, Wuelfing *et al.* 2000).

This kind of functionalization further helps in providing technical advantages for applying gold nanoparticles in biological systems, which have been well recognized (Kreuter 1996). The covalent binding between gold nanoparticles and biomolecules can be easily achieved by self-assembled thiolated molecules onto the nanoparticle surface (Mathai Mammen 1998). Multiple carbohydrate ligands have been assembled on linear polymers (Gestwicki and Kiessling 2002), two-dimensional gold surface (Mann, Kanai *et al.* 1998; Liang, Smith *et al.* 2000; Lin, Yeh *et al.* 2002) and liposomes (Kingery-Wood, Williams *et al.* 1992) to enhance carbohydrate-protein interactions.

Biofunctionalization

Coupling reactions commonly include carbodimiimide, ionic and maleimide coupling, click chemistry, disulfide bridges and histidine tagged proteins (Thanh and Green 2010). Various compounds have been used to functionalize nanoparticles in order to make their surface interactions compatible with the cellular environment. Chitosan also has been found to be biocompatible and used in various applications (Lewin, Carlesso *et al.* 2000; Wang, Zhang *et al.* 2006; Chairam, Poolperm *et al.* 2009). Bacterial and mammalian cell surfaces are rich in phospholipids. This has been exploited by bioconjugating nanoparticles with phospholipids. Biotin-avidin interactions have been investigated for drug delivery and hyperthermic applications (Giri, Guha Thakurta *et al.*

2005; Senarath-Yapa, Phimphivong *et al.* 2007). Signal transduction at cellular level has been attempted by functionalization of nanoparticles with peptide sequences (Lewin, Carlesso *et al.* 2000; Torchilin 2008). Proteins are vital biomolecules that determine the fate of the nanoparticle in vivo. They not only determine the specific binding interactions of nanoparticles but also determine the toxic effects when inside the cell. Biodetection of antigens with the help of antibody tagged nanoparticles has shown specific and non-specific interactions (Dyal, Loos *et al.* 2003; Gupta, Berry *et al.* 2003; Lin, Chou *et al.* 2006; Bonasio, Carman *et al.* 2007). Protein corona of nanoparticles has shown that nanoparticles are less cytotoxic and enhance cellular uptake (Konerackᡠ, Kopčanský *et al.* 1999; Huh, Jun *et al.* 2005).

Carbohydrates have been extensively used in microbial detection (Berry, Wells *et al.* 2003; Chairam and Somsook 2008; Chompoosor, Han *et al.* 2008). Carbohydrates on the host surface play an important role for the pathogen binding and colonization. The carbohydrate-protein interaction primarily decides adhesion of the pathogen leading to internalization (Sharon and Lis 1989; Karlsson, Angström *et al.* 1992). Facilitating anti-adhesion to fight microbial antibiotic resistance is one such approach in nanotechnology (Balland, Pinto-Alphandary *et al.* 1996; Sondi and Salopek-Sondi 2004; Naruse 2005; Shi, Neoh *et al.* 2006). Initial phase of pathogenesis involves attachment of bacterial to the mucosal surfaces. Bacterial surfaces consist of adhesins that are characteristic for each bacterial and often determine the attachment to host cell membrane receptors. There are fimbrial adhesins often located on the fimbriae of the bacteria. The fimbrial adhesins can result in specific binding interactions with the host cell surface. There are different

kinds of fimbrial adhesion mechanisms of which are type 1 (mannosylated) and P-fimbriated adhesion (galabiose). The primary sugar specificity of bacteria dictates its fimbrial adhesion mechanism to the host cells (Wentworth, Austin *et al.* 1991; Mitchell, Houles *et al.* 2002; Loris, Tielker *et al.* 2003). Basing on this principle, engineered nanoparticles that are functionalized with carbohydrates have been used in drug delivery, biodiagnosis, biodetection and other biomedical applications (Varshney, Yang *et al.* 2005).

Characterization

Once synthesized, the nanoparticles need to be characterized in order to understand their surface chemistry for further applications and controlled nanoparticle synthesis. It is important to determine the particles size, shape, crystalline structure, fractal dimensions, pore size, surface area, surface plasmon resonance, electrodynamic surface chemistry and stability of the synthesized nanoparticles (Priyadarshini, Gopinath *et al.* 2013). Different techniques are available that are being used extensively for the above studies such as transmission and scanning electron microscopy (TEM/SEM), UV-Vis spectroscopy, powder X-ray diffractometry (XRD), atomic force microscopy (AFM), X-ray photoelectron spectroscopy (XPS), dynamic light scattering (DLS) and Fourier transform infrared spectroscopy (FTIR), thermal gravimetric analysis (TGA) and nanoparticle shape and configuration analysis by electron topography analysis (Khomutov and Gubin 2002; Yeo, Lee *et al.* 2003; Chimentao, Kirm *et al.* 2004; Zhang, Tanha *et al.* 2004; Agasti, You *et al.* 2008; Vilchis-Nestor, Sánchez-Mendieta *et al.*

2008). These tools have made easy qualitative and quantitative identification of nanoparticle behavior in vitro feasible.

Gold nanoparticles

Gold nanoparticles at nanoscale (~100 nm or less) exhibit brilliant colors. The dielectric constant for gold is in the visible region of the electromagnetic spectrum (El-Sayed 2001). The plasmon frequency of nanoparticles is uniquely sensitive to the dielectric interaction in the liquid they are dispersed. Any surface modification or aggregation leads to the shift in surface plasmon resulting in a color change (El-Sayed 2001; Murphy 2002; Kelly, Coronado *et al.* 2003; Burda, Chen *et al.* 2005; Rosi and Mirkin 2005). This property is mainly used for chemical sensing and imaging applications as coupling of plasmons due to shift in plasmon frequency makes it possible to optically track the nanoparticles (Daniel and Astruc 2004; Rosi and Mirkin 2005). Not only they can synthesized in different shapes and sizes, their optical properties make them an excellent candidate in drug encapsulation, gene therapy, biodiagnosis, bioimaging and anticancer applications (Dhar, Daniel *et al.* 2009; Yen, Hsu *et al.* 2009). Therefore, gold nanoparticles that belong to the noble metal family exhibit interesting properties making them the most suitable candidate for nanomedicine.

Silver nanoparticles, in contrast, have been used predominantly as anti-bacterial agents in food, health, and textile industries (Gupta and Silver 1998; Bosetti, Massè *et al.* 2002; Vivek, Thangam *et al.* 2012). Owing to their broad spectrum of antimicrobial activity and surface plasmon resonance effects, silver nanoparticles (AgNPs) have gained

much popularity. They have also been used for several environmental applications (Abou El-Nour, Eftaiha *et al.* 2010). Research has shown that cotton fibers are highly anti-bacterial against *Escherichia coli* (Yeo, Lee *et al.* 2003; Chen and Chiang 2008). Among the wide range of applications from simple water purification in home appliances to disinfection of biomedical devices, wound dressings, bond prostheses and heart valves and biosensing are a few (Dubas and Pimpan 2008; Xu, Yang *et al.* 2008). Due to their electrochemical properties, they have been incorporated in sensors that offer faster and lower detection limits. It has been showed that the bleaching of the organic dyes by application of silver nanoparticles can be enhanced at room temperatures (Köhler, Abahmane *et al.* 2008). Silver nanoparticles have been used as biological labels because of their fluorescent emissions from silver nano clusters (Mulvaney 1996; El-Sayed 2001; Kelly, Coronado *et al.* 2002; Berciaud, Cognet *et al.* 2005; Kossyrev, Yin *et al.* 2005; Jia, Ma *et al.* 2008).

Cytotoxicity of Gold nanoparticles

Use of gold nanoparticles in various applications largely deals with their uptake, cellular toxicity, biocompatibility and ultimately their disposal from biological systems. This is very important in regard to their clinical applications. There is a subtle difference between cytotoxicity, cellular damage and genotoxicity of nanoparticles. Nanoparticles can be capable of causing cellular damage but not exhibiting any toxicity. The cells might initiate processes to combat the cellular damage but might not effectively replicate. Citrate-capped gold nanoparticles of 13 nm were found to be toxic to skin cells but

inhibited cell proliferation (Nadine Pernodet 2006). Based on the different types of cell lines the cytotoxicity has been reported to vary. Citrate-capped nanospheres of gold are not cytotoxic to baby hamster kidney and human liver cells, but cytotoxic to human lung cancer cell line(Patra, Banerjee *et al.* 2007). In general, they have been found to be nontoxic in low doses and but toxic as dosage increases. Gold nanoparticles are taken up by human leukemia cells (Nikoobakht and El-Sayed 2001; Connor, Mwamuka *et al.* 2005; Sau and Murphy 2005). The reduction in the amount of harmful reactive oxygen species (ROS) to the immune system cells has been reported (Shukla, Thomas *et al.* 2005). Gold nanometers of 50 nm were reported to be taken up quickly by HeLa, a human cervical cancer cell line (Chithrani, Ghazani *et al.* 2006). Studies have also shown that gold nanoparticles of 14-100 nm have been taken up by cells but have no cytotoxic effects (Chithrani, Jelveh *et al.* 2010). The surface modifications, net surface charge, size and the physical dimensions of the gold nanoparticles have been shown to play an important role in the difference in cytotoxic effects (Connor, Mwamuka *et al.* 2005). Whereas due to the widespread use of silver nanoparticles in medicine, the body exposure to these nanoparticles is more compared to gold nanoparticles. Decreased mitochondrial function, DNA damage, induced cell apoptosis, and necrotic cell death for several types of cells have been reported (AshaRani, Low Kah Mun *et al.* 2009).

Particle stability plays an important role in uptake and cytotoxic effects. Zeta potentials of particles reflect the net surface charge of particles. Charged particles (cationic or anionic) display different rates of uptake when compared to particles whose zeta potential values are close to zero (Roser, Fischer *et al.* 1998). Cytotoxicity includes

the induction of immunological response in the cells that can lead to proinflammatory response which leads to gene expression. Often this leads to onset of endocytosis – one of the vital mechanisms by which macrophages expunge foreign material in the cell. Phagocytosis, pinocytosis and receptor mediated endocytosis are integral parts of endocytic mechanisms of the cell (Kruth, Jones *et al.* 2005). In general, it has been shown that phagocytosis occurs for most nanoparticles that are larger than 100 nm and pinocytosis occurs for nanoparticles less than 100 nm. However, protein coated nanoparticles with albumin or transferrin can not only enter the cells via a receptor mediated endocytic pathway but also escape into cellular organelles (Chithrani and Chan 2007). The protein corona is a fascinating approach where by uptake of nanoparticles by pinocytosis can be avoided and receptor based endocytosis can be achieved. The former simply would lead to aggregation in the cell vesicle, whereas the latter is taken up actively via a coated vesicle (Chithrani, Ghazani *et al.* 2006).

The protein corona not only helps in the uptake of the nanoparticles but also brings about less cytotoxicity in the cells. Due to the protein coating around the nanoparticles, the negative surface charges interact with the receptor proteins that changes the conformation of the cell surface receptors and allows the negatively charged proteins to be more readily absorbed on to positively charged substances (albumin, fibrinogen, fetal bovine serum, transferrin etc.). Therefore, in other words, protein corona shows promising effects that can steer the nanoparticle's fate in the cell. However it has been shown that higher the immunological responses in gold nanoparticles than silver nanoparticles could be related to their uptake mechanisms (Podila, Vedantam *et al.*

2012). The surface zeta potentials impact the aggregation of nanoparticles based on their hydrophobicity or hydrophilicity. Differences in zeta potentials has been attributed to different cytotoxicity profiles of gold and silver nanoparticles (Lundqvist, Stigler *et al.* 2011).

Applications of NPs

Nanomedicine is a science that is fairly developed and requires decades of research in order to understand the potential treatment options nanotechnology offers in personalized treatment of diseases. The benefits are such as accuracy, speed of treatment, efficacy and safety. There has been great amount of short term and long term research that has been done in this regard. Commercial nanomedicine is at its early stages of development. There has been a great deal of development of nanovectors and site targeted nanosystems. Multifunctionality of nanocantilevers, nanowires and nanotubes has made it possible to detect molecular signals in real time. These kinds of systems help in the detection, diagnosis and prognosis of cancer.

Early detection systems that detect the early stages of cancer will help in the treatment of the disease. This being one of the most critical point, it is important to achieve full recovery of the disease. In the current treatment methods, it is possible to treat cancer in an effective manner once detected early and not metastasized. Many nano-based technologies are being studied for prognosis of the tumor stages in cancer. It is important to find least invasive methods for the same. Once it is detected, the effective tumor targeting technique is crucial for treatment. There has been plenty of research done

with nanoparticles that have acted as carriers for releasing drugs in targeted drug delivery method. This kind of approach will help opt for treatment options by passing the surgery and the chemotherapy route. Since there is overwhelming information in this regard this section of the review will pivot mainly around the biomedical technology that is of therapeutic value and promising enough to better patient care.

Nanomedical applications

The future of nanomedicine is steering towards eliminating bacterial infections in patients by passing the use of antibiotics, surgery at the cellular level, repairing damaged tissues and cells. The biggest hurdle is to engineer nanoparticles that can get around biological barriers. The tight junctions between cells, the blood-brain barrier, the filtration systems in the body and there are various bio-physical barriers that slow down the extravasation of vascularly injected agents (Grossman and McNeil 2012).

Targeting has been approached by two modalities in cancer nanotechnology, active and passive. Directly involves linking of nanoparticles directly to the ligands that identify and associate with the tumor specific cells. The antibody conjugated approach is one such where the nanoparticles is made to localize with cell surface proteins. Specific targeting of blood vessels has been demonstrated (Akerman, Chan *et al.* 2002; Wu, Liu *et al.* 2003; Paciotti, Myer *et al.* 2004; Nida, Rahman *et al.* 2005). The passive approach takes advantage of the nano size of the nanoparticles and the tumor vasculature. Since the tumor vasculature is unlike the normal endothelium with wide fenestrations, the large pore sizes allow the passage of nanoparticles into the tumor. It is seen that nanoparticles

do reach inside the tumor and this brings about the advantage of tailoring a nanoparticle linked to various drugs, proteins and polymers to treat the tumor (Hobbs, Monsky *et al.* 1998; Schaeffer, Tan *et al.* 2008; Seigneuric, Markey *et al.* 2010; Shao, Gao *et al.* 2011; Mukerjee, Ranjan *et al.* 2012; Lee, Qian *et al.* 2013; Stefanick, Ashley *et al.* 2013).

It has been demonstrated that nanoparticles coated with albumin and bound to drug paclitaxel was more efficient in treating metastatic breast cancer pateints (Gradishar, Tjulandin *et al.* 2005). Abraxane was approved by the FDA in 2005 for the treatment of metastatic breast cancer, which consists of paclitaxel nanoparticles that are bound to albumin molecules. This combination allows it to cross the vascular endothelium barrier across blood vessels and hence 50% greater clinical dosages of the drug. In a study conducted in rats, poloxamer 188-coated PLGA nanoparticles enable delivery of doxorubicin across the blood-brain barrier in the therapeutically effective concentrations to treat glioblastoma (Wohlfart, Khalansky *et al.* 2011). Various nanosystems also have been reported to cross the blood brain barrier which include thiamine coated nanoparticles (Lockman, Oyewumi *et al.* 2003), in situ transport of nanoparticles across the blood brain barrier and drug delivery (Koziara, Lockman *et al.* 2003).

Enzymatic digestion of the drug coated, the delayed release of the drug, penetration of the orally administered biomolecular agents thought the intestinal barrier, has led to development of synthetic nanoparticles that can be biological, therapeutic and clinical agents (Chen, Torchilin *et al.* 1996; Wu, Akaike *et al.* 1998; Martin and Grove 2001; Tao, Lubeley *et al.* 2003; Lundqvist, Stigler *et al.* 2011; Li, Nielsen *et al.* 2012). The cells of the reticuloendothelial system act as one of the biological and immunological

barriers to targeting of nanoparticle-encapsulated drugs. Surface modifications do aid in tackling this impediment and one such polymer is polyethylene glycol (PEG). It has been demonstrated that Peg increases the circulatory half-life to hours/days when used in tumor targeting (Klibanov, Maruyama *et al.* 1991; Park 2002; Park, Benz *et al.* 2004; Deng, Ke *et al.* 2012).

Another approach has been tagging antibodies to nanovectors to treat various tumors. Carbon nanotubes, dendrimers and gold nanoparticles have been used for treatment and it has been seen that the particles when bound to protein sensitize the tumor tissue and initiate an immunogenic response towards themselves (Lee, Parthasarathy *et al.* 2001; Lee, Parthasarathy *et al.* 2004; Desai 2012). The tumor tissue itself acts as a biophysical barrier sometimes. The increased osmotic pressure in the lesions, the metastatic nature of the tumor and presence of dead cells blocking the release of the drug are few. The diffusion of the drug loaded agents into the tumor results in imbalance in pressure and risk the effusion of the therapeutic agent out of the site of injection altogether. There has also been research showing that nanoparticles provide spatiotemporal control of drug release and enhanced tissue penetration (Netti, Baxter *et al.* 1995; Sarntinoranont, Rooney *et al.* 2003; Stylianopoulos, Martin *et al.* 2012; Tong, Hemmati *et al.* 2012).

Targeting efficacy is an important aspect in cancer lesions and tumors. The tumor microenvironment and tumor angiogenesis present the biggest hurdle to targeted drug delivery systems. Most of the chemotherapeutic agents lead to systemic toxicity with potential side effects. The leaky vaculature of the tumor tissue helps in enhanced

permeability. Hence, the nanocarriers allow active targeting by successfully binding to the surface receptors of the cancer cells. Directional targeting of the nanosystems has been shown that delivery of cytotoxic agents to the tumor site might lead to the breaking down of the lesions into small neoplasms (Sinek, Frieboes *et al.* 2004; Danhier, Feron *et al.* 2010).

Controlled release of the drug in the treatment of prostate cancer has been demonstrated that resulted in therapeutic advantage, reduction of side effects of chemotherapy, regularity of dosing and targeted delivery. Drug delivery systems when administered need to be selective and effective. For which it is important to determine the right amount of drug, right time and right target in the body. Direct injection of the nanosystems loaded with drugs is mostly the preferred choice. The binding affinity of the particles to the cells is affected by binding force and growth rate with time. The hemodynamics play an important role in the viscoelastic properties of the particle targeting the cell (Decuzzi, Lee *et al.* 2004).

For example, to enhance the increased uptake of anticancer agents, folic acid has been employed as a targeting ligand and found to increase their cellular uptake within target cells since the folate receptor is overexpressed on the surface of tumor cells (Zwicke, Mansoori *et al.* 2012). One such study in human breast cancer cells MCF-7 indicated that the paclitaxel loaded folic acid micelle is a successful anticancer-targeted drug-delivery system for effective cancer chemotherapy (Wang, Chen *et al.* 2012). Biodistribution and tumor retention in vivo studies carried out in human nasopharyngeal carcinoma tumor-bearing athymic mice , indicate that intracellular targeting of

nanoparticles by folate is enhanced (Oyewumi, Yokel *et al.* 2004). On the other hand there has been research showing that development of nanoscale drug delivery could be engineered in such a manner that would not only allow intracellular delivery also have an imaging capability for tracking the uptake of the material introduced. Growth inhibitory oligonucleotides have been linked to dendrimers and introduced to treat breast, ovarian and prostate cell lines effectively (Santhakumaran, Thomas *et al.* 2004). Identification systems consisting of oliguncleotide-bound gold and magnetic nanoparticles have been developed that carry a predefined sequence, this system hence becomes more sensitive than PCR (Nam, Stoeva *et al.* 2004; Vetrone, Huarng *et al.* 2012).

Gold nanoparticles in conjuction with microcantilevers have been developed that can detect single mismatch errors in DNA molecule (Su, Li *et al.* 2003). Hence, there has been technology that has been developed to detect specific molecular changes in cancer cells, but the sensitivity behind each innovation is mainly that matters the most. Another approach of theragnostic nanotechnology involves siRNA therapy of tumor tissue. This therapy is nothing but gene therapy that involves use of nucleotide drugs for clinical purpose instead of the conventional chemical drugs. This approach has dual advantages of treatment as well as tracking/imaging of the treatment. siRNAs bound to RNA-induced silencing complex (RISC) with silencing activity is introduced that brings about degradation of the target RNA sequence (mRNA). Hence, in cancer cells there is over expression of proteins. By employing this technique the siRNA can be introduced in the target cell cytosol and suppress specific surface proteins. Many nanosystems like metals, lipids and polymers have been used (Lee, Lee *et al.* 2010; Zarbin, Montemagno *et al.*

2010; da Paz, Santos Mde *et al.* 2012; Lee, Kim *et al.* 2012; Balasubramanian, Ravindran Girija *et al.* 2013). This concept is fairly new and is diagnostic in nature and one can monitor the therapeutic treatment at the same time. By this it is easy for clinicians to administer, monitor and alter the treatment depending on the patient response.

In summary there are opportunities to for developing entirely new therapeutics which can be safer and more effective in the diagnostic areas to fight cancer. The most promising area being the drug encapsulated nanoparticles. Various platforms that have emerged using nanotechnology have successfully shown treatment efficacy, safety and practicality of the approach is the key to target tumors for treatment of cancer. Multifunctional nanocarriers that combine chemotherapy, radiation and drug therapy are shaping the future of cancer treatment. These technologies sure move the clinical application forward, but the accelerating development should also be in compliance with FDA and be promising in innovation. There has been a dearth of research in this regard as discussed above. There is a paradigm shift in treatment approaches of passive and active treatment of cancer. It will be exciting to know if nanomedicine becomes a mode of treatment to improve the longevity of the cancer patients in future.

Biomedical applications

Various biomedical applications of NPs include use as drug carriers, gene delivery, gene therapy, labeling and tracking agents, hyperthermic agents and magnetic resonance imaging (MRI) agents. All the applications are based on the chemical modification of the nanoparticle surface which defines the biomolecular interactions in

vitro or in vivo. Drug delivery has been the most studied platform to launch drug coated nanoparticles to achieve localized treatment in vitro and in vivo. Drug delivery systems are necessary for increasing the efficacy of biodistribution of the drug (Laurent, Forge *et al.* 2008; Sun, Lee *et al.* 2008; Berry 2009). Nanoparticles have been used for transport of drugs and genes, via passive, active and direct means. This has been attributed to the small nature of NPs which allows them to cross cellular barriers/membranes. The increased surface area of nanoparticles to volume ratio allows increased loading of drugs (Parak, Gerion *et al.* 2003; Alivisatos 2004). These properties make nanoparticles an excellent vehicle for diagnostic and therapeutic uses in biomedical applications. Different nanoparticles have been used for imaging. Such studies have shed considerable light and shown insight in understand the biochemical processes in vitro and in vivo. The photophysical properties of gold nanoparticles have used in sensing, biodiagnostics, imaging, sensing, biodetection, treatment of cancer and targeting cancer cell markers. Labeling of cellular proteins, cellular tracking, pathogen detection, anti-toxin treatment and fluorescence resonance energy transfer (FRET) techniques have taken advantage of size, surface plasmon resonance pattern, photophysical properties of nanoparticles (Maenosono, Yoshida *et al.* ; Lyon, Musick *et al.* 1998; Storhoff, Elghanian *et al.* 1998; Yguerabide and Yguerabide 1998; He, Musick *et al.* 2000; Zhao, Brook *et al.* 2008; Agasti, Chompoosor *et al.* 2009; Nam, Won *et al.* 2009; Xu, Wang *et al.* 2009; Wang and Irudayaraj 2010).

In conclusion, bionanomaterials have been studied and a great depth of biomolecular research has been achieved. But scientists really need to answer the key

question - what is the fate of nanoparticles once introduced into the human body system for biomedical applications? There has been a shift in the treatment modalities with nanotechnology towards specific, targeted, localized and detection mechanisms in biomedical applications. There is an increase in the interest of biosensor development, biodetection, bioterrorism, nano-chip based innovations, site specific tumor treatment and so on and so forth. The basic underlying current of developing these technologies is to provide rapid screening methods, specificity, multi-modal treatment approaches, lower the cost of patient care, treat infections, fight cancer and hence, engineer therapeutic approaches that can increase the life span with quality care treatment. Therefore, use of nanotechnology in health care has shown prospective future but there are ethical issues associated with the technology that also need attention.

Objectives

Nanoparticles are unique materials that have been studied extensively since they are at nano scale. This allows for larger surface area of the particle to show increased range of possible interactions with bio-organic materials that are present on the cell surface. The interactions also depend on the methods used for synthesizing the particles, their size and their cytotoxic effects *in vivo*. The main aim of this study was to study and compare the different antimicrobial and cytotoxic profiles of commercial nanoparticles and green synthetic nanoparticles. The second aim of this investigation sheds light on effects of protein corona on cytotoxicity in different cancer cells. Thirdly, this study attempts to study the size based differences in uptake of sugar functionalized and plain

gold nanoparticles. Once the degree of uptake and cytotoxicity of different kinds of NPs based on surface chemistry and size is determined, the last part of this dissertation aims at investigating the use of carbohydrate functionalized gold nanoparticles to bring about competitive binding in prostate cancer cells in the presence of urinary tract infection causing bacteria *E. coli* ORN178. Overall, this study attempts to investigate the cytotoxicity of nanoparticles based on their mechanisms of synthesis, size and surface functionalization.

Literature Cited

Abou El-Nour, K. M. M., A. a. Eftaiha, *et al.* (2010). "Synthesis and applications of silver nanoparticles." Arabian Journal of Chemistry **3**(3): 135-140.

Agasti, S. S., A. Chompoosor, *et al.* (2009). "Photoregulated Release of Caged Anticancer Drugs from Gold Nanoparticles." Journal of the American Chemical Society **131**(16): 5728-5729.

Agasti, S. S., C.-C. You, *et al.* (2008). "Structural control of the monolayer stability of water-soluble gold nanoparticles." Journal of Materials Chemistry **18**(1): 70-73.

Aguila, A. and R. Murray (2000). "Monolayer-protected clusters with fluorescent dansyl ligands." Langmuir **16**(14): 5949-5954.

Ahmad, N., S. Sharma, *et al.* (2010). "Rapid synthesis of silver nanoparticles using dried medicinal plant of basil." Colloids Surf B Biointerfaces **81**(1): 81-86.

Aizpurua, J., P. Hanarp, *et al.* (2003). "Optical Properties of Gold Nanorings." Physical Review Letters **90**(5): 057401.

Akerman, M. E., W. C. Chan, *et al.* (2002). "Nanocrystal targeting in vivo." Proc Natl Acad Sci U S A **99**(20): 12617-12621.

Alexis, F., J.-W. Rhee, *et al.* (2008). "New frontiers in nanotechnology for cancer treatment." Urologic Oncology: Seminars and Original Investigations **26**(1): 74-85.

Alivisatos, P. (2004). "The use of nanocrystals in biological detection." Nat Biotech **22**(1): 47-52.

Amendola, V. and M. Meneghetti (2007). "Controlled size manipulation of free gold nanoparticles by laser irradiation and their facile bioconjugation." Journal of Materials Chemistry **17**(44): 4705-4710.

Amendola, V., M. Meneghetti, *et al.* (2011). "Top-down synthesis of multifunctional iron oxide nanoparticles for macrophage labelling and manipulation." Journal of Materials Chemistry **21**(11): 3803-3813.

Amendola, V., S. Polizzi, *et al.* (2006). "Laser ablation synthesis of gold nanoparticles in organic solvents." J Phys Chem B **110**(14): 7232-7237.

Ankamwar, B. (2010). "Biosynthesis of Gold Nanoparticles (Green-gold) Using Leaf Extract of Terminalia Catappa." E-Journal of Chemistry **7**(4): 1334-1339.

Ankamwar, B., C. Damle, *et al.* (2005). "Biosynthesis of Gold and Silver Nanoparticles Using Emblica Officinalis Fruit Extract, Their Phase Transfer and Transmetallation in an Organic Solution." Journal of Nanoscience and Nanotechnology **5**(10): 1665-1671.

Arnida, A. Malugin, *et al.* (2010). "Cellular uptake and toxicity of gold nanoparticles in prostate cancer cells: a comparative study of rods and spheres." Journal of Applied Toxicology **30**(3): 212-217.

AshaRani, P. V., G. Low Kah Mun, *et al.* (2009). "Cytotoxicity and genotoxicity of silver nanoparticles in human cells." ACS Nano **3**(2): 279-290.

Aslan, K., J. R. Lakowicz, *et al.* (2004). "Nanogold-plasmon-resonance-based glucose sensing." Anal Biochem **330**(1): 145-155.

Aslan, K., J. R. Lakowicz, *et al.* (2004). "Tunable plasmonic glucose sensing based on the dissociation of Con A-aggregated dextran-coated gold colloids." Analytica Chimica Acta **517**(1–2): 139-144.

Bakshi, M. S., F. Possmayer, *et al.* (2008). "Aqueous-Phase Room-Temperature Synthesis of Gold Nanoribbons: Soft Template Effect of a Gemini Surfactant." The Journal of Physical Chemistry C **112**(22): 8259-8265.

Balasubramanian, S., A. Ravindran Girija, *et al.* (2013). "Multifunctional Carboxymethyl cellulose based magnetic nanovector as theragnostic system for folate receptor targeted chemotherapy, imaging and hyperthermia against cancer." Langmuir.

Balland, O., H. Pinto-Alphandary, *et al.* (1996). "Intracellular distribution of ampicillin in murine macrophages infected with Salmonella typhimurium and treated with (3H) ampicillin-loaded nanoparticles." Journal of Antimicrobial Chemotherapy **37**(1): 105-115.

Bao, H., E. Wang, *et al.* (2006). "One-Pot Synthesis of CdTe Nanocrystals and Shape Control of Luminescent CdTe–Cystine Nanocomposites." Small **2**(4): 476-480.

Basavaraj V Chivde, K. V. B., Rajabhau (2011). "ANXIOLYTIC ACTIVITY OF TABERNAEMONTANA DIVARICATA (LINN) R. Br. FLOWERS EXTRACT IN MICE." International Journal of Pharma and Bio Sciences **2**(3): 65-72.

Bashir, S. and A. H. Gilani (2008). "Studies on the antioxidant and analgesic activities of Aztec marigold (Tagetes erecta) flowers." Phytotherapy Research **22**(12): 1692-1694.

Bawendi, M., M. Steigerwald, *et al.* (1990). "The quantum mechanics of larger semiconductor clusters (" quantum dots")." Annual Review of Physical Chemistry **41**(1): 477-496.

Berciaud, S., L. Cognet, *et al.* (2005). "Observation of Intrinsic Size Effects in the Optical Response of Individual Gold Nanoparticles." Nano Letters **5**(3): 515-518.

Berry, C. C. (2009). "Progress in functionalization of magnetic nanoparticles for applications in biomedicine." Journal of Physics D: Applied Physics **42**(22): 224003.

Berry, C. C., S. Wells, *et al.* (2003). "Dextran and albumin derivatised iron oxide nanoparticles: influence on fibroblasts in vitro." Biomaterials **24**(25): 4551-4557.
Bonasio, R., C. V. Carman, *et al.* (2007). "Specific and covalent labeling of a membrane protein with organic fluorochromes and quantum dots." Proceedings of the National Academy of Sciences **104**(37): 14753-14758.
Bosetti, M., A. Massè, *et al.* (2002). "Silver coated materials for external fixation devices: in vitro biocompatibility and genotoxicity." Biomaterials **23**(3): 887-892.
Brewer, S. H., W. R. Glomm, *et al.* (2005). "Probing BSA Binding to Citrate-Coated Gold Nanoparticles and Surfaces." Langmuir **21**(20): 9303-9307.
Brust, M., J. Fink, *et al.* (1995). "Synthesis and reactions of functionalised gold nanoparticles." Journal of the Chemical Society, Chemical Communications **1995**(16): 1655-1656.
Brust, M., M. Walker, *et al.* (1994). "Synthesis of thiol-derivatised gold nanoparticles in a two-phase liquid–liquid system." Journal of the Chemical Society, Chemical Communications **1994**(7): 801-802.
Brust, M., M. Walker, *et al.* (1994). "Synthesis of thiol-derivatised gold nanoparticles in a two-phase Liquid-Liquid system." Journal of the Chemical Society, Chemical Communications **0**(7): 801-802.
Bueno-Alejo, C. J., C. D'Alfonso, *et al.* (2012). "Ultraclean derivatized monodisperse gold nanoparticles through laser drop ablation customization of polymorph gold nanostructures." Langmuir **28**(21): 8183-8189.
Buffat, P. and J. P. Borel (1976). "Size effect on the melting temperature of gold particles." Physical Review A **13**(6): 2287-2298.
Burda, C., X. Chen, *et al.* (2005). "Chemistry and properties of nanocrystals of different shapes." Chem. Rev **105**(4): 1025-1102.
Capriotti, A. L., G. Caracciolo, *et al.* (2011). "Differential analysis of "protein corona" profile adsorbed onto different nonviral gene delivery systems." Anal Biochem **419**(2): 180-189.
Capriotti, A. L., G. Caracciolo, *et al.* (2011). "DNA affects the composition of lipoplex protein corona: a proteomics approach." Proteomics **11**(16): 3349-3358.
Caracciolo, G., D. Pozzi, *et al.* (2011). "Evolution of the protein corona of lipid gene vectors as a function of plasma concentration." Langmuir **27**(24): 15048-15053.
Cedervall, T., I. Lynch, *et al.* (2007). "Understanding the nanoparticle–protein corona using methods to quantify exchange rates and affinities of proteins for nanoparticles." Proceedings of the National Academy of Sciences **104**(7): 2050-2055.
Chairam, S., C. Poolperm, *et al.* (2009). "Starch vermicelli template-assisted synthesis of size/shape-controlled nanoparticles." Carbohydrate Polymers **75**(4): 694-704.
Chairam, S. and E. Somsook (2008). "Starch vermicelli template for synthesis of magnetic iron oxide nanoclusters." Journal of Magnetism and Magnetic Materials **320**(15): 2039-2043.
Chandran, S. P., M. Chaudhary, *et al.* (2006). "Synthesis of Gold Nanotriangles and Silver Nanoparticles Using Aloevera Plant Extract." Biotechnology Progress **22**(2): 577-583.

Charbonniere, L. J., J.-L. Rehspringer, *et al.* (2008). "Highly luminescent water-soluble lanthanide nanoparticles through surface coating sensitization." New Journal of Chemistry **32**(6): 1055-1059.

Chen, C.-Y. and C.-L. Chiang (2008). "Preparation of cotton fibers with antibacterial silver nanoparticles." Materials Letters **62**(21–22): 3607-3609.

Chen, H., V. Torchilin, *et al.* (1996). "Lectin-bearing polymerized liposomes as potential oral vaccine carriers." Pharm Res **13**(9): 1378-1383.

Chen, J., T. Herricks, *et al.* (2005). "Polyol Synthesis of Platinum Nanostructures: Control of Morphology through the Manipulation of Reduction Kinetics." Angewandte Chemie International Edition **44**(17): 2589-2592.

Chen, L. X., T. Liu, *et al.* (2002). "Fe2O3 Nanoparticle Structures Investigated by X-ray Absorption Near-Edge Structure, Surface Modifications, and Model Calculations." The Journal of Physical Chemistry B **106**(34): 8539-8546.

Chen, S. and K. Kimura (1999). "Synthesis and Characterization of Carboxylate-Modified Gold Nanoparticle Powders Dispersible in Water." Langmuir **15**(4): 1075-1082.

Chimentao, R. J., I. Kirm, *et al.* (2004). "Different morphologies of silver nanoparticles as catalysts for the selective oxidation of styrene in the gas phase." Chemical Communications **0**(7): 846-847.

Chithrani, B. D. and W. C. W. Chan (2007). "Elucidating the Mechanism of Cellular Uptake and Removal of Protein-Coated Gold Nanoparticles of Different Sizes and Shapes." Nano Lett **7**(6): 1542-1550.

Chithrani, B. D., A. A. Ghazani, *et al.* (2006). "Determining the Size and Shape Dependence of Gold Nanoparticle Uptake into Mammalian Cells." Nano Lett **6**(4): 662-668.

Chithrani, B. D., A. A. Ghazani, *et al.* (2006). "Determining the Size and Shape Dependence of Gold Nanoparticle Uptake into Mammalian Cells." Nano letters **6**(4): 662-668.

Chithrani, D. B., S. Jelveh, *et al.* (2010). "Gold Nanoparticles as Radiation Sensitizers in Cancer Therapy." Radiation Research **173**(6): 719-728.

Chompoosor, A., G. Han, *et al.* (2008). "Charge dependence of ligand release and monolayer stability of gold nanoparticles by biogenic thiols." Bioconjug Chem **19**(7): 1342-1345.

Cliffel, D., F. Zamborini, *et al.* (2000). "Mercaptoammonium-monolayer-protected, water-soluble gold, silver, and palladium clusters." Langmuir **16**(25): 9699-9702.

Collier, C., T. Vossmeyer, *et al.* (1998). "Nanocrystal superlattices." Annual Review of Physical Chemistry **49**(1): 371-404.

Connor, E., J. Mwamuka, *et al.* (2005). "Gold nanoparticles are taken up by human cells but do not cause acute cytotoxicity." Small **1**(3): 325-327.

Connor, E. E., J. Mwamuka, *et al.* (2005). "Gold nanoparticles are taken up by human cells but do not cause acute cytotoxicity." Small **1**(3): 325-327.

da Paz, M. C., F. Santos Mde, *et al.* (2012). "Anti-CEA loaded maghemite nanoparticles as a theragnostic device for colorectal cancer." Int J Nanomedicine **7**: 5271-5282.

Danhier, F., O. Feron, *et al.* (2010). "To exploit the tumor microenvironment: Passive and active tumor targeting of nanocarriers for anti-cancer drug delivery." J Control Release **148**(2): 135-146.

Daniel, M.-C. and D. Astruc (2003). "Gold Nanoparticles: Assembly, Supramolecular Chemistry, Quantum-Size-Related Properties, and Applications toward Biology, Catalysis, and Nanotechnology." Chemical Reviews **104**(1): 293-346.

Daniel, M. and D. Astruc (2004). "Gold nanoparticles: assembly, supramolecular chemistry, quantum-size-related properties, and applications toward biology, catalysis, and nanotechnology." Chemical reviews **104**(1): 293-346.

Daou, T. J., S. Begin-Colin, *et al.* (2007). "Phosphate Adsorption Properties of Magnetite-Based Nanoparticles." Chemistry of Materials **19**(18): 4494-4505.

Dastjerdi, R., M. Montazer, *et al.* (2009). "A new method to stabilize nanoparticles on textile surfaces." Colloids and Surfaces A: Physicochemical and Engineering Aspects **345**(1–3): 202-210.

David A, S. (2003). "Plasmon resonant particles for biological detection." Current Opinion in Biotechnology **14**(1): 13-22.

Decuzzi, P., S. Lee, *et al.* (2004). "Adhesion of microfabricated particles on vascular endothelium: a parametric analysis." Annals of biomedical engineering **32**(6): 793-802.

Deng, L., X. Ke, *et al.* (2012). "A MSLN-targeted multifunctional nanoimmunoliposome for MRI and targeting therapy in pancreatic cancer." Int J Nanomedicine **7**: 5053-5065.

Desai, N. (2012). "Challenges in development of nanoparticle-based therapeutics." Aaps J **14**(2): 282-295.

Devi, J. S. and B. V. Bhimba (2012). "Silver nanoparticles: Antibacterial activity against wound isolates & invitro cytotoxic activity on Human Caucasian colon adenocarcinoma." Asian Pacific Journal of Tropical Disease **2, Supplement 1**(0): S87-S93.

Dhar, S., W. L. Daniel, *et al.* (2009). "Polyvalent Oligonucleotide Gold Nanoparticle Conjugates as Delivery Vehicles for Platinum(IV) Warheads." Journal of the American Chemical Society **131**(41): 14652-14653.

Diegoli, S., A. L. Manciulea, *et al.* (2008). "Interaction between manufactured gold nanoparticles and naturally occurring organic macromolecules." Science of The Total Environment **402**(1): 51-61.

Dizdaroglu, M. (1992). "Oxidative damage to DNA in mammalian chromatin." Mutat Res **275**(3-6): 331-342.

Dobrovolskaia, M. A., A. K. Patri, *et al.* (2009). "Interaction of colloidal gold nanoparticles with human blood: effects on particle size and analysis of plasma protein binding profiles." Nanomedicine: Nanotechnology, Biology and Medicine **5**(2): 106-117.

Dubas, S. T. and V. Pimpan (2008). "Humic acid assisted synthesis of silver nanoparticles and its application to herbicide detection." Materials Letters **62**(17–18): 2661-2663.

Dwek, R. A. (1996). "Glycobiology: Toward Understanding the Function of Sugars." Chem Rev **96**(2): 683-720.

Dwivedi, A. D. and K. Gopal (2010). "Biosynthesis of silver and gold nanoparticles using Chenopodium album leaf extract." Colloids and Surfaces A: Physicochemical and Engineering Aspects **369**(1–3): 27-33.

Dyal, A., K. Loos, *et al.* (2003). "Activity of Candida rugosa Lipase Immobilized on γ-Fe2O3 Magnetic Nanoparticles." Journal of the American Chemical Society **125**(7): 1684-1685.

El-Sayed, M. (2001). "Some interesting properties of metals confined in time and nanometer space of different shapes." Accounts of Chemical Research **34**(4): 257-264.

El-Sayed, M. A. (2001). "Some Interesting Properties of Metals Confined in Time and Nanometer Space of Different Shapes." Accounts of Chemical Research **34**(4): 257-264.

Esumi, K., T. Hosoya, *et al.* (2000). "Formation of Gold and Silver Nanoparticles in Aqueous Solution of Sugar-Persubstituted Poly(amidoamine) Dendrimers." Journal of Colloid and Interface Science **226**(2): 346-352.

Freund, P. L. and M. Spiro (1985). "Colloidal catalysis: the effect of sol size and concentration." The Journal of Physical Chemistry **89**(7): 1074-1077.

Gardea-Torresdey, J. L., J. G. Parsons, *et al.* (2002). "Formation and Growth of Au Nanoparticles inside Live Alfalfa Plants." Nano Letters **2**(4): 397-401.

Gestwicki, J. E. and L. L. Kiessling (2002). "Inter-receptor communication through arrays of bacterial chemoreceptors." Nature **415**(6867): 81-84.

Giri, J., S. Guha Thakurta, *et al.* (2005). "Preparation and characterization of phospholipid stabilized uniform sized magnetite nanoparticles." Journal of Magnetism and Magnetic Materials **293**(1): 62-68.

Gittins, D. I. and F. Caruso (2001). "Spontaneous Phase Transfer of Nanoparticulate Metals from Organic to Aqueous Media." Angewandte Chemie International Edition **40**(16): 3001-3004.

Gnanadesigan, M., M. Anand, *et al.* (2011). "Biosynthesis of silver nanoparticles by using mangrove plant extract and their potential mosquito larvicidal property." Asian Pacific Journal of Tropical Medicine **4**(10): 799-803.

Gnanaprakash, G., S. Mahadevan, *et al.* (2007). "Effect of initial pH and temperature of iron salt solutions on formation of magnetite nanoparticles." Materials Chemistry and Physics **103**(1): 168-175.

Gnanaprakash, G., J. Philip, *et al.* (2007). "Effect of Digestion Time and Alkali Addition Rate on Physical Properties of Magnetite Nanoparticles." The Journal of Physical Chemistry B **111**(28): 7978-7986.

Gopinath, V., D. MubarakAli, *et al.* (2012). "Biosynthesis of silver nanoparticles from Tribulus terrestris and its antimicrobial activity: A novel biological approach." Colloids and Surfaces B: Biointerfaces **96**(0): 69-74.

Gradishar, W. J., S. Tjulandin, *et al.* (2005). "Phase III Trial of Nanoparticle Albumin-Bound Paclitaxel Compared With Polyethylated Castor Oil–Based Paclitaxel in Women With Breast Cancer." Journal of Clinical Oncology **23**(31): 7794-7803.

Grossman, J. H. and S. E. McNeil (2012). "Nanotechnology in Cancer Medicine." Physics Today **65**(8): 38-42.

Grunwaldt, J.-D., C. Kiener, *et al.* (1999). "Preparation of Supported Gold Catalysts for Low-Temperature CO Oxidation via "Size-Controlled" Gold Colloids." Journal of Catalysis **181**(2): 223-232.

Gupta, A. and S. Silver (1998). "Molecular Genetics: Silver as a biocide: Will resistance become a problem?" Nat Biotech **16**(10): 888-888.

Gupta, A. K., C. Berry, *et al.* (2003). "Receptor-mediated targeting of magnetic nanoparticles using insulin as a surface ligand to prevent endocytosis." NanoBioscience, IEEE Transactions on **2**(4): 255-261.

Gupta, N., H. P. Singh, *et al.* (2010). "Single-pot synthesis: Plant mediated gold nanoparticles catalyzed reduction of methylene blue in presence of stannous chloride." Colloids and Surfaces A: Physicochemical and Engineering Aspects **367**(1–3): 102-107.

Hadden, W. L., R. H. Watkins, *et al.* (1999). "Carotenoid Composition of Marigold (Tagetes erecta) Flower Extract Used as Nutritional Supplement." Journal of Agricultural and Food Chemistry **47**(10): 4189-4194.

Haes, A. J. and R. P. Van Duyne (2002). "A Nanoscale Optical Biosensor: Sensitivity and Selectivity of an Approach Based on the Localized Surface Plasmon Resonance Spectroscopy of Triangular Silver Nanoparticles." Journal of the American Chemical Society **124**(35): 10596-10604.

Hainfeld, J. F., D. N. Slatkin, *et al.* (2004). "The use of gold nanoparticles to enhance radiotherapy in mice." Physics in Medicine and Biology **49**(18): N309.

Harris, S. L., P. A. Spears, *et al.* (2001). "Characterization of Escherichia coli Type 1 Pilus Mutants with Altered Binding Specificities." J. Bacteriol. **183**(13): 4099-4102.

Haynes, C. L. and R. P. Van Duyne (2001). "Nanosphere Lithography: A Versatile Nanofabrication Tool for Studies of Size-Dependent Nanoparticle Optics." The Journal of Physical Chemistry B **105**(24): 5599-5611.

He, L., M. D. Musick, *et al.* (2000). "Colloidal Au-Enhanced Surface Plasmon Resonance for Ultrasensitive Detection of DNA Hybridization." Journal of the American Chemical Society **122**(38): 9071-9077.

Heller, M. and M. J. Heller (2006). "Nanotechnology for cancer diagnostics and therapeutics." Nanomedicine: Nanotechnology, Biology and Medicine **2**(4): 301.

Hobbs, S. K., W. L. Monsky, *et al.* (1998). "Regulation of transport pathways in tumor vessels: role of tumor type and microenvironment." Proc Natl Acad Sci U S A **95**(8): 4607-4612.

Hong, R., G. Han, *et al.* (2006). "Glutathione-Mediated Delivery and Release Using Monolayer Protected Nanoparticle Carriers." Journal of the American Chemical Society **128**(4): 1078-1079.

Hu, J. J., N. Dubin, *et al.* (1995). "The effects of hydrogen peroxide on DNA repair activities." Mutation Research/DNA Repair **336**(2): 193-201.

Huh, Y.-M., Y.-w. Jun, *et al.* (2005). "In Vivo Magnetic Resonance Detection of Cancer by Using Multifunctional Magnetic Nanocrystals." Journal of the American Chemical Society **127**(35): 12387-12391.

Hung, C.-S., J. Bouckaert, *et al.* (2002). "Structural basis of tropism of Escherichia coli to the bladder during urinary tract infection." Molecular Microbiology **44**(4): 903-915.

Jacob, J., T. Mukherjee, *et al.* (2012). "A simple approach for facile synthesis of Ag, anisotropic Au and bimetallic (Ag/Au) nanoparticles using cruciferous vegetable extracts." Materials Science and Engineering: C **32**(7): 1827-1834.

Jacob, J. A., N. Biswas, *et al.* (2011). "Effect of plant-based phenol derivatives on the formation of Cu and Ag nanoparticles." Colloids and Surfaces B: Biointerfaces **87**(1): 49-53.

Jagajjanani Rao, K. and S. Paria (2013). "Green synthesis of silver nanoparticles from aqueous Aegle marmelos leaf extract." Materials Research Bulletin **48**(2): 628-634.

Jain, K. K. (2005). "Nanotechnology in clinical laboratory diagnostics." Clin Chim Acta **358**(1-2): 37-54.

Jain, T. K., M. Varshney, *et al.* (1989). "Structural studies of Aerosol OT reverse micellar aggregates by FT-IR spectroscopy." The Journal of Physical Chemistry **93**(21): 7409-7416.

Jeyaraj, M., G. Sathishkumar, *et al.* (2013). "Biogenic silver nanoparticles for cancer treatment: An experimental report." Colloids and Surfaces B: Biointerfaces **106**(0): 86-92.

Jha, A. K., K. Prasad, *et al.* (2009). "Plant system: Nature's nanofactory." Colloids and Surfaces B: Biointerfaces **73**(2): 219-223.

Jia, X., X. Ma, *et al.* (2008). "Direct formation of silver nanoparticles in cuttlebone-derived organic matrix for catalytic applications." Colloids and Surfaces A: Physicochemical and Engineering Aspects **330**(2–3): 234-240.

Jiang, W., Y. S. KimBetty, *et al.* (2008). "Nanoparticle-mediated cellular response is size-dependent." Nat Nano **3**(3): 145-150.

Joumaa, N., P. Toussay, *et al.* (2008). "Surface modification of iron oxide nanoparticles by a phosphate-based macromonomer and further encapsulation into submicrometer polystyrene particles by miniemulsion polymerization." Journal of Polymer Science Part A: Polymer Chemistry **46**(1): 327-340.

Karlsson, K., J. Angström, *et al.* (1992). "Microbial interaction with animal cell surface carbohydrates." APMIS. Supplementum **27**: 71.

Kasten, B. B., T. C. Liu, *et al.* (2013). "Targeting prostate cancer cells with PSMA inhibitor-guided gold nanoparticles." Bioorganic & Medicinal Chemistry Letters **23**(2): 565-568.

Katsnelson, B. A., L. I. Privalova, *et al.* (2013). "Comparative in vivo assessment of some adverse bioeffects of equidimensional gold and silver nanoparticles and the attenuation of nanosilver's effects with a complex of innocuous bioprotectors." Int J Mol Sci **14**(2): 2449-2483.

Kelly, K., E. Coronado, *et al.* (2003). "The optical properties of metal nanoparticles: the influence of size, shape, and dielectric environment." Journal of Physical Chemistry B-Condensed Phase **107**(3): 668-677.

Kelly, K. L., E. Coronado, *et al.* (2002). "The Optical Properties of Metal Nanoparticles: The Influence of Size, Shape, and Dielectric Environment." The Journal of Physical Chemistry B **107**(3): 668-677.

Khan, Z., O. Bashir, *et al.* (2012). "Effects of ionic surfactants on the morphology of silver nanoparticles using Paan (Piper betel) leaf petiole extract." Colloids Surf B Biointerfaces **98**: 85-90.

Khomutov, G. B. and S. P. Gubin (2002). "Interfacial synthesis of noble metal nanoparticles." Materials Science and Engineering: C **22**(2): 141-146.

Kingery-Wood, J. E., K. W. Williams, *et al.* (1992). "The agglutination of erythrocytes by influenza virus is strongly inhibited by liposomes incorporating an analog of sialyl gangliosides." Journal of the American Chemical Society **114**(18): 7303-7305.

Klein, E. A. and I. M. Thompson (2012). "Chemoprevention of prostate cancer: an updated view." World J Urol **30**(2): 189-194.

Klibanov, A. L., K. Maruyama, *et al.* (1991). "Activity of amphipathic poly(ethylene glycol) 5000 to prolong the circulation time of liposomes depends on the liposome size and is unfavorable for immunoliposome binding to target." Biochim Biophys Acta **25**(2): 142-148.

Köhler, J. M., L. Abahmane, *et al.* (2008). "Preparation of metal nanoparticles with varied composition for catalytical applications in microreactors." Chemical Engineering Science **63**(20): 5048-5055.

Kometani, N., M. Tsubonishi, *et al.* (2001). "Preparation and Optical Absorption Spectra of Dye-Coated Au, Ag, and Au/Ag Colloidal Nanoparticles in Aqueous Solutions and in Alternate Assemblies." Langmuir **17**(3): 578-580.

Koneracká, M., P. Kopčanský, *et al.* (1999). "Immobilization of proteins and enzymes to fine magnetic particles." Journal of Magnetism and Magnetic Materials **201**(1–3): 427-430.

Kossyrev, P. A., A. Yin, *et al.* (2005). "Electric Field Tuning of Plasmonic Response of Nanodot Array in Liquid Crystal Matrix." Nano Letters **5**(10): 1978-1981.

Koziara, J. M., P. R. Lockman, *et al.* (2003). "In situ blood-brain barrier transport of nanoparticles." Pharm Res **20**(11): 1772-1778.

Kreuter, J. (1996). "Nanoparticles and microparticles for drug and vaccine delivery." Journal of Anatomy **189**(3): 503-506.

Krogfelt, K. A., H. Bergmans, *et al.* (1990). "Direct evidence that the FimH protein is the mannose-specific adhesin of Escherichia coli type 1 fimbriae." Infect. Immun. **58**(6): 1995-1998.

Kruth, H. S., N. L. Jones, *et al.* (2005). "Macropinocytosis is the endocytic pathway that mediates macrophage foam cell formation with native low density lipoprotein." J Biol Chem **280**(3): 2352-2360.

Kumar, S., N. Harrison, *et al.* (2007). "Plasmonic Nanosensors for Imaging Intracellular Biomarkers in Live Cells." Nano Letters **7**(5): 1338-1343.

Kumar, V., S. C. Yadav, *et al.* (2010). "Syzygium cumini leaf and seed extract mediated biosynthesis of silver nanoparticles and their characterization." Journal of Chemical Technology & Biotechnology **85**(10): 1301-1309.

Kuntz, S., U. Wenzel, *et al.* (1999). "Comparative analysis of the effects of flavonoids on proliferation, cytotoxicity, and apoptosis in human colon cancer cell lines." European Journal of Nutrition **38**(3): 133-142.

Laurent, S., D. Forge, *et al.* (2008). "Magnetic Iron Oxide Nanoparticles: Synthesis, Stabilization, Vectorization, Physicochemical Characterizations, and Biological Applications." Chemical Reviews **108**(6): 2064-2110.

Lazarides, A. A. and G. C. Schatz (1999). "DNA-Linked Metal Nanosphere Materials: Structural Basis for the Optical Properties." The Journal of Physical Chemistry B **104**(3): 460-467.

Lee, G. Y., W. P. Qian, *et al.* (2013). "Theranostic Nanoparticles with Controlled Release of Gemcitabine for Targeted Therapy and MRI of Pancreatic Cancer." ACS Nano **12**: 12.

Lee, K., H. Lee, *et al.* (2010). "Heparin immobilized gold nanoparticles for targeted detection and apoptotic death of metastatic cancer cells." Biomaterials **31**(25): 6530-6536.

Lee, M. H., J. Y. Kim, *et al.* (2012). "Direct fluorescence monitoring of the delivery and cellular uptake of a cancer-targeted RGD peptide-appended naphthalimide theragnostic prodrug." J Am Chem Soc **134**(30): 12668-12674.

Lee, S., R. Parthasarathy, *et al.* (2001). "Recognition Properties of Antibodies to PAMAM Dendrimers and Their Use in Immune Detection of Dendrimers." Biomedical Microdevices **3**(1): 53-59.

Lee, S. C., R. Parthasarathy, *et al.* (2004). "Biochemical and immunological properties of cytokines conjugated to dendritic polymers." Biomed Microdevices **6**(3): 191-202.

Lehmann, J., A. Natarajan, *et al.* (2008). "Short communication: nanoparticle thermotherapy and external beam radiation therapy for human prostate cancer cells." Cancer Biother Radiopharm **23**(2): 265-271.

Letsinger, R. L., R. Elghanian, *et al.* (2000). "Use of a Steroid Cyclic Disulfide Anchor in Constructing Gold Nanoparticle−Oligonucleotide Conjugates." Bioconjugate Chemistry **11**(2): 289-291.

Lewin, M., N. Carlesso, *et al.* (2000). "Tat peptide-derivatized magnetic nanoparticles allow in vivo tracking and recovery of progenitor cells." Nat Biotechnol **18**(4): 410-414.

Lewinski, N., V. Colvin, *et al.* (2008). "Cytotoxicity of Nanoparticles." Small **4**(1): 26-49.

Li, P., H. M. Nielsen, *et al.* (2012). "Oral delivery of peptides and proteins using lipid-based drug delivery systems." Expert Opin Drug Deliv **9**(10): 1289-1304.

Li, W.-R., X.-B. Xie, *et al.* (2010). "Antibacterial activity and mechanism of silver nanoparticles on Escherichia coli." Applied Microbiology and Biotechnology **85**(4): 1115-1122.

Liang, M. N., S. P. Smith, *et al.* (2000). "Measuring the forces involved in polyvalent adhesion of uropathogenic Escherichia coli to mannose-presenting surfaces." Proceedings of the National Academy of Sciences of the United States of America **97**(24): 13092-13096.

Lin, C., Y. Yeh, *et al.* (2002). "Selective binding of mannose-encapsulated gold nanoparticles to type 1 pili in Escherichia coli." Journal of the American Chemical Society **124**(14): 3508-3509.

Lin, C. C., Y. C. Yeh, *et al.* (2002). "Selective binding of mannose-encapsulated gold nanoparticles to type 1 pili in Escherichia coli." J Am Chem Soc **124**(14): 3508-3509.

Lin, C. C., Y. C. Yeh, *et al.* (2003). "Quantitative analysis of multivalent interactions of carbohydrate-encapsulated gold nanoparticles with concanavalin A." Chem Commun (Camb)(23): 2920-2921.

Lin, P.-C., P.-H. Chou, *et al.* (2006). "Ethylene Glycol-Protected Magnetic Nanoparticles for a Multiplexed Immunoassay in Human Plasma." Small **2**(4): 485-489.

Lockman, P. R., M. O. Oyewumi, *et al.* (2003). "Brain uptake of thiamine-coated nanoparticles." J Control Release **93**(3): 271-282.

Longmire, M., P. L. Choyke, *et al.* (2008). "Clearance properties of nano-sized particles and molecules as imaging agents: considerations and caveats." Nanomedicine **3**(5): 703-717.

López-Cartes, C., T. C. Rojas, *et al.* (2005). "Gold Nanoparticles with Different Capping Systems: An Electronic and Structural XAS Analysis." The Journal of Physical Chemistry B **109**(18): 8761-8766.

Loris, R., D. Tielker, *et al.* (2003). "Structural Basis of Carbohydrate Recognition by the Lectin LecB from Pseudomonas aeruginosa." Journal of Molecular Biology **331**(4): 861-870.

Lu, L. T., L. D. Tung, *et al.* (2008). "Size and shape control for water-soluble magnetic cobalt nanoparticles using polymer ligands." Journal of Materials Chemistry **18**(21): 2453-2458.

Lundqvist, M., J. Stigler, *et al.* (2011). "The Evolution of the Protein Corona around Nanoparticles: A Test Study." ACS Nano **5**(9): 7503-7509.

Lyon, L. A., M. D. Musick, *et al.* (1998). "Colloidal Au-Enhanced Surface Plasmon Resonance Immunosensing." Analytical Chemistry **70**(24): 5177-5183.

Machado, S., S. L. Pinto, *et al.* (2013). "Green production of zero-valent iron nanoparticles using tree leaf extracts." Sci Total Environ **5**: 1-8.

Maenosono, S., R. Yoshida, *et al.* Evaluation of genotoxicity of amine-terminated water-dispersible FePt nanoparticles in the Ames test and in vitro chromosomal aberration test, J Toxicol Sci. 2009 Jun;34(3):349-54.

Maiorano, G., S. Sabella, *et al.* (2010). "Effects of Cell Culture Media on the Dynamic Formation of Protein−Nanoparticle Complexes and Influence on the Cellular Response." ACS Nano **4**(12): 7481-7491.

Mandeville, J. B., J. J. A. Marota, *et al.* (1998). "Dynamic functional imaging of relative cerebral blood volume during rat forepaw stimulation." Magnetic Resonance in Medicine **39**(4): 615-624.

Mann, D. A., M. Kanai, *et al.* (1998). "Probing Low Affinity and Multivalent Interactions with Surface Plasmon Resonance: Ligands for Concanavalin A." Journal of the American Chemical Society **120**(41): 10575-10582.

Martin, F. and C. Grove (2001). "Microfabricated Drug Delivery Systems: Concepts to Improve Clinical Benefit." Biomedical Microdevices **3**(2): 97-108.

Mathai Mammen, S.-K. C., George M. Whitesides, (1998). "Polyvalent Interactions in Biological Systems: Implications for Design and Use of Multivalent Ligands and Inhibitors." Angewandte Chemie International Edition **37**(20): 2754-2794.

Mayya, K. S., V. Patil, *et al.* (1997). "On the Stability of Carboxylic Acid Derivatized Gold Colloidal Particles: The Role of Colloidal Solution pH Studied by Optical Absorption Spectroscopy." Langmuir **13**(15): 3944-3947.

Mehra, R. K. and D. R. Winge (1991). "Metal ion resistance in fungi: Molecular mechanisms and their regulated expression." Journal of Cellular Biochemistry **45**(1): 30-40.

Menéndez-Manjón, A., B. N. Chichkov, *et al.* (2010). "Influence of Water Temperature on the Hydrodynamic Diameter of Gold Nanoparticles from Laser Ablation." The Journal of Physical Chemistry C **114**(6): 2499-2504.

Mirshafiee, V., M. Mahmoudi, *et al.* (2013). "Protein corona significantly reduces active targeting yield." Chem Commun (Camb) **49**(25): 2557-2559.

Mitchell, E., C. Houles, *et al.* (2002). "Structural basis for oligosaccharide-mediated adhesion of Pseudomonas aeruginosa in the lungs of cystic fibrosis patients." Nature Structural Biology **9**: 918-921.

Miura, H., K. Nishimura, *et al.* (2001). "Effects of hepatocyte growth factor on E-cadherin-mediated cell-cell adhesion in DU145 prostate cancer cells." Urology **58**(6): 1064-1069.

Mohan, J. C., G. Praveen, *et al.* (2013). "Functionalised gold nanoparticles for selective induction of in vitro apoptosis among human cancer cell lines." Journal of Experimental Nanoscience **8**(1): 32-45.

MubarakAli, D., N. Thajuddin, *et al.* (2011). "Plant extract mediated synthesis of silver and gold nanoparticles and its antibacterial activity against clinically isolated pathogens." Colloids and Surfaces B: Biointerfaces **85**(2): 360-365.

Mukerjee, A., A. P. Ranjan, *et al.* (2012). "Combinatorial nanoparticles for cancer diagnosis and therapy." Curr Med Chem **19**(22): 3714-3721.

Mukherjee, S., V. Sushma, *et al.* (2012). "Green chemistry approach for the synthesis and stabilization of biocompatible gold nanoparticles and their potential applications in cancer therapy." Nanotechnology **23**(45).

Mulvaney, P. (1996). "Surface Plasmon Spectroscopy of Nanosized Metal Particles." Langmuir **12**(3): 788-800.

Murphy, C., T. Sau, *et al.* (2005). "Anisotropic metal nanoparticles: synthesis, assembly, and optical applications." J. Phys. Chem. B **109**(29): 13857-13870.

Murphy, C. J. (2002). "MATERIALS SCIENCE: Nanocubes and Nanoboxes." Science **298**(5601): 2139-2141.

Murphy, C. J. (2008). "Sustainability as an emerging design criterion in nanoparticle synthesis and applications." Journal of Materials Chemistry **18**(19): 2173-2176.

Murray, C. B., C. R. Kagan, *et al.* (2000). "SYNTHESIS AND CHARACTERIZATION OF MONODISPERSE NANOCRYSTALS AND CLOSE-PACKED NANOCRYSTAL ASSEMBLIES." Annual Review of Materials Science **30**(1): 545-610.

Muto, H., K. Miyajima, *et al.* (2008). "Mechanism of laser-induced size reduction of gold nanoparticles as studied by single and double laser pulse excitation." Journal of Physical Chemistry C **112**(15): 5810-5815.

N.T.K. Thanh, V. F. P., L.D. Tung, D.G (2004). 70–76.

Nadine Pernodet, X. F., Yuan Sun, Asya Bakhtina, Aditi Ramakrishnan, Jonathan Sokolov, Abraham Ulman, Miriam Rafailovich, (2006). "Adverse Effects of Citrate/Gold Nanoparticles on Human Dermal Fibroblasts." Small **2**(6): 766-773.

Nam, J., N. Won, *et al.* (2009). "pH-Induced Aggregation of Gold Nanoparticles for Photothermal Cancer Therapy." Journal of the American Chemical Society **131**(38): 13639-13645.

Nam, J. M., S. I. Stoeva, *et al.* (2004). "Bio-bar-code-based DNA detection with PCR-like sensitivity." J Am Chem Soc **126**(19): 5932-5933.

Naruse, Y. (2005). "Theoretical concept of physical antibiotics using nanoparticles." Japanese Journal of Applied Physics **44**(5A): 3330-3332.

Nativo, P., I. A. Prior, *et al.* (2008). "Uptake and Intracellular Fate of Surface-Modified Gold Nanoparticles." ACS Nano **2**(8): 1639-1644.

Netti, P. A., L. T. Baxter, *et al.* (1995). "Time-dependent behavior of interstitial fluid pressure in solid tumors: implications for drug delivery." Cancer Res **55**(22): 5451-5458.

Nida, D. L., M. S. Rahman, *et al.* (2005). "Fluorescent nanocrystals for use in early cervical cancer detection." Gynecol Oncol **99**(3 Suppl 1): 1.

Nikkon, F., M. R. Habib, *et al.* (2011). "Tagetes erecta Linn. and its mosquitocidal potency against Culex quinquefasciatus." Asian Pacific Journal of Tropical Biomedicine **1**(3): 186-188.

Nikoobakht, B. and M. A. El-Sayed (2001). "Evidence for Bilayer Assembly of Cationic Surfactants on the Surface of Gold Nanorods." Langmuir **17**(20): 6368-6374.

Nune, S. K., P. Gunda, *et al.* (2009). "Nanoparticles for biomedical imaging." Expert Opinion on Drug Delivery **6**(11): 1175-1194.

Ofek I and R.J. Doyle (1994). Bacterial adhesion to cells and tissues, London: Chapman & Hall.

Ofek, I., D. Mirelman, *et al.* (1977). "Adherence of Escherichia coli to human mucosal cells mediated by mannose receptors." Nature **265**(5595): 623-625.

Oyewumi, M. O., R. A. Yokel, *et al.* (2004). "Comparison of cell uptake, biodistribution and tumor retention of folate-coated and PEG-coated gadolinium nanoparticles in tumor-bearing mice." J Control Release **95**(3): 613-626.

P.C. Chen, S.C. Mwakwari, *et al.* (2008). "Gold nanoparticles: from nanomedicine to nanosensing." Nanotech. Sci. Appl. **1**: 45–66.

Paciotti, G. F., L. Myer, *et al.* (2004). "Colloidal gold: a novel nanoparticle vector for tumor directed drug delivery." Drug Deliv **11**(3): 169-183.

Pan, Y., S. Neuss, *et al.* (2007). "Size-dependent cytotoxicity of gold nanoparticles." Small **3**(11): 1941-1949.
Parak, W. J., D. Gerion, *et al.* (2003). "Biological applications of colloidal nanocrystals." Nanotechnology **14**(7): R15.
Parak, W. J., T. Pellegrino, *et al.* (2002). "Conformation of Oligonucleotides Attached to Gold Nanocrystals Probed by Gel Electrophoresis." Nano Letters **3**(1): 33-36.
Park, J.-H., K.-H. Im, *et al.* (2005). "Preparation and characterization of magnetic chitosan particles for hyperthermia application." Journal of Magnetism and Magnetic Materials **293**(1): 328-333.
Park, J. W. (2002). "Liposome-based drug delivery in breast cancer treatment." Breast Cancer Res **4**(3): 95-99.
Park, J. W., C. C. Benz, *et al.* (2004). "Future directions of liposome- and immunoliposome-based cancer therapeutics." Semin Oncol **31**(6 Suppl 13): 196-205.
Park, M. V., A. M. Neigh, *et al.* (2011). "The effect of particle size on the cytotoxicity, inflammation, developmental toxicity and genotoxicity of silver nanoparticles." Biomaterials **32**(36): 9810-9817.
Patra, H. K., S. Banerjee, *et al.* (2007). "Cell selective response to gold nanoparticles." Nanomedicine: Nanotechnology, Biology and Medicine **3**(2): 111-119.
Paul, R., C. M. Ewing, *et al.* (1997). "The cadherin cell-cell adhesion pathway in prostate cancer progression." Br J Urol **1**: 37-43.
Philip, D. (2010). "Green synthesis of gold and silver nanoparticles using Hibiscus rosa sinensis." Physica E: Low-dimensional Systems and Nanostructures **42**(5): 1417-1424.
Philip, D. (2010). "Rapid green synthesis of spherical gold nanoparticles using Mangifera indica leaf." Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy **77**(4): 807-810.
Philip, D., C. Unni, *et al.* (2011). "Murraya Koenigii leaf-assisted rapid green synthesis of silver and gold nanoparticles." Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy **78**(2): 899-904.
Podila, R. and J. M. Brown (2013). "Toxicity of engineered nanomaterials: a physicochemical perspective." J Biochem Mol Toxicol **27**(1): 50-55.
Podila, R., P. Vedantam, *et al.* (2012). "Evidence for Charge-Transfer-Induced Conformational Changes in Carbon Nanostructure–Protein Corona." The Journal of Physical Chemistry C **116**(41): 22098-22103.
Podsiadlo, P., S. Paternel, *et al.* (2005). "Layer-by-Layer Assembly of Nacre-like Nanostructured Composites with Antimicrobial Properties." Langmuir **21**(25): 11915-11921.
Polito, L., M. Colombo, *et al.* (2008). "Resolving the structure of ligands bound to the surface of superparamagnetic iron oxide nanoparticles by high-resolution magic-angle spinning NMR spectroscopy." J Am Chem Soc **130**(38): 12712-12724.
Porter, M. D., T. B. Bright, *et al.* (1987). "Spontaneously organized molecular assemblies. 4. Structural characterization of n-alkyl thiol monolayers on gold by

optical ellipsometry, infrared spectroscopy, and electrochemistry." Journal of the American Chemical Society **109**(12): 3559-3568.

Prathna, T. C., N. Chandrasekaran, *et al.* (2011). "Biomimetic synthesis of silver nanoparticles by Citrus limon (lemon) aqueous extract and theoretical prediction of particle size." Colloids Surf B Biointerfaces **82**(1): 152-159.

Priyadarshini, S., V. Gopinath, *et al.* (2013). "Synthesis of anisotropic silver nanoparticles using novel strain, Bacillus flexus and its biomedical application." Colloids Surf B Biointerfaces **102**: 232-237.

Qu, L., L. Gu, *et al.* (2005). "Galactosylated Polymeric Nanoparticles: Synthesis and Adhesion Interactions with Escherichia coli." Journal of Biomedical Nanotechnology **1**: 61-67.

Raghunandan, D., M. D. Bedre, *et al.* (2010). "Rapid biosynthesis of irregular shaped gold nanoparticles from macerated aqueous extracellular dried clove buds (Syzygium aromaticum) solution." Colloids and Surfaces B: Biointerfaces **79**(1): 235-240.

Rai, M., A. Yadav, *et al.* (2012). Silver Nanoparticles as Nano-Antimicrobials: Bioactivity, Benefits and Bottlenecks. Nano-Antimicrobials. N. Cioffi and M. Rai, Springer Berlin Heidelberg**:** 211-224.

Rai, M., A. Yadav, *et al.* (2009). "Silver nanoparticles as a new generation of antimicrobials." Biotechnology Advances **27**(1): 76-83.

Rana, S., A. Bajaj, *et al.* (2012). "Monolayer coated gold nanoparticles for delivery applications." Advanced Drug Delivery Reviews **64**(2): 200-216.

Rideal, E. (1951). "Size and shape factor in colloidal systems. General introduction." Discussions of the Faraday Society **11**(0): 9-10.

Robinson, I., C. Alexander, *et al.* (2007). "One-step synthesis of monodisperse water-soluble 'dual-responsive' magnetic nanoparticles." Chemical Communications **0**(44): 4602-4604.

Rogach, A. L. (2000). "Nanocrystalline CdTe and CdTe(S) particles: wet chemical preparation, size-dependent optical properties and perspectives of optoelectronic applications." Materials Science and Engineering: B **69–70**(0): 435-440.

Roser, M., D. Fischer, *et al.* (1998). "Surface-modified biodegradable albumin nano- and microspheres. II: effect of surface charges on in vitro phagocytosis and biodistribution in rats." European Journal of Pharmaceutics and Biopharmaceutics **46**(3): 255-263.

Rosi, N. and C. Mirkin (2005). "Nanostructures in biodiagnostics." Chem. Rev **105**(4): 1547–1562.

Rosi, N. L. and C. A. Mirkin (2005). "Nanostructures in biodiagnostics." Chem Rev **105**(4): 1547-1562.

Sahoo, Y., H. Pizem, *et al.* (2001). "Alkyl Phosphonate/Phosphate Coating on Magnetite Nanoparticles: A Comparison with Fatty Acids." Langmuir **17**(25): 7907-7911.

Sambrook, J. and D. Russell (2001). Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press.

Sambrook, J. and D. W. Russell (2001). Molecular cloning : a laboratory manual. Cold Spring Harbor, N.Y., Cold Spring Harbor Laboratory Press.

Sanghi, R. and P. Verma (2009). "Biomimetic synthesis and characterisation of protein capped silver nanoparticles." Bioresource Technology **100**(1): 501-504.
Santhakumaran, L. M., T. Thomas, *et al.* (2004). "Enhanced cellular uptake of a triplex-forming oligonucleotide by nanoparticle formation in the presence of polypropylenimine dendrimers." Nucleic Acids Res **32**(7): 2102-2112.
Sarntinoranont, M., F. Rooney, *et al.* (2003). "Interstitial stress and fluid pressure within a growing tumor." Ann Biomed Eng **31**(3): 327-335.
Sau, T. K. and C. J. Murphy (2005). "Self-Assembly Patterns Formed upon Solvent Evaporation of Aqueous Cetyltrimethylammonium Bromide-Coated Gold Nanoparticles of Various Shapes." Langmuir **21**(7): 2923-2929.
Schaeffer, N., B. Tan, *et al.* (2008). "Fluorescent or not? Size-dependent fluorescence switching for polymer-stabilized gold clusters in the 1.1-1.7 nm size range." Chemical Communications **0**(34): 3986-3988.
Schaffler, M., M. Semmler-Behnke, *et al.* (2013). "Serum protein identification and quantification of the corona of 5, 15 and 80 nm gold nanoparticles." Nanotechnology **24**(26): 265103.
Schmitt, J., G. Decher, *et al.* (1997). "Metal nanoparticle/polymer superlattice films: Fabrication and control of layer structure." Advanced Materials **9**(1): 61-65.
Seigneuric, R., L. Markey, *et al.* (2010). "From nanotechnology to nanomedicine: applications to cancer research." Curr Mol Med **10**(7): 640-652.
Selim, M. E. and A. A. Hendi (2012). "Gold Nanoparticles Induce Apoptosis in MCF-7 Human Breast Cancer Cells." Asian Pacific Journal of Cancer Prevention **13**(4): 1617-1620.
Senarath-Yapa, M. D., S. Phimphivong, *et al.* (2007). "Preparation and Characterization of Poly(lipid)-Coated, Fluorophore-Doped Silica Nanoparticles for Biolabeling and Cellular Imaging." Langmuir **23**(25): 12624-12633.
Sergeev, G. (2003). "Cryochemistry of Metal Nanoparticles." Journal of Nanoparticle Research **5**(5-6): 529-537.
Sergeev, G. B. and T. I. Shabatina (2008). "Cryochemistry of nanometals." Colloids and Surfaces A: Physicochemical and Engineering Aspects **313–314**(0): 18-22.
Shankar, S. S., A. Ahmad, *et al.* (2003). "Bioreduction of chloroaurate ions by geranium leaves and its endophytic fungus yields gold nanoparticles of different shapes." Journal of Materials Chemistry **13**(7): 1822-1826.
Shankar, S. S., A. Rai, *et al.* (2004). "Rapid synthesis of Au, Ag, and bimetallic Au core–Ag shell nanoparticles using Neem (Azadirachta indica) leaf broth." Journal of Colloid and Interface Science **275**(2): 496-502.
Shankar, S. S., A. Rai, *et al.* (2005). "Controlling the Optical Properties of Lemongrass Extract Synthesized Gold Nanotriangles and Potential Application in Infrared-Absorbing Optical Coatings." Chemistry of Materials **17**(3): 566-572.
Shankar, S. S., A. Rai, *et al.* (2004). "Biological synthesis of triangular gold nanoprisms." Nat Mater **3**(7): 482-488.
Shao, L., Y. Gao, *et al.* (2011). "Semiconductor quantum dots for biomedicial applications." Sensors **11**(12): 11736-11751.

Sharon, N. (2006). "Carbohydrates as future anti-adhesion drugs for infectious diseases." Biochimica et Biophysica Acta (BBA) - General Subjects **1760**(4): 527-537.

Sharon, N. and H. Lis (1989). "Lectins as cell recognition molecules." Science **246**(4927): 227-234.

Sharon, N. and I. Ofek (2000). "Safe as mother's milk: Carbohydrates as future anti-adhesion drugs for bacterial diseases." Glycoconjugate Journal **17**(7): 659-664.

Sheny, D. S., J. Mathew, *et al.* (2011). "Phytosynthesis of Au, Ag and Au–Ag bimetallic nanoparticles using aqueous extract and dried leaf of Anacardium occidentale." Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy **79**(1): 254-262.

Shi, Z., K. Neoh, *et al.* (2006). "Antibacterial and mechanical properties of bone cement impregnated with chitosan nanoparticles." Biomaterials **27**(11): 2440-2449.

Shon, Y., C. Mazzitelli, *et al.* (2001). "Unsymmetrical disulfides and thiol mixtures produce different mixed monolayer-protected gold clusters." Langmuir **17**(25): 7735-7741.

Shukla, R., T. P. Thomas, *et al.* (2005). "Tumor angiogenic vasculature targeting with PAMAM dendrimer-RGD conjugates." Chem Commun (Camb)(46): 5739-5741.

Simons, B. L., P. T. J. Willemsen, *et al.* (1990). "Structure, localization and function of FanF, a minor component of K99 fibrillae of enterotoxigenic Escherichia coii." Molecular Microbiology **4**(12): 2041-2050.

Simons, L. H., P. T. J. Willemsen, *et al.* (1991). "Localization and function of FanH and FanG, minor components of K99 fimbriae of enterotoxigenic Escherichia coli." Microbial Pathogenesis **11**(5): 325-336.

Sinek, J., H. Frieboes, *et al.* (2004). "Two-dimensional chemotherapy simulations demonstrate fundamental transport and tumor response limitations involving nanoparticles." Biomed Microdevices **6**(4): 297-309.

Singh, A., R. Shukla, *et al.* (2011). "Cytotoxicity and Cellular Internalization Studies of Biogenic Gold Nanotriangles in Animal Cell Lines." International Journal of Green Nanotechnology **3**(4): 251-263.

Singh, R. P. and P. Ramarao (2012). "Cellular uptake, intracellular trafficking and cytotoxicity of silver nanoparticles." Toxicol Lett **213**(2): 249-259.

Sobhan, M. A., V. K. A. Sreenivasan, *et al.* (2012). "Non-specific internalization of laser ablated pure gold nanoparticles in pancreatic tumor cell." Colloids and Surfaces B: Biointerfaces **92**(0): 190-195.

Sokurenko, E. V., V. Chesnokova, *et al.* (1997). "Diversity of the Escherichia coli type 1 fimbrial lectin. Differential binding to mannosides and uroepithelial cells." J Biol Chem **272**(28): 17880-17886.

Sondi, I. and B. Salopek-Sondi (2004). "Silver nanoparticles as antimicrobial agent: a case study on *E. coli* as a model for Gram-negative bacteria." Journal of Colloid and Interface Science **275**(1): 177-182.

Stefanick, J. F., J. D. Ashley, *et al.* (2013). "A Systematic Analysis of Peptide Linker Length and Liposomal Polyethylene Glycol Coating on Cellular Uptake of Peptide-Targeted Liposomes." ACS Nano **19**: 19.

Stöber, W., A. Fink, *et al.* (1968). "Controlled growth of monodisperse silica spheres in the micron size range." Journal of Colloid and Interface Science **26**(1): 62-69.
Storhoff, J. J., R. Elghanian, *et al.* (1998). "One-Pot Colorimetric Differentiation of Polynucleotides with Single Base Imperfections Using Gold Nanoparticle Probes." Journal of the American Chemical Society **120**(9): 1959-1964.
Storhoff, J. J., A. A. Lazarides, *et al.* (2000). "What Controls the Optical Properties of DNA-Linked Gold Nanoparticle Assemblies?" Journal of the American Chemical Society **122**(19): 4640-4650.
Stouwdam, J. W., J. Shan, *et al.* (2006). "Photostability of Colloidal PbSe and PbSe/PbS Core/Shell Nanocrystals in Solution and in the Solid State." The Journal of Physical Chemistry C **111**(3): 1086-1092.
Stylianopoulos, T., J. D. Martin, *et al.* (2012). "Causes, consequences, and remedies for growth-induced solid stress in murine and human tumors." Proc Natl Acad Sci U S A **109**(38): 15101-15108.
Su, M., S. Li, *et al.* (2003). "Microcantilever resonance-based DNA detection with nanoparticle probes." Applied Physics Letters **82**(20): 3562-3564.
Sudimack, J. and R. J. Lee (2000). "Targeted drug delivery via the folate receptor." Advanced Drug Delivery Reviews **41**(2): 147-162.
Sun, C., J. S. H. Lee, *et al.* (2008). "Magnetic nanoparticles in MR imaging and drug delivery." Advanced Drug Delivery Reviews **60**(11): 1252-1265.
Sun, S., C. B. Murray, *et al.* (2000). "Monodisperse FePt Nanoparticles and Ferromagnetic FePt Nanocrystal Superlattices." Science **287**(5460): 1989-1992.
Svarovsky, S. A., Z. Szekely, *et al.* (2005). "Synthesis of gold nanoparticles bearing the Thomsen-Friedenreich disaccharide: a new multivalent presentation of an important tumor antigen." Tetrahedron: Asymmetry **16**(2): 587-598.
Sweeney, S. F., G. H. Woehrle, *et al.* (2006). "Rapid Purification and Size Separation of Gold Nanoparticles via Diafiltration." Journal of the American Chemical Society **128**(10): 3190-3197.
Tadmor, R., R. E. Rosensweig, *et al.* (2000). "Resolving the Puzzle of Ferrofluid Dispersants." Langmuir **16**(24): 9117-9120.
Takae, S., Y. Akiyama, *et al.* (2005). "Ligand Density Effect on Biorecognition by PEGylated Gold Nanoparticles: Regulated Interaction of RCA120 Lectin with Lactose Installed to the Distal End of Tethered PEG Strands on Gold Surface." Biomacromolecules **6**(2): 818-824.
Tao, S. L., M. W. Lubeley, *et al.* (2003). "Bioadhesive poly(methyl methacrylate) microdevices for controlled drug delivery." J Control Release **88**(2): 215-228.
Templeton, A., W. Wuelfing, *et al.* (2000). "Monolayer-protected cluster molecules." Accounts of Chemical Research **33**(1): 27-36.
Templeton, A. C., J. J. Pietron, *et al.* (2000). "Solvent Refractive Index and Core Charge Influences on the Surface Plasmon Absorbance of Alkanethiolate Monolayer-Protected Gold Clusters." The Journal of Physical Chemistry B **104**(3): 564-570.
Thakkar, K. N., S. S. Mhatre, *et al.* (2010). "Biological synthesis of metallic nanoparticles." Nanomedicine: Nanotechnology, Biology and Medicine **6**(2): 257-262.

Thanh, N. T. K. and L. A. W. Green (2010). "Functionalisation of nanoparticles for biomedical applications." Nano Today **5**(3): 213-230.
Tong, R., H. D. Hemmati, *et al.* (2012). "Photoswitchable nanoparticles for triggered tissue penetration and drug delivery." J Am Chem Soc **134**(21): 8848-8855.
Torchilin, V. P. (2008). "Cell penetrating peptide-modified pharmaceutical nanocarriers for intracellular drug and gene delivery." Peptide Science **90**(5): 604-610.
Ulman, A. (1996). "Formation and Structure of Self-Assembled Monolayers." Chemical Reviews **96**(4): 1533-1554.
V, J. J. P. a. L. (2012). "Screening of phytochemical constituents and antimicrobial activity of traditional medicinal plants." Int. J. Res. Pharm. Sci **3**(3): 461-465.
Varki, A. (1993). "Biological roles of oligosaccharides: all of the theories are correct." Glycobiology **3**: 1297 - 1340.
Varshney, M., L. Yang, *et al.* (2005). "Magnetic nanoparticle-antibody conjugates for the separation of Escherichia coli O157: H7 in ground beef." Journal of food protection **68**(9): 1804-1811.
Vedantam, P., G. Huang, *et al.* (2013). "Size-dependent cellular toxicity and uptake of commercial colloidal gold nanoparticles in DU-145 cells." Cancer Nanotechnology: 1-8.
Vedantam, P., T.-R. Tzeng, *et al.* (2012). "Binding of Escherichia coli to Functionalized Gold Nanoparticles." Plasmonics **7**(2): 301-308.
Verma, A. and F. Stellacci (2010). "Effect of Surface Properties on Nanoparticle–Cell Interactions." Small **6**(1): 12-21.
Vetrone, S. A., M. C. Huarng, *et al.* (2012). "Detection of non-PCR amplified S. enteritidis genomic DNA from food matrices using a gold-nanoparticle DNA biosensor: a proof-of-concept study." Sensors **12**(8): 10487-10499.
Vijay Singh, L. K., G.S.Chakraborthy, Avijit Mazumder (2011). "Pharmacological and Phytochemical Findings of *Tecoma stans*- A Review." JAPHR **1**(3): 75-81.
Vilchis-Nestor, A. R., V. Sánchez-Mendieta, *et al.* (2008). "Solventless synthesis and optical properties of Au and Ag nanoparticles using Camellia sinensis extract." Materials Letters **62**(17–18): 3103-3105.
Vivek, R., R. Thangam, *et al.* (2012). "Green biosynthesis of silver nanoparticles from Annona squamosa leaf extract and its in vitro cytotoxic effect on MCF-7 cells." Process Biochemistry **47**(12): 2405-2410.
Wang, C. and J. Irudayaraj (2010). "Multifunctional Magnetic–Optical Nanoparticle Probes for Simultaneous Detection, Separation, and Thermal Ablation of Multiple Pathogens." Small **6**(2): 283-289.
Wang, F., Y. Chen, *et al.* (2012). "Folate-mediated targeted and intracellular delivery of paclitaxel using a novel deoxycholic acid-O-carboxymethylated chitosan-folic acid micelles." Int J Nanomedicine **7**: 325-337.
Wang, F., Y. Zhang, *et al.* (2006). "One-pot synthesis of chitosan/LaF 3 :Eu 3+ nanocrystals for bio-applications." Nanotechnology **17**(5): 1527.
Wang, S., S. Sato, *et al.* (2003). "Preparation of Hexagonal-Close-Packed Colloidal Crystals of Hydrophilic Monodisperse Gold Nanoparticles in Bulk Aqueous Solution." Chemistry of Materials **15**(12): 2445-2448.

Wang, Y. and N. Herron (1991). "Nanometer-sized semiconductor clusters: materials synthesis, quantum size effects, and photophysical properties." The Journal of Physical Chemistry **95**(2): 525-532.

Wang, Y. and N. Herron (1996). "X-ray photoconductive nanocomposites." Science **273**(5275): 632.

Weisbecker, C. S., M. V. Merritt, *et al.* (1996). "Molecular Self-Assembly of Aliphatic Thiols on Gold Colloids." Langmuir **12**(16): 3763-3772.

Wentworth, J., F. Austin, *et al.* (1991). "Cytoplasmic lectins contribute to the adhesion of Pseudomonas aeruginosa." Biofouling **4**(1): 99-104.

Westerlund-Wikström, B. and T. K. Korhonen (2005). "Molecular structure of adhesin domains in Escherichia coli fimbriae." International Journal of Medical Microbiology **295**(6-7): 479-486.

White, M. A., J. A. Johnson, *et al.* (2006). "Toward the Syntheses of Universal Ligands for Metal Oxide Surfaces: Controlling Surface Functionality through Click Chemistry." Journal of the American Chemical Society **128**(35): 11356-11357.

Wijaya, E., C. Lenaerts, *et al.* (2011). "Surface plasmon resonance-based biosensors: From the development of different SPR structures to novel surface functionalization strategies." Current Opinion in Solid State and Materials Science **15**(5): 208-224.

Wilson, R., Z. Xiaojing, *et al.* (2009). "Gold nanoparticle sensitize radiotherapy of prostate cancer cells by regulation of the cell cycle." Nanotechnology **20**(37): 375101.

Wohlfart, S., A. S. Khalansky, *et al.* (2011). "Efficient Chemotherapy of Rat Glioblastoma Using Doxorubicin-Loaded PLGA Nanoparticles with Different Stabilizers." PLoS One **6**(5): e19121.

Wu, J., T. Akaike, *et al.* (1998). "Modulation of enhanced vascular permeability in tumors by a bradykinin antagonist, a cyclooxygenase inhibitor, and a nitric oxide scavenger." Cancer Res **58**(1): 159-165.

Wu, X., H. Liu, *et al.* (2003). "Immunofluorescent labeling of cancer marker Her2 and other cellular targets with semiconductor quantum dots." Nat Biotechnol **21**(1): 41-46.

Wu, X. R., T. T. Sun, *et al.* (1996). "In vitro binding of type 1-fimbriated Escherichia coli to uroplakins Ia and Ib: relation to urinary tract infections." Proceedings of the National Academy of Sciences of the United States of America **93**(18): 9630-9635.

Xu, C., B. Wang, *et al.* (2009). "Dumbbell-like Au−Fe3O4 Nanoparticles for Target-Specific Platin Delivery." Journal of the American Chemical Society **131**(12): 4216-4217.

Xu, C., K. Xu, *et al.* (2004). "Dopamine as A Robust Anchor to Immobilize Functional Molecules on the Iron Oxide Shell of Magnetic Nanoparticles." Journal of the American Chemical Society **126**(32): 9938-9939.

Xu, X., Q. Yang, *et al.* (2008). "Fabrication of biodegradable electrospun poly(L-lactide-co-glycolide) fibers with antimicrobial nanosilver particles." J Nanosci Nanotechnol **8**(10): 5066-5070.

Yee, C., M. Scotti, *et al.* (1999). "One-Phase Synthesis of Thiol-Functionalized Platinum Nanoparticles." Langmuir **15**(13): 4314-4316.
Yen, H. J., S. H. Hsu, *et al.* (2009). "Cytotoxicity and immunological response of gold and silver nanoparticles of different sizes." Small **5**(13): 1553-1561.
Yeo, S., H. Lee, *et al.* (2003). "Preparation of nanocomposite fibers for permanent antibacterial effect." Journal of Materials Science **38**(10): 2143-2147.
Yguerabide, J. and E. E. Yguerabide (1998). "Light-Scattering Submicroscopic Particles as Highly Fluorescent Analogs and Their Use as Tracer Labels in Clinical and Biological Applications: I. Theory." Analytical Biochemistry **262**(2): 137-156.
Yilmaz, M., H. Turkdemir, *et al.* (2011). "Biosynthesis of silver nanoparticles using leaves of Stevia rebaudiana." Materials Chemistry and Physics **130**(3): 1195-1202.
Yonezawa, T., K. Yasui, *et al.* (2000). "Controlled Formation of Smaller Gold Nanoparticles by the Use of Four-Chained Disulfide Stabilizer." Langmuir **17**(2): 271-273.
Zaki, S., M. F. El Kady, *et al.* (2011). "Biosynthesis and structural characterization of silver nanoparticles from bacterial isolates." Materials Research Bulletin **46**(10): 1571-1576.
Zarbin, M. A., C. Montemagno, *et al.* (2010). "Nanotechnology in ophthalmology." Canadian Journal of Ophthalmology / Journal Canadien d'Ophtalmologie **45**(5): 457-476.
Zhang, J., C. D. Geddes, *et al.* (2004). "Complexation of polysaccharide and monosaccharide with thiolate boronic acid capped on silver nanoparticle." Analytical Biochemistry **332**(2): 253-260.
Zhang, J., J. Tanha, *et al.* (2004). "Pentamerization of Single-domain Antibodies from Phage Libraries: A Novel Strategy for the Rapid Generation of High-avidity Antibody Reagents." Journal of Molecular Biology **335**(1): 49-56.
Zhang, X., J. Z. Xing, *et al.* (2008). "Enhanced radiation sensitivity in prostate cancer by gold-nanoparticles." Clin Invest Med **31**(3): E160-167.
Zhao, W., M. A. Brook, *et al.* (2008). "Design of Gold Nanoparticle-Based Colorimetric Biosensing Assays." ChemBioChem **9**(15): 2363-2371.
Zolnik, B. S., Á. González-Fernández, *et al.* (2010). "Minireview: Nanoparticles and the Immune System." Endocrinology **151**(2): 458-465.
Zwicke, G. L., G. A. Mansoori, *et al.* (2012). "Utilizing the folate receptor for active targeting of cancer nanotherapeutics." Nano Rev **3**(10): 7.

CHAPTER TWO

APOPTOSIS INDUCED CELL DEATH BY PROTEIN COATED GOLD AND SILVER NANOPARTICLES

Introduction

The ability to tailor and engineer nanomaterials with desired physicochemical properties has resulted in numerous applications in chemical, biological, and medical fields. Particularly, nanomaterials have shown promising solutions for cancer diagnosis, therapy, molecular targeting, drug delivery and many other biomedical applications (Thakkar, Mhatre *et al.* 2010). In view of this, the United States National Cancer Institute (NCI) launched the 'Alliance for Nanotechnology in Cancer' in 2004 to accelerate research in cancer nanotechnology and promptly achieve clinical applications. Nanoparticles (NPs) are being widely used for oxidative therapy against cancer (Kumar, Harrison *et al.* 2007). NP-based oxidative cancer therapy involves the elimination of free oxygen radicals that are involved in multistage carcinogenic process. Any oxidative DNA damage by free oxygen radicals and suppressive effects on DNA repair can propel carcinogen activation ensuing in tumor promotion (Hu, Dubin *et al.* 1995). Furthermore, oxidative damage can initiate programmed cell death or apoptosis. As a result of apoptosis, the DNA machinery is halted due to modifications produced in cell chromatin, leading to the release of apoptotic bodies.

The reactive oxygen species (ROS) can cause DNA modifications in the histones and thereby change the conformation of the DNA-binding sites. As a result, DNA molecule is further exposed to oxygen radicals that destabilize the helix leading to cell

death (Dizdaroglu 1992). It is clear that there exist a plethora of interrelated mechanisms in cancer cells, which can be exploited for employing different treatment strategies. Accordingly, various strategies such as chemotherapy, hyperthermia, and radiation therapy have been developed (Sudimack and Lee 2000). Many in vitro studies have studied the effects of noble metal nanoparticles on apoptosis in order to develop novel NP based oxidative therapeutic strategies against cancer. However, the effects of non-specific binding of proteins to NPs or NP-surface fouling on apoptosis have not yet been well investigated.

Surface fouling and subsequent formation of so-called 'protein corona' can mediate the uptake of the NPs via receptor-mediated endocytosis. The main blood plasma proteins involved (both directly and indirectly) in surface fouling are albumins, fibronectins, complement proteins, fibrinogen, immunoglobulins and apolipoprotein. Surface fouling has been found to result in phagocytosis of the nanostructure by monocytes/macrophages ultimately promoting an immune response and inflammation. In the presence of protein corona, particle-cell interaction depends on the type of coated protein(s), electronic charge, and protein-lipid interactions [10-12]. It is well known that the adsorption of proteins onto the NP surface occurs mainly due to a gain in conformational entropy (DS) due to protein unfolding. Therefore, any conformational changes that occur in the protein upon binding NPs can also cause adverse physiological response. In light of this, it is pertinent to study the effects of protein corona formation on the physiological response of NPs.

Here, the effects of protein corona formation on the physiological response of gold and silver NPs (GNPs and AgNPs) in three different cells lines breast (MCF-7), colorectal (HT-29), and prostate cancer cells (DU-145) was studied. The dependence of physicochemical properties on the formation of albumin and fetal bovine serum protein corona using UV-Visible spectrophotometry, dynamic light scattering, and gel electrophoresis was investigated. The results indicated that apoptosis is dependent on the size of the NPs. More importantly, it was seen that protein corona causes significant changes in the induction of apoptosis due to several factors such as reduced surface charge, change in protein conformation, and increased agglomeration.

Materials and methods

DU-145 cells were grown in Dulbecco's modified Eagle's 100 medium (DMEM) modified to contain Earles Balanced Salt Solution, non-essential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate, and 1,500 mg/L sodium bicarbonate. It was supplemented with fetal bovine serum to a final concentration of 10 %, 100 UI/ml penicillin G, and 100 μg/ml streptomycin and the cells were grown in a humidified incubator with 5 % at 37 °C. All the media components were purchased from Promega, USA. The HT-29 cells were grown in McCoy's medium (ATCC, USA). The human MCF-7 cells were cultured in Eagle's Minimum Essential Medium (ATCC, USA).

The commercial gold nanoparticles (GNPs): 20 and 80 nm AgNPs and 20 nm GNPs were purchased from Ted Pella Inc., USA while 80 nm GNPs were prepared using

a laser ablation technique. For ease of discussion, we labeled NPs as X-GNP/X-AgNP where X indicates the NP size. All NPs were characterized using a Hitachi H-7600 transmission electron microscope.

For studying the effects of protein coated NPs on apoptosis, all NPs were coated with Fetal Bovine Serum (FBS) and bovine serum albumin (ALB) using the following procedure. All the samples were incubated in ALB or FBS for 1 h at 37°C. Subsequently, the samples were washed by centrifuging at 10000 x g for 10 mins. The pellets containing protein coated NPs were washed thrice with nanopure water in order to remove any loosely bound proteins. Finally, all NPs were re-suspended in nanopure water. SDS-PAGE was performed for all the samples using a 4-20% gel (Biorad, USA). The gel was stained by Coomassie blue stain. A Smart Protein standard (Genscript, USA) was used to identify and analyze the presence of BSA and FBS bound to the samples. The hydrodynamic sizes of the plain NPs and protein-bound NP samples were measured using dynamic light scattering (DLS) using Malvern Zetasizer and UV-Vis spectroscopy (Biotek Synergy H2, USA).

Cell death detection ELISA assay

Apoptosis was measured using a photometric enzyme immunoassay available in the Cell Death Detection ELISA kit (Roche chemicals, USA). Briefly, the kit is an in vitro technique to measures the cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) after induced cell death. The anti-histone antibody reacts with the histones H1, H2A, H2B, H3 and H4. Addition of anti-DNA peroxidase binds to single

and double-stranded DNA. Hence, the ELISA allows the detection of mono and oligonucleosomes by measuring the optical absorbance at 405 nm. For all the studies, cells were diluted with culture medium to obtain a 1 x 105 cells/ml concentration in a 96-well plate. Samples containing the commercial plain 20 nm GNPs, 20 nm AgNPs, 80 nm GNPs and 80 AgNPs and the corresponding protein coated (FBS & ALB) NPs were added to the cells. The 96- well plates were incubated for 24 h. The cells were then centrifuged at 200 x g for 5 min. After discarding the supernatant the cell pellet was resuspended in cell culture medium.

Statistical analysis

All experiments were carried out in triplicates with results expressed as mean±standard error. Statistically significant differences were calculated using the two-tailed unpaired one-way analysis of variance (ANOVA) with p values of ≤ 0.05, <0.01, and <0.001 considered significant using Prism 5.0 (GraphPad Software, CA, USA).

Results

Characterization of NPs

As shown in Figure 2.1, the TEM images clearly showed that all the NPs exhibited spherical shape with a well-defined diameter. Further, the DLS studies indicated that the hydrodynamic size of NPs was slightly higher than the dehydrated diameter obtained from TEM. All the pristine NP suspensions exhibited high zeta

potential suggesting long-term stability (Table 2.1 and 2.2). Upon protein coating, the hydrodynamic size of all the NPs increased at least by ~10 nm. Furthermore, FBS coating resulted in a higher increase in hydrodynamic size compared to ALB possibly due to the adsorption of multiple proteins. The magnitude of zeta potential of protein coated NPs decreased considerably indicating that the NPs agglomerate more rapidly due to the formation of protein corona.

The UV-Vis absorption spectra displayed the characteristic surface plasmon resonance peak (SPR) at ~520-550 nm for GNPs and 400-420 nm for AgNPs (Figure 2.2). Interestingly, the SPR peak showed an evident upshift for all the protein coated NPs. Such an observation may be interpreted as follows. In metal NPs, the characteristic peak of SPR is dependent on the size, shape, and dielectric function (ε) of the NPs and its surrounding medium. The interaction of GNPs/AgNPs with protein molecules resulted in a further change of ε leading to an upshift in the extinction coefficient maximum and the SPR peaks.

The percentage of apoptosis caused by 20-GNPs and 80-GNPs are depicted in Figure 2.3. It was seen that 20-GNPs caused significant ($P<0.001$) apoptosis in MCF-7 and DU-145 cells when compared to HT-29 cells. The protein coated 20-GNPs in general indicated decrease in the percentage of apoptotic cells in MCF-7 cells when compared plain 20-GNPs (Figure 2.3a). On the other hand, 80-GNPs caused significant cytotoxicity in DU-145 cells compared to MCF-7 and HT-29 cells (Figure 2.3b). Significant apoptosis was characteristically higher in MCF-7 and DU-145 cells when compared to the HT-29 cells.

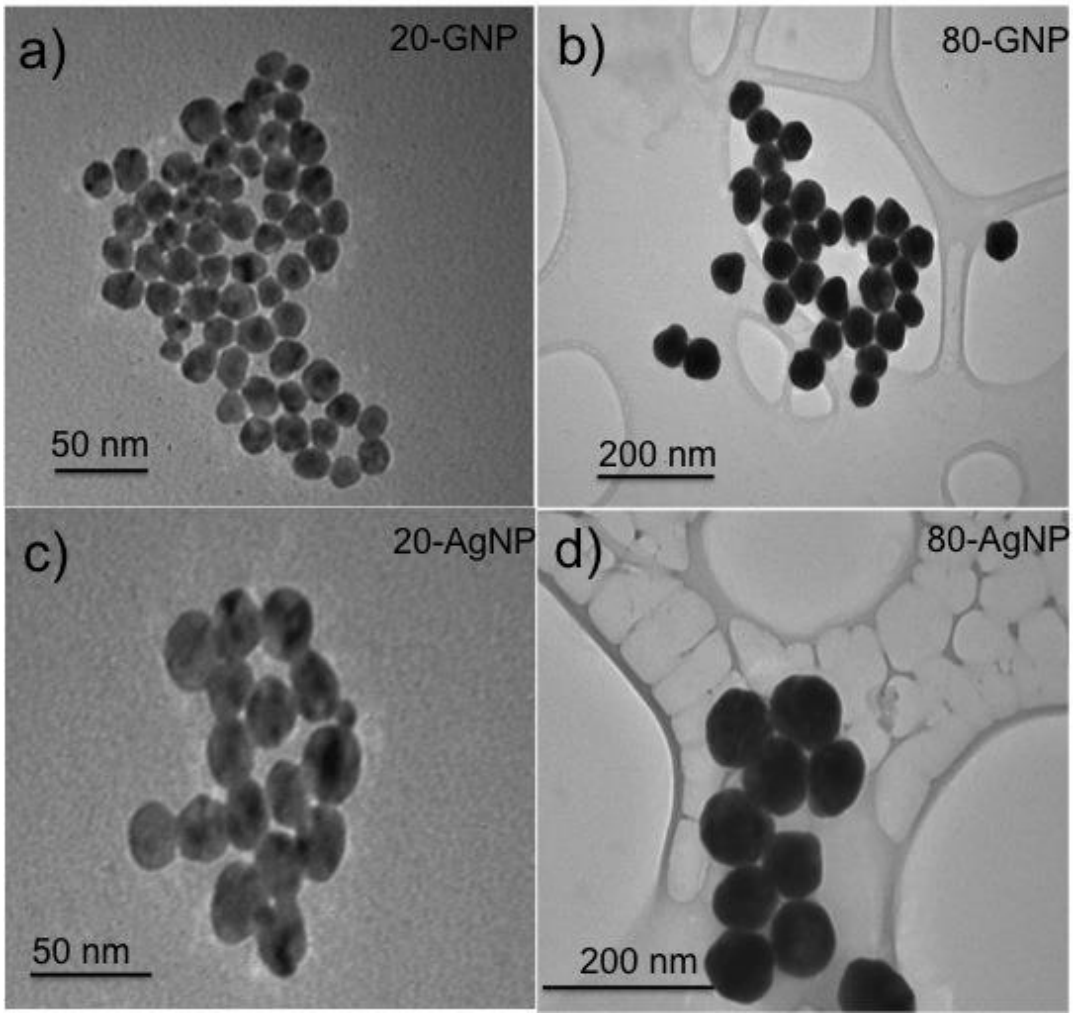

Figure 2.1. TEM micrographs of GNPs and AgNPs

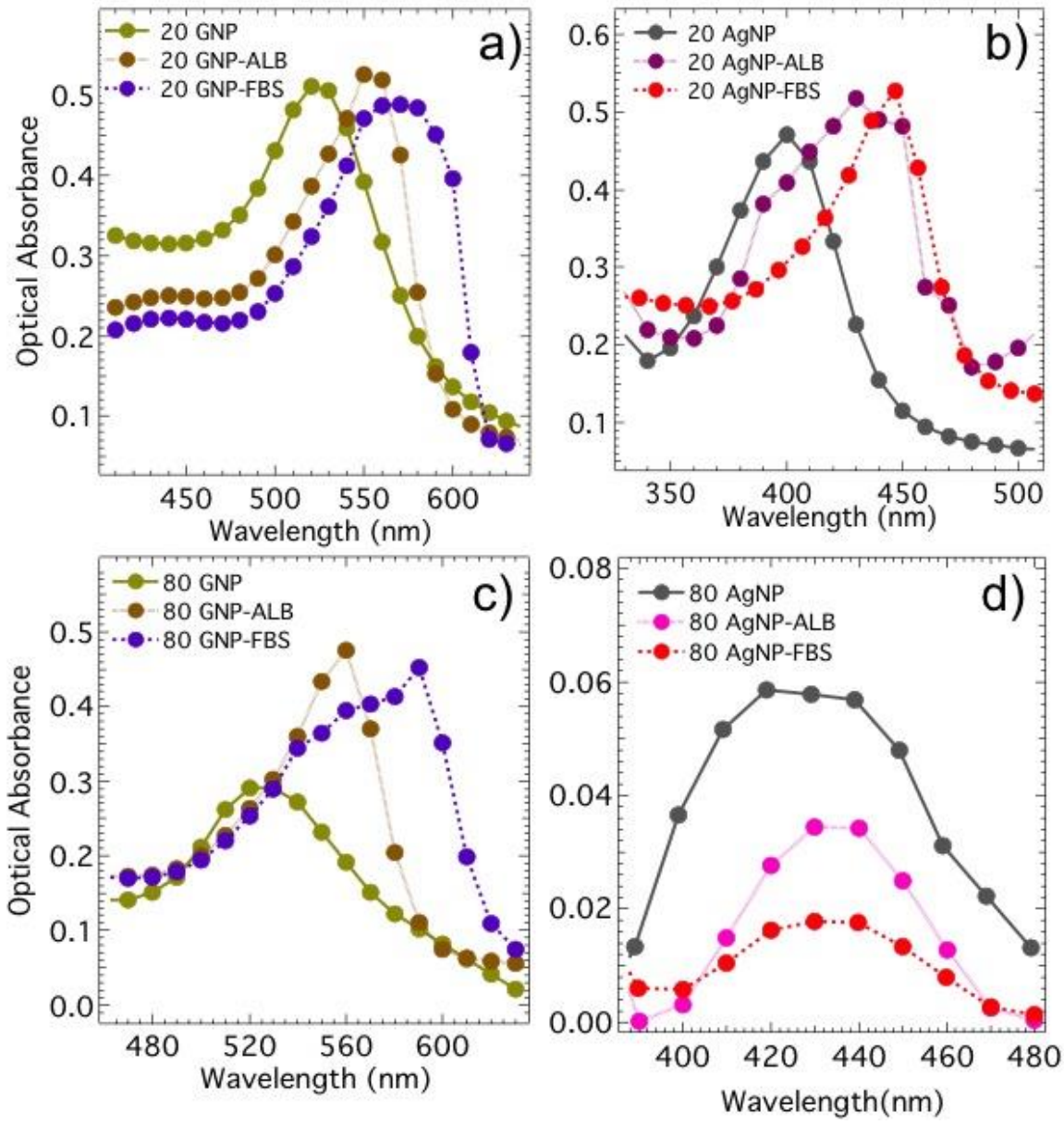

Figure 2.2. UV-Vis absorption spectra of plain, ALB and FBS bound GNPs and AgNPs

Table 2.1. DLS measurements of GNPs

Sample	Size (nm)	Zeta Potential (mV)
20 GNP	20	-54.4
20 -GNP-ALB	30	-10.5
20 -GNP-FBS	41	-9.99
80-GNP	80	-28.8
80 -GNP-ALB	99	-8.65
80 -GNP-FBS	109	-9.51

Table 2.2. DLS measurements of AgNPs

Sample	Size (nm)	Zeta Potential (mV)
20 AgNP	20	-43.2
20 -AgNP-ALB	33	-13.9
20 -AgNP-FBS	43	-10.2
80 AgNP	80	-48.45
80-AgNP-ALB	110	-14.0
80-AgNP-FBS	123	-9.59

The ALB-80-GNPs caused 20% apoptosis in MCF-7 and DU-145 cells than FBS-80-GNPs that caused significant apoptosis of about 25% in MCF-7 cells and only 8% cytotoxicity in DU-145 cells. It is interesting to note that the HT-29 cells did show significantly low apoptotic cells when treated with ALB/FBS-80-GNPs (Figure 2.3b).

Effect of protein corona on apoptosis

As shown in Figure 2.4 the 20-AgNPs and 80-AgNPs showed significant ($P<0.001$) cytotoxic effects in MCF-7 and DU-145 cells. The MCF-7 cells had significantly higher levels of apoptosis when treated with 20/80-AgNPs and as well as 20/80-ALB-AgNPs when compared to DU-145 and HT-29 cells (Figure 2.4a&b). In case of HT-29 cells, there was a significant decrease in cytotoxicity when the cells were treated with plain AgNPs and FBS-AgNPs. Interestingly the 80-AgNPs brought about higher cytotoxicity than the 20-AgNPs. On the contrary, the FBS-80-AgNPs exhibited higher apoptosis in DU-145 cells when compared to ALB-80-AgNPs (Figure 2.4b).

The plain 80-GNPs showed significant apoptotic effects in HT-29 and DU-145 cells rather than the multi-drug resistant (MDR) MCF-7 cells.

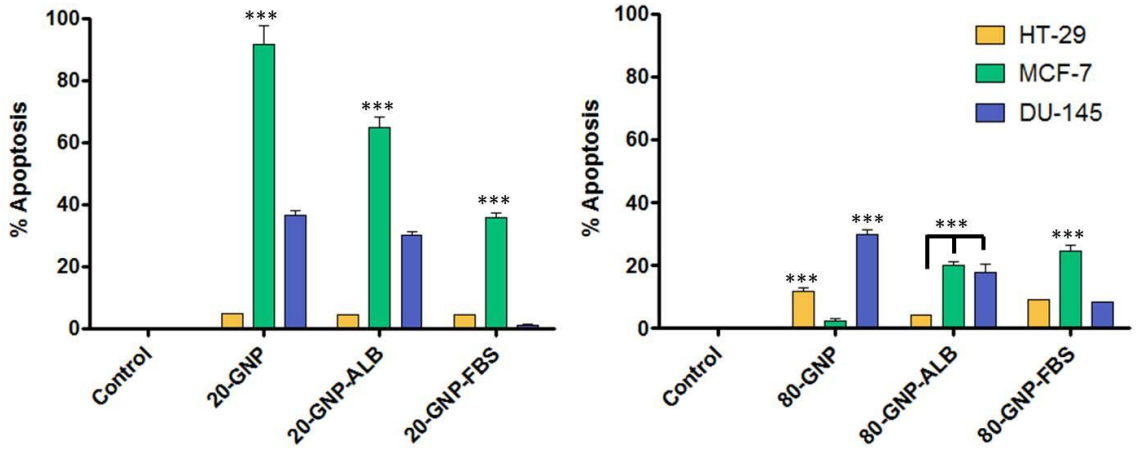

Figure 2.3. Induction of apoptosis by as measured by Cell Death Detection ELISA in the presence of GNPs

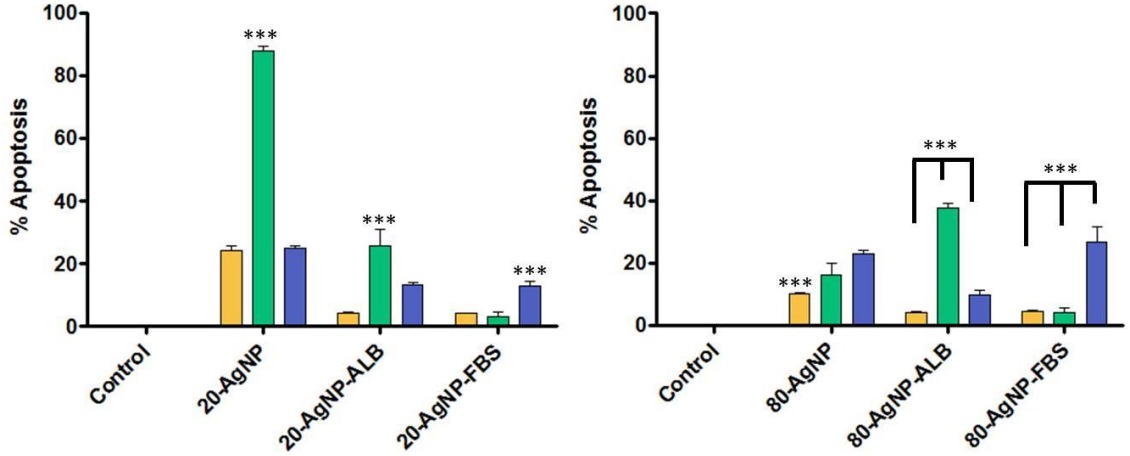

Figure 2.4. Induction of apoptosis by as measured by Cell Death Detection ELISA in the presence of AgNPs

Discussion

Nanoparticles have been shown to enter the cell and often tend to accumulate in various parts of the cellular components depending on their route of entry. Particles of 30-80 nm in particular not only enter the cellular components but also elicit endocytosis that often results in a cascading chain reaction that leads the cell to shut down its machinery (Podila and Brown 2013). The nuclear entry of mono-dispersed NPs through the nuclear pore has been demonstrated and attributed to the onset of reactive oxygen species resulting ultimately in DNA damage (Heller and Heller 2006). This DNA damage is an important factor in cancer treatment. The propensity for cancer has been linked to apoptosis, followed by chromatin condensation, pyknosis, nuclear membrane blebbing and ultimately fragmentation of cell into apoptotic bodies. The aim of this investigation was to study the stability of plain and protein coated NPs, their biocompatibility and measure the amount of DNA damage in terms of apoptosis by using a cell death detection ELISA kit.

The plain and the ALB/FBS coated NPs were found to be stable. The zeta potential values of the protein coated NPs indicated aggregation due to protein corona. The TEM micrographs confirmed spherical NPs. The DLS measurements showed an increase in the hydrodynamic size of the protein coated NPs. The cell death detection ELISA showed interesting results. The plain GNPs and AgNPs showed significant apoptosis in MCF-7 cells compared to HT-29 and DU-145 cells. The effect of protein corona showed significant apoptosis in MCF-7 and DU-145 cells. The surface chemistry of the NPs is important for nanoparticles-cell interactions. In this study we used

commercial NPs that are citrate capped and GNPs synthesized by laser ablation. Studies have shown even though citrate functionalized GNPs do not bring about reduction in MCF-7, buy they induced apoptosis in lung cancer cells.

However, the number of apoptotic cells was found to increase as the concentration of citrate capped GNPs was increased (Mohan, Praveen *et al.* 2013). Selim *et al.* (2012) showed that GNPs caused cytotoxicity by inducing significant upregulation of p53, bax, caspase-3 & caspase-9 in MCF-7 cells (Selim and Hendi 2012). On the other hand, it has been demonstrated that 20-30 nm GNPs synthesized by laser ablation were completely non-toxic to pancreatic cancer cells after 24 h of exposure. However, they were found to enter cells and reach the cytoplasm by endocytosis without any surface modification (Sobhan, Sreenivasan *et al.* 2012). Katsnelson *et al.* (2013) used nanogold and nanosilver of 50 nm prepared by laser ablation process in water to demonstrate both the particles were significantly bioactive in pulmonary phagocytosis in rats (Katsnelson, Privalova *et al.* 2013). They also demonstrated that nanosilver compared to nanogold caused enhanced toxicity and genotoxicity which was measured in histological specimens of spleen, kidney and liver of rats. Also, there is vast evidence that AgNPs can enter the systemic circulation and cause cytotoxic effects such as hepatotoxicity and induce inflammatory effects (Park, Neigh *et al.* 2011). Many studies have demonstrated that AgNPs are capable of inducing apoptosis, causing membrane damage resulting in cell death and releasing oxygen reactive species (Rani *et al.* 2009). The ability for the AgNPs to degrade internally in a cell and release Ag ions has been attributed to the ability of these particles to induce cell death. This intracellular release of Ag ions is responsible in

eliciting cytotoxic effects that cause apoptosis. Singh and Ramarao (2012) demonstrated that AgNPs were taken up by phagocytosis in murine macrophages and were found to be localized in the cytoplasm. They also reported that the AgNPs induced apoptosis because of the intracellular dissolution of Ag ions which was 50 times faster than in water (Singh and Ramarao 2012).

Many studies of targeted nuclear delivery of NPs have exploited the protein corona to determine the biokinetics of the conjugated particles in biological systems (Schaffler, Semmler-Behnke *et al.* 2013). It has been shown that smaller NPs have more proteins coated due to their large surface area. However, as size increases, less protein is bound to its surface(Mirshafiee, Mahmoudi *et al.* 2013). Not only does the protein corona mask the NP and make it inaccessible to the cell while being endocytosed it also causes NPs coated with protein to prevent agglomeration when the protein is stable and uniformly charged (Schaffler, Semmler-Behnke *et al.* 2013). The zeta potential values in this study have shown increase in net surface charge there by indicating more protein bound to the NPs. FBS compared to ALB is a complex serum consisting of multiple proteins when compared to ALB. The DLS measurements indicate that the FBS coated NPs were of relatively higher hydrodynamic size when compared to the ALB coated NPs. This could be attributed to the composition of the proteins in FBS that are also found in plasma. Capriotti *et al.* (2013) have shown that polyanionic DNA is associated to the lipoplex surface and can interact with basic plasma proteins (Capriotti, Caracciolo *et al.* 2011; Capriotti, Caracciolo *et al.* 2011; Caracciolo, Pozzi *et al.* 2011). Protein

functionalized GNPs have been successfully shown as a tunable platform for specific binding to prostate cancer cells (Kasten, Liu *et al.* 2013).

In this investigation bigger sized plain 80-GNPs did enhance toxic effects in DU-145 cells when compared to 20-GNPs and 20/80nm AgNPs. It was also seen that the percentage of apoptosis was significantly low in HT-29 cells compared to the MCF-7 and DU-145 cells when they were treated with ALB-80-GNPs. On the other hand, FBS-80-GNPs induced significantly higher levels of apoptosis in MCF-7 cells when compared to the commercial NPs. This difference in apoptosis could be attributed to the different lengths of the protein ALB and FBS coated to the NP. The protein chain that is readily accessible to the cell environment can undergo conformational changes in its secondary structures and bring about different biomolecular interactions by means of electron charge transfer and destabilize the cellular components by initiating different downstream processes.

The charge variation does not only destruct the interaction between the drugs and the carriers, but also cause the disassembly of the carriers themselves. Hence, there is a need to develop vectors that trigger the release of the drug inside the cells than outside the cells. Gene therapy not only involves delivering such a mutant that attacks the basic DNA machinery by using the cancer cells as its basis of treatment but alternatively can also be introduced in a vector to bring about a desired effect by controlled release in a tumor (Alexis, Rhee *et al.* 2008). Advances in apoptotic research have shown that protein coated NPs are less susceptible to cause cytotoxic effects in cells when compared to bare NPs (Podila, Vedantam *et al.* 2012; Vedantam, Huang *et al.* 2013). The protein

corona has served as a mask for the NPs to escape endocytic digestion, makes the NPs biocompatible due to conformational changes and enhances stability of the NP in the cancer cell, makes the NPs a good vector to enter the cells and cause desired release of drugs and lastly, brings about synergistic effects in treatment modalities (Lundqvist, Stigler *et al.* 2011; Podila and Brown 2013). The ALB/FBS 80-AgNPs interestingly caused high levels of apoptotic cells in MCF-7 and DU-145 cells than HT-29 cells. Hence, these particles can be differentially introduced in the cancer cells to bring about cell death. The 80-GNPs show promising future when used as a vector for gene therapy in a tumor. The protein coated NPs can be used as a vector for drug delivery for drugs like DOX and Palitaxel in cancer cells, which could successfully enter a cancer cell and initiate a controlled release. Therefore, not only they can be used for targeted therapy but also not trigger higher levels of apoptotic cells, which ensures the therapeutic effect in the cancer cells.

In conclusion two different kinds of NPS that were synthesized commercially and by laser ablation method were studied. The NPs were coated with ALB and FBS to study the effect of protein corona in the MCF-7, HT-29 and DU-145 cells. The increase or decrease of percentage of apoptotic cells caused by the different NPs was measured. Cell death was measured colorimetrically detecting the extent of DNA damage caused by the modifications in conformational changes in histones. It was evident that the laser ablation particles caused significantly different levels of apoptosis in the three cells lines when compared to the commercially synthesized citrate capped GNPs and AgNPs. Also the

laser ablation particles show potentially futuristic use in cancer therapy and biomedical applications by exploiting their unique chemically active surface chemistry.

Literature Cited

Alexis, F., J.-W. Rhee, *et al.* (2008). "New frontiers in nanotechnology for cancer treatment." Urologic Oncology: Seminars and Original Investigations **26**(1): 74-85.

AshaRani, P. V., G. Low Kah Mun, *et al.* (2009). "Cytotoxicity and genotoxicity of silver nanoparticles in human cells." ACS Nano **3**(2): 279-290.

Capriotti, A. L., G. Caracciolo, *et al.* (2011). "Differential analysis of "protein corona" profile adsorbed onto different nonviral gene delivery systems." Anal Biochem **419**(2): 180-189.

Capriotti, A. L., G. Caracciolo, *et al.* (2011). "DNA affects the composition of lipoplex protein corona: a proteomics approach." Proteomics **11**(16): 3349-3358.

Caracciolo, G., D. Pozzi, *et al.* (2011). "Evolution of the protein corona of lipid gene vectors as a function of plasma concentration." Langmuir **27**(24): 15048-15053.

Dizdaroglu, M. (1992). "Oxidative damage to DNA in mammalian chromatin." Mutat Res **275**(3-6): 331-342.

Heller, M. and M. J. Heller (2006). "Nanotechnology for cancer diagnostics and therapeutics." Nanomedicine: Nanotechnology, Biology and Medicine **2**(4): 301.

Hu, J. J., N. Dubin, *et al.* (1995). "The effects of hydrogen peroxide on DNA repair activities." Mutation Research/DNA Repair **336**(2): 193-201.

Kasten, B. B., T. C. Liu, *et al.* (2013). "Targeting prostate cancer cells with PSMA inhibitor-guided gold nanoparticles." Bioorganic & Medicinal Chemistry Letters **23**(2): 565-568.

Katsnelson, B. A., L. I. Privalova, *et al.* (2013). "Comparative in vivo assessment of some adverse bioeffects of equidimensional gold and silver nanoparticles and the attenuation of nanosilver's effects with a complex of innocuous bioprotectors." Int J Mol Sci **14**(2): 2449-2483.

Kumar, S., N. Harrison, *et al.* (2007). "Plasmonic Nanosensors for Imaging Intracellular Biomarkers in Live Cells." Nano Letters **7**(5): 1338-1343.

Lundqvist, M., J. Stigler, *et al.* (2011). "The Evolution of the Protein Corona around Nanoparticles: A Test Study." ACS Nano **5**(9): 7503-7509.

Mirshafiee, V., M. Mahmoudi, *et al.* (2013). "Protein corona significantly reduces active targeting yield." Chem Commun (Camb) **49**(25): 2557-2559.

Mohan, J. C., G. Praveen, *et al.* (2013). "Functionalised gold nanoparticles for selective induction of in vitro apoptosis among human cancer cell lines." Journal of Experimental Nanoscience **8**(1): 32-45.

Park, M. V., A. M. Neigh, *et al.* (2011). "The effect of particle size on the cytotoxicity, inflammation, developmental toxicity and genotoxicity of silver nanoparticles." Biomaterials **32**(36): 9810-9817.

Podila, R. and J. M. Brown (2013). "Toxicity of engineered nanomaterials: a physicochemical perspective." J Biochem Mol Toxicol **27**(1): 50-55.

Podila, R., P. Vedantam, *et al.* (2012). "Evidence for Charge-Transfer-Induced Conformational Changes in Carbon Nanostructure–Protein Corona." The Journal of Physical Chemistry C **116**(41): 22098-22103.

Reed, J. C. Mechanisms of apoptosis, Am J Pathol. 2000 Nov;157(5):1415-30.

Schaffler, M., M. Semmler-Behnke, *et al.* (2013). "Serum protein identification and quantification of the corona of 5, 15 and 80 nm gold nanoparticles." Nanotechnology **24**(26): 265103.

Selim, M. E. and A. A. Hendi (2012). "Gold Nanoparticles Induce Apoptosis in MCF-7 Human Breast Cancer Cells." Asian Pacific Journal of Cancer Prevention **13**(4): 1617-1620.

Singh, R. P. and P. Ramarao (2012). "Cellular uptake, intracellular trafficking and cytotoxicity of silver nanoparticles." Toxicol Lett **213**(2): 249-259.

Sobhan, M. A., V. K. A. Sreenivasan, *et al.* (2012). "Non-specific internalization of laser ablated pure gold nanoparticles in pancreatic tumor cell." Colloids and Surfaces B: Biointerfaces **92**(0): 190-195.

Sudimack, J. and R. J. Lee (2000). "Targeted drug delivery via the folate receptor." Advanced Drug Delivery Reviews **41**(2): 147-162.

Thakkar, K. N., S. S. Mhatre, *et al.* (2010). "Biological synthesis of metallic nanoparticles." Nanomedicine: Nanotechnology, Biology and Medicine **6**(2): 257-262.

Vedantam, P., G. Huang, *et al.* (2013). "Size-dependent cellular toxicity and uptake of commercial colloidal gold nanoparticles in DU-145 cells." Cancer Nanotechnology: 1-8.

CHAPTER THREE

SYNERGISTIC COLLUSION OF GREEN SYNTHESIS NANOPARTICLES WITH ANTIBIOTICS AS EFFECTIVE PHARMACOLOGICAL AGENTS

Introduction

The field of nanotechnology is progressing at a rapid rate due to the remarkable potential of nanomaterials for numerous applications (Mukherjee, Sushma *et al.* 2012). The ability to manufacture a wide variety of nanomaterials presents us with vast opportunities for bio-medical applications such as drug and gene delivery (Nune, Gunda *et al.* 2009). Currently, wet chemical synthesis techniques are being widely used for producing metal nanoparticles mainly due to their simplicity, high growth rate and throughput. These chemical methods extensively employ hazardous organic reagents that can result in adverse biological response in living organisms, and the environment at large. Therefore, researchers have begun to replace harsh chemical synthetic procedures with clean, non-toxic and environmentally acceptable ‘‘green chemistry’’ methods. In green synthesis methods, NPs are synthesized using an environmentally benign solvent, eco-friendly reducing and natural capping agents. In the last five years, many researchers have utilized extracts from biological systems such as microorganisms and plants to produce metal NPs. Importantly, the use of plant biomass and extracts has gained much popularity over other biological green methods due to its simplicity, ready scalability, and low reaction time.

Thus far, green extracts from neem leaf, geranium, lemongrass, tamarind, Aloe vera and many other plants have been used to synthesize noble metal NPs with varying shapes, sizes, and core-shell structure. These green extracts are known to contain such phytochemicals as terpenoids, flavonoids, phenol derivatives, di-hydric phenols that can mimic reducing and capping agents (Jha, Prasad *et al.* 2009; Ahmad, Sharma *et al.* 2010; Kumar, Yadav *et al.* 2010). The main advantage of green synthesis NPs is that there is no need for artificial chemical stabilizing agents, external heat or any other active process. For example, Au NPs synthesized using wet chemical method often use heat, stirring, and citrate functional groups for producing stable aqueous suspension. In contrast, green synthesis NPs are naturally coated with phytochemicals that lend them high surface charge required for achieving a stable aqueous suspension. The presence of such phytochemicals on the NP surface could then be used to achieve antimicrobial action from NPs. While there has been much focus on utilizing NPs directly as antimicrobial agents, a concomitant need for highly efficient, broad-spectrum drugs demands a NP-biomedical drug composite that can treat multi-drug resistant bacteria (Jagajjanani Rao and Paria 2013). To this end, we prepared NP-drug composites and investigated the synergistic effects of noble metal NPs on the antimicrobial activity of traditional drugs.

Biogenic Ag and Au NPs were synthesized using the floral extract of *Tecoma stans* and *Tagetes erectus*. *Tecoma stans* has been known for its anti-inflammatory, antimicrobial, antifungal, antispasmodic and cytotoxic activities and has been considered as a potent curative for diabetes mellitus (Vijay Singh 2011; V 2012). On the other hand *Tagetes erecta* has been used in the treatment of eye diseases, rheumatism, cough,

bleeding piles, and ulcers and is known for its insecticidal properties. The phytochemical content of these flowers is known for its potent antimicrobial and cytotoxic activity and has been found to be high in antioxidant activity (Hadden, Watkins *et al.* 1999; Bashir and Gilani 2008; Basavaraj V Chivde 2011; Nikkon, Habib *et al.* 2011).

This study utilizes green synthesis noble metal NPs to ameliorate the strength of traditional such antimicrobial drugs as Vancomycin, tetracycline and ampicillin. Interestingly, it was observed that the biogenic NPs act synergistically with the standard drugs albeit their inherent low antibacterial activity. Synergistic effects in terms of drug-NP interactions and NP catalysis was observed. Results suggest that the biogenic NP-drug composite could open new avenues for the development of drugs for multi-drug resistant bacteria.

Materials and methods

Silver nitrate (AgNO3) $_3$and Chloroauric acid ($HAuCl_4.3H_2O$) were procured from Sigma-Aldrich chemicals and were used without any further purification. Fully blossomed flowers of *Tecoma stans* and *Tagetes erecta* were collected from the university garden (Figure 3.1).

Figure 3.1. Floral images of a) *Tagetes erecta* and b) *Tecoma stans*

Preparation of the floral extract

In the first step, flowers of *Tecoma stans* and *Tagetes erecta* were collected, washed and cleansed with de-ionized water. One gram of the flower petals were added to 10 ml of millipore water and the mixture was refluxed for 10 minutes at room temperature. The mixture was then filtered using a macro porous Whatman filter paper. Subsequently, the extract was centrifuged at 4500-5000 rpm for 15 min and the supernatant was used for synthesis of silver and gold nanoparticles. The extract was used within 5 hours of its preparation, to maintain its activity and for the reproducibility of the results.

Bio-synthesis of Ag and Au nanoparticles using the extracts

Various amounts (0.5 ml - 2.5 ml) of floral extracts were added to 2.5 ml (2mM) $AgNO_3$ solution. In all our experiments, the total volume of the solutions was maintained at 5 ml by adding millipore water. The solution was then allowed to stand quiescently for 24 hrs. After 24 hrs, the solution was centrifuged at 6000 rpm for 10 min and the supernatant containing extract was removed and, the pellet was re-suspended in 3 ml millipore water. This process was repeated three times. In the final step, the pellet was re-suspended in 1 ml of millipore water to get a relatively more concentrated and pure colloidal solution of silver nanoparticles. For the synthesis of gold nanoparticles, the same procedure was followed but with $HAuCl_4.3H_2O$.

Characterization of nanoparticles

AgNPs and GNPs were characterized using transmission electron microscopy, UV-Vis spectroscopy and dynamic light scattering measurements. All UV spectra were recorded on a Shimadzu 2450 PC UV-Vis spectrophotometer. The hydrodynamic size and zeta potential were determined by using Brookhaven Particle Analyzer 90 instrument. The shape and size distribution were determined using transmission electron microscope - Hitachi H 7600 instrument operated at an accelerating voltage of 75-120 kV. The nanoparticles obtained from the *Tecoma stans* was referred as sample AgNP-TS and GNP-TS. The nanoparticles obtained from *Tagetes erecta* was labeled as AgNP-TE and GNP-TE respectively.

Antimicrobial activity

The microorganisms *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* and *Escherichia coli* DH5α SK+ were used for the investigation of anti-microbial activity. Vancomycin, Ampicillin and Tetracycline were the standard antibiotics of choice. The zones of inhibition (ZOI) were obtained using disk diffusion assay (DDA). For DDA, overnight cultures of bacterial strains *E. faecalis*, *S. aureus* ATCC 25923 and *E. coli* DH5α SK+ were washed with sterile saline. The optical density was adjusted to 0.122 (1×10^7 cells/ml). Spread plating of a 100 µl aliquot of the cultures was performed on Tryptic Soy Agar plates (TSA). After plating the three bacteria, antibiotic discs TE (Tetracycline 30 µg/ml), VA (Vancomycin 30 µg/ml) and AM (Ampicillin 10 µg/ml) were dipped in the respective extracts and placed on the TSA plates. In addition to that, a

plain disc dipped in plain extract (E) containing nanoparticles (Au and Ag) was placed on the plates. The plates were incubated at 37°C overnight. The zones of inhibition were measured and compared to the TSA plate containing the antibiotic discs that were not dipped in the extract which served as a standard. The diameter of the commercial antibiotic discs (6 mm) was nullified from the measured zones of inhibition. This way the synergistic effect of the extract on the antibiotic discs on the three different pathogens was analyzed.

Cytotoxic effects of green synthetic particles

The cytotoxic effect of the samples on human colorectal adenocarcinoma cells (HT-29) and human breast cancer cells (MCF-7) cell lines was investigated. The cell lines were purchased from ATCC, USA. The cells were cultured in McCoy's medium (HT-29) and Eagle's modified medium (MCF-7) procured from ATCC, USA. The cell lines were maintained in a humidified incubator with 5 % at 37 °C.

Both the cells lines were seeded (1×10^5 cells/well) into a 96-well plate and incubated at 37°C overnight to allow the cells to adhere to the 96-well plate. Both the cell lines were exposed to the GNP-TS/TE and AgNP-TS/TE at 1:4 ratios. The plates were further incubated for 24 h at 37°C. After exposure of the nanoparticles to the two difference cell lines, the membrane cytotoxicity/damage was determined by the release of lactate dehydrogenase (LDH) from cells with a damaged membrane using the CytoTox-ONE™ Homogeneous Membrane Integrity Assay kit from Promega, USA. Briefly, the cytotoxicity of the particles is measured by amount of leakage of cell components from

cytoplasm into the surrounding culture medium, indicating the number of nonviable cells. The assay essentially measures the release of LDH into the culture medium due to the enzymatic coupling that converts the substrate resazurin to resorufin. This rapid assay hence, directly measures the LDH released in assay wells without damaging the healthy cells.

Statistical analysis

All experiments were carried out in triplicates with results expressed as mean±standard error. Statistically significant differences were calculated one-way analysis of variance (ANOVA) with p values of ≤ 0.05 and <0.001 considered significant using Prism 5.0 (GraphPad Software, CA, USA).

Results and discussion

Characterization of biogenic nanoparticles

Figure 3.2 shows the time evolution of surface plasmon resonance (SPR) peak from AgNPs (or GNPs) formed during the reaction between $AgNO_3$ (or $HAuCl_4$) in the presence of *Tecoma stans* and *Tagetes erecta* extracts. A clear surface plasmon resonance (SPR) peak at 430 nm (for AgNPs) and 540 nm (for GNPs) suggests that the salts are effectively reduced by the floral extracts to form AgNPs and GNPs. As evident from Figure 3.2, the SPR peak intensity saturated within 90 mins implying the completion of reaction or formation of NPs. Clearly, the SPR intensity increases very rapidly for GNPs compared to AgNPs for both the extracts. Such an observation concurs with the

difference in the reduction potential of Au and Ag. The reduction of Ag salts is expected to be more difficult and slower than Au NPs due to their lower reduction potential (Ag^+/Ag^0 = 0.80 V and Au^{3+}/Au^0 = 1.50 V versus SCE).

The detailed electron microscopy studies revealed that both Ag and GNPs are spherical in shape with a size ~30-40 nm (Figure 3.3). However, the light scattering experiments showed that the NPs agglomerated with mean sizes larger than the dehydrated size, as indicated by TEM (see Table 3.1). This could be possibly attributed low zeta potential arising from the lack of capping agents in our synthesis procedure. Nonetheless, we observed that the AgNPs and GNPs from both the extracts formed relatively stable aqueous suspension due to the presence of flavonones and terpenoids on the surface of NPs. In the absence of other strong ligating agents, flavonones or terpenoids could be adsorbed on the surface of NPs possibly by interaction through carbonyl groups or π –electrons.

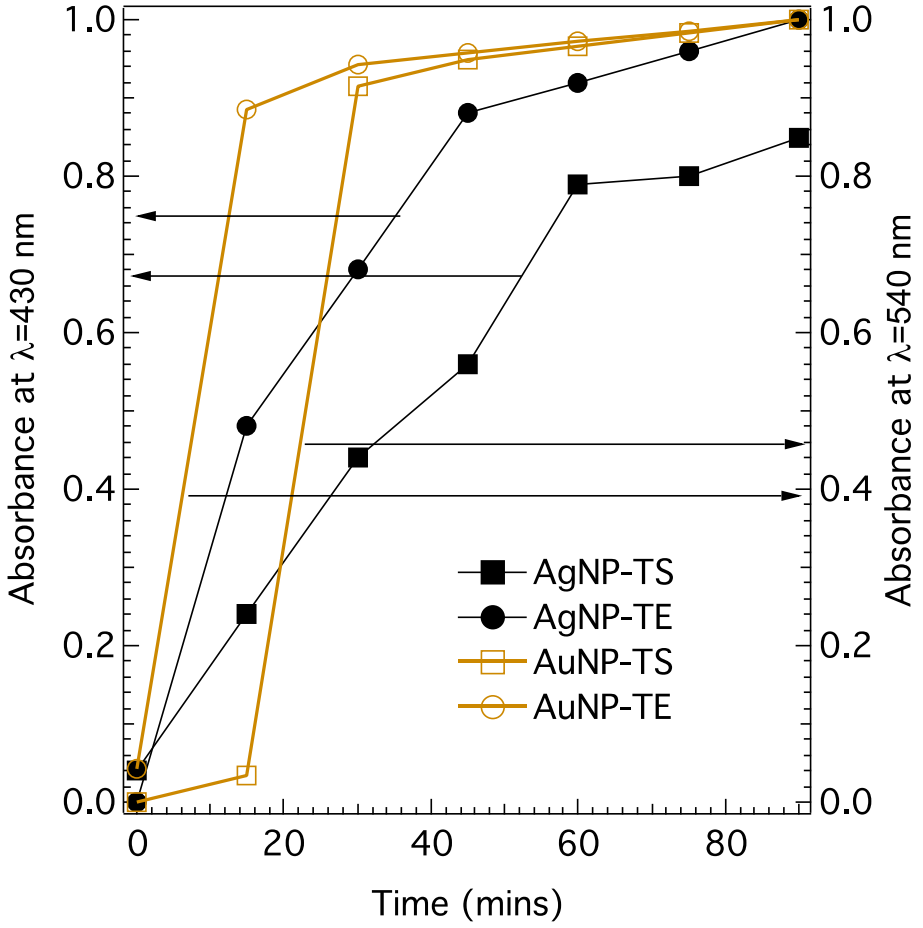

Figure 3.2. The time evolution of surface plasmon resonance peak during the formation of AgNP and GNP in the presence of *Tacoma stans* and *Tagetus erecta*.

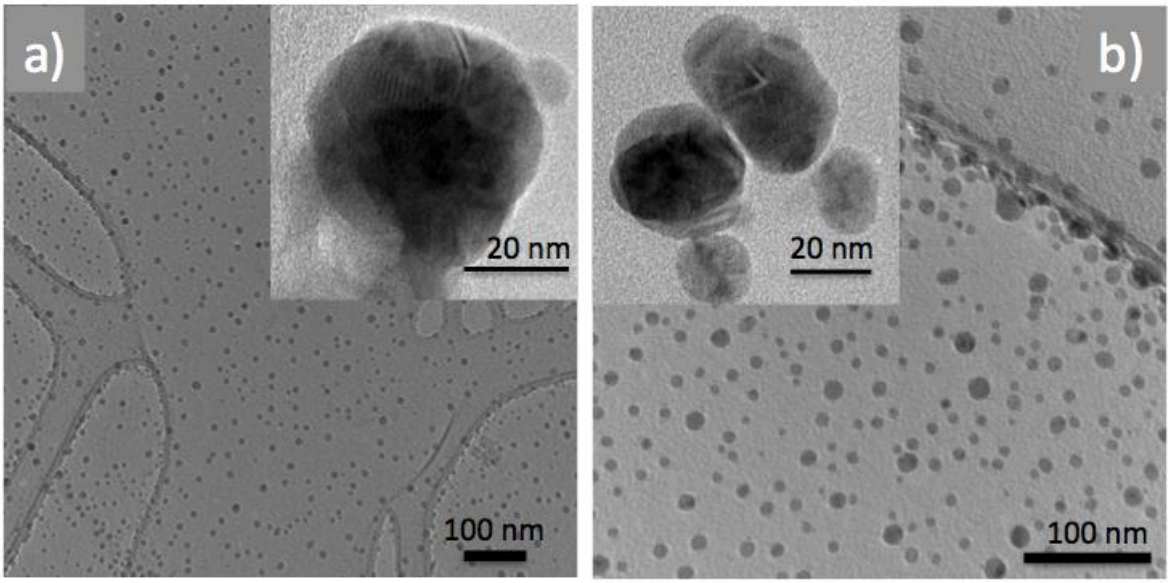

Figure 3.3: Typical transmission electron micrograph of AgNPs prepared using *Tacoma Stans* (a) and GNPs prepared using *Tagetus erecta* (b) show that NPs exhibited spherical shape with a mean size ~30-40 nm. The inset shows lattice planes of individual NPs suggesting that the samples are highly crystalline.

.

Table 3.1 Hydrodynamic size distribution and zeta-potentials of biogenic nanoparticles

Flower extract	AgNPs		AuNPs	
	Size (nm)	Zeta potential (mV)	Size (nm)	Zeta- potential (mV)
Tecoma stans	80.4	-18.3	77.7	-31.1
Tagetes erecta	80.9	-30.1	75.8	-13.9

Antimicrobial activity

The zones of inhibition (ZOI) in the disc diffusion assay for the three select pathogenic bacteria with respect to AgNPs and GNPs synthesized from both floral extracts are shown in Figure 3.4. In case of AgNP- TS, there was a significant increase ($p<0.001$) in the ZOI for AE (Ampicillin dipped in extract) against *E. coli* when compared to the standard Ampicllin. The same trend was also observed in case of AgNP-TE. A significant increase ($p<0.001$) in ZOI by was also observed in case of TE (Tetracycline dipped in extract) against *E. faecalis* when compared to the standard antibiotic The AgNPs did not however show significant increase in ZOIs in case of other treatments against the select pathogenic microorganisms.

The GNP-TS showed significant increase ($p<0.001$) in ZOI for Vancomycin disc dipped in extract only against *E. coli* whereas a significant increase ($p<0.001$) in ZOI for Tetracycline dipped in extract against *E. faecalis* was observed when compared to the ZOI of the plain antibiotic disc. The same trend was also observed with respect to GNP-TS (Figure 3.4). Interestingly, GNP-TE showed significant increase ($p<0.001$) in the ZOI for AE (Ampicillin dipped in extract) against *E. coli* when compared to GNP-TS.

On the whole, there was a very small ZOI by the plain extract against the three pathogens. The extracts containing AgNPs and GNPs did enhance the antimicrobial activity of the antibiotic by increase of zones of inhibition by different percentages against the select pathogens. There seems to be a synergistic effect that brings about increase in the diameter of ZOIs against the three pathogens. On the contrary it was also

observed that in some instances the antibiotic discs dipped in extract showed significant decrease in ZOI when compared to the standard antibiotics.

As mentioned earlier, three standard antibiotic discs (Vancomycin, Tetracycline and Ampicillin) to test the synergistic effects of biogenic nanoparticles. Vancomycin and Ampicillin drugs inhibit the proper cell wall synthesis of bacteria. However, the mechanism of action in Vancomycin limits it to be effective only in gram-positive bacteria while Ampicillin is an effective for both gram-positive and negative bacteria. Tetracycline is a broad-spectrum antibiotic that hampers bacterial cell growth by inhibiting the translational process. For example studies have shown that biosynthetic AgNPs from *Hypnea sp*. are more bactericidal against gram-positive (*S. aureus*) and gram-negative bacteria (*E. coli*) isolated from wound specimens. (Devi and Bhimba 2012). AgNP-TS extract by itself had antimicrobial effects against the three pathogens whereas the GNP-TS extract did not have any effect at all (Figure 3.4). The AgNP-TE extract exhibited antimicrobial activity only against *E. coli* and *S. aureus* and not against *E. faecalis*. The GNP-TE extract did not show any antimicrobial activity against all the three pathogens.

The extract exhibited antagonist and synergistic effects along with the antibiotic discs against the three pathogens. The AgNP-TS/TE showed the antibiotic effect was enhanced in case of Tetracycline and Ampicillin whereas antagonistic effect was seen in case of Vancomycin when used with extract (Figure 3.5). In case of the GNP-TS the antibiotic effect was enhanced. However, decrease of antimicrobial activity was observed in case of Vancomycin and Tetracycline against *E. coli* and *E. faecalis*. Interestingly, no

zone of inhibition was observed in case of Ampicillin against *E. coli* but there was a zone of inhibition against *E. coli* with Ampicillin disc dipped in the GNP-TE extract. This phenomenon is indicative of the fact that there is some sort of interference in the mechanism of action of the antibiotic disc against the pathogens in presence of the extract that synergistically enhances or antagonistically decreases the activity of the drug. The synergistic effects of the extracts containing nanoparticles could be due to the presence of the various phytochemicals. Overall bio-synthetic nanoparticles hence, showed anti-bacterial activity against gram positive bacteria (*E. faecalis*, *S. aureus* ATCC 25923) as well as gram negative bacteria (*E. coli*).

In case of *Tagetes erecta*, the anti-bacterial activity may be due to the presence of flavanoids and terpenoids (Basavaraj V Chivde 2011). The antimicrobial activity of extract of *Tecoma stans* can be attributed to the presence of alkaloids, phenols and tannins that are high in phenol content (Vijay Singh 2011; V 2012).

Cytotoxicity of biogenic nanoparticles

The AgNPs and GNPs from the two different flowers did not show any significant cytotoxicity against HT-29 cells. However, AgNP-TE showed significant cytotoxicity ($P < 0.001$) when compared to other samples (Figure 3.5). Interestingly, AgNP-TS significant ($P < 0.05$) cytotoxicity when compared to the GNP-TS extracted from the same flower. On the whole there was no significant cytotoxicity from the particles from both flowers against HT-29 cells, exhibited significant membrane damage in MCF-7 cells. Such anti-cancer effects can be used to develop nano drug carriers for treatment of

specific cancer cells. Hence, the GNP-TS/TE did not show any significant membrane damage in HT-29 cells. Whereas, the AgNP-TS did bring about significant cytotoxicity in MCF-7 cells when compared to the GNP-TS from the same extract of *Tecoma stans*. It is however necessary to purify the sample further and quantify the number of nanoparticles per ml, which will shed more light on the antimicrobial activity and cytotoxicity. Since the biosynthesis did not involve chemicals, the particles could be further purified and potentially used in medical and biomedical applications.

In addition to that it has been shown that flavonoids of the flavone, flavonol, flavanone, and isoflavone classes exhibit anti-proliferative effects in HT-29 and various cancer cell lines. The chemical composition in most cases dictates the mechanism of induction of apoptosis (Kuntz, Wenzel *et al.* 1999). Singh *et al.* have shown that the biogenic GNPs synthesized from lemon grass can be internalized by cells and reach the cytoplasm (Singh, Shukla *et al.* 2011).

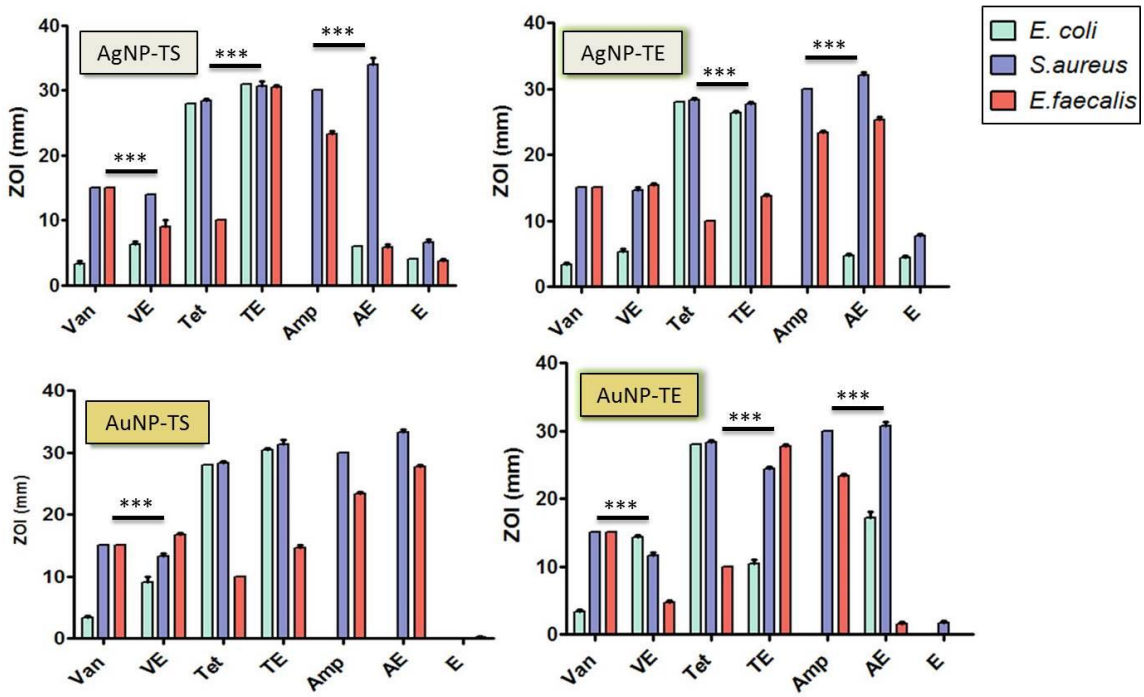

Figure. 3.4 Histograms showing the Zones of Inhibition against pathogenic bacteria in the presence of antibiotics loaded with AgNPs and GNPs synthesized using the floral extracts of *Tecoma stans* and *Tagetes erecta*

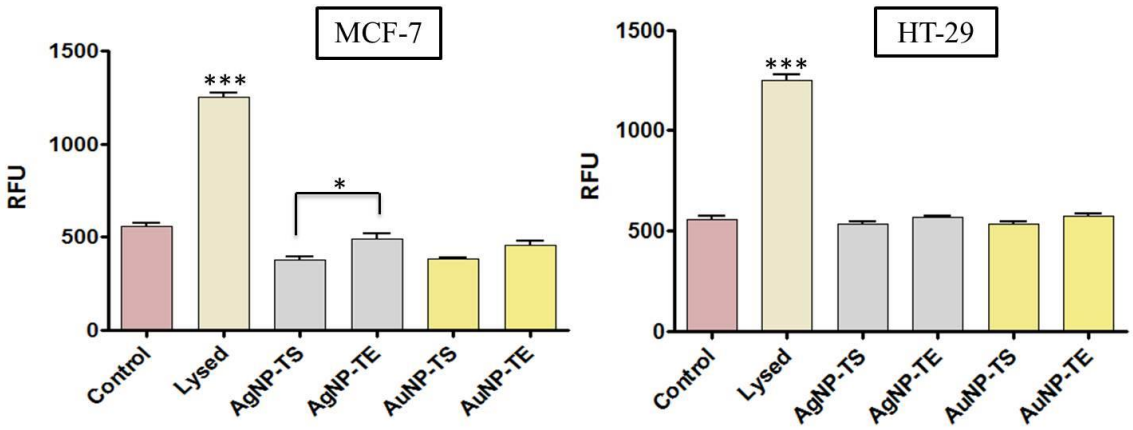

Figure. 3.5 Effect of membrane damage by biogenic nanoparticles against a) HT-29 cells b) MCF-7 cells *p<0.05, ***p<0.001

It is interesting to note that they had biogenic nanotriangles unlike mono-dispersed spherical particles. It not only shows that the nanoparticles synthesized from biosynthesis methods not only can be used as drug carriers and vectors but they are also biocompatible. In a recent study it was seen that biogenic AgNPs showed cytotoxic effects against MCF-7 cells by bringing about an induction of apoptosis and cell membrane integrity (Jeyaraj, Sathishkumar *et al.* 2013). Devi *et al.* have shown significant cytotoxicity against HT-29 cells by biogenic AgNPs synthesized from attributing to the presence of alkaloids present in extract Hypnea sp. (Devi and Bhimba 2012).

In order to cause cytotoxic effects the surface particles properties play a crucial role in dictating the fate of the biogenic particles in biological systems. GNPs have found to exhibit low core toxicity in general. The surface monolayer decides the ability to parametrically control the particle's surface properties which can be used as efficient vectors in biomedicine (Rana, Bajaj *et al.* 2012).

Theoretically the diameter calculated from the TEM images indicates the diameter of the particle in stable position and only of the inorganic core of the particle. Hence, the hydrodynamic size on the other hand is bigger. However, it is the hydrodynamic size which is an important parameter that also determines the biological activity of the particle in in vitro systems. But since the particles are not completely mono-dispersed and uniform in nature it will be interesting to investigate further more in optimizing size-dependent synthesis that will result in more defined particles that can be of significant use in biological applications. The antimicrobial properties of the particles however show

promising results suggesting that if controlled size-dependent synthesis of these particles can be achieved then they can be used for specific antimicrobial applications.

In summary, silver and gold nanoparticles on time scales faster than the conventional methods using the floral extracts of *Tecoma stans* and *Tagetes erecta* were synthesized. The nanoparticles obtained were largely spherical and mono-disperse with diameters in the range of ~25nm. Importantly, the nanoparticles can be tuned to the desired geometry by altering the extract concentration. Furthermore, these bio-genic nanoparticles were used to test their antimicrobial activity against pathogenic organisms *S. aureus*, *E. faecalis* and *E.coli*. The enhanced antimicrobial activity can largely be attributed due to the bio-genic nature and catalytic properties of the extract. Even though the particles did not show pronounced cytotoxic effects against HT-29 cells, the extracts were found to have significant effect in reduction of MCF-7.

Literature Cited

Ahmad, N., S. Sharma, *et al.* (2010). "Rapid synthesis of silver nanoparticles using dried medicinal plant of basil." Colloids Surf B Biointerfaces **81**(1): 81-86.

Basavaraj V Chivde, K. V. B., Rajabhau (2011). "ANXIOLYTIC ACTIVITY OF TABERNAEMONTANA DIVARICATA (LINN) R. Br. FLOWERS EXTRACT IN MICE." International Journal of Pharma and Bio Sciences **2**(3): 65-72.

Bashir, S. and A. H. Gilani (2008). "Studies on the antioxidant and analgesic activities of Aztec marigold (Tagetes erecta) flowers." Phytotherapy Research **22**(12): 1692-1694.

Devi, J. S. and B. V. Bhimba (2012). "Silver nanoparticles: Antibacterial activity against wound isolates & invitro cytotoxic activity on Human Caucasian colon adenocarcinoma." Asian Pacific Journal of Tropical Disease **2, Supplement 1**(0): S87-S93.

Hadden, W. L., R. H. Watkins, *et al.* (1999). "Carotenoid Composition of Marigold (Tagetes erecta) Flower Extract Used as Nutritional Supplement." Journal of Agricultural and Food Chemistry **47**(10): 4189-4194.

Jacob, J. A., N. Biswas, *et al.* (2011). "Effect of plant-based phenol derivatives on the formation of Cu and Ag nanoparticles." Colloids and Surfaces B: Biointerfaces **87**(1): 49-53.

Jagajjanani Rao, K. and S. Paria (2013). "Green synthesis of silver nanoparticles from aqueous Aegle marmelos leaf extract." Materials Research Bulletin **48**(2): 628-634.

Jeyaraj, M., G. Sathishkumar, *et al.* (2013). "Biogenic silver nanoparticles for cancer treatment: An experimental report." Colloids and Surfaces B: Biointerfaces **106**(0): 86-92.

Jha, A. K., K. Prasad, *et al.* (2009). "Plant system: Nature's nanofactory." Colloids and Surfaces B: Biointerfaces **73**(2): 219-223.

Kumar, V., S. C. Yadav, *et al.* (2010). "Syzygium cumini leaf and seed extract mediated biosynthesis of silver nanoparticles and their characterization." Journal of Chemical Technology & Biotechnology **85**(10): 1301-1309.

Kuntz, S., U. Wenzel, *et al.* (1999). "Comparative analysis of the effects of flavonoids on proliferation, cytotoxicity, and apoptosis in human colon cancer cell lines." European Journal of Nutrition **38**(3): 133-142.

Mukherjee, S., V. Sushma, *et al.* (2012). "Green chemistry approach for the synthesis and stabilization of biocompatible gold nanoparticles and their potential applications in cancer therapy." Nanotechnology **23**(45).

Nikkon, F., M. R. Habib, *et al.* (2011). "Tagetes erecta Linn. and its mosquitocidal potency against Culex quinquefasciatus." Asian Pacific Journal of Tropical Biomedicine **1**(3): 186-188.

Nune, S. K., P. Gunda, *et al.* (2009). "Nanoparticles for biomedical imaging." Expert Opinion on Drug Delivery **6**(11): 1175-1194.

Raghunandan, D., M. D. Bedre, *et al.* (2010). "Rapid biosynthesis of irregular shaped gold nanoparticles from macerated aqueous extracellular dried clove buds (Syzygium aromaticum) solution." Colloids and Surfaces B: Biointerfaces **79**(1): 235-240.

Rana, S., A. Bajaj, *et al.* (2012). "Monolayer coated gold nanoparticles for delivery applications." Advanced Drug Delivery Reviews **64**(2): 200-216.

Singh, A., R. Shukla, *et al.* (2011). "Cytotoxicity and Cellular Internalization Studies of Biogenic Gold Nanotriangles in Animal Cell Lines." International Journal of Green Nanotechnology **3**(4): 251-263.

Thakkar, K. N., S. S. Mhatre, *et al.* (2010). "Biological synthesis of metallic nanoparticles." Nanomedicine: Nanotechnology, Biology and Medicine **6**(2): 257-262.

V, J. J. P. a. L. (2012). "Screening of phytochemical constituents and antimicrobial activity of traditional medicinal plants." Int. J. Res. Pharm. Sci **3**(3): 461-465.

Vijay Singh, L. K., G.S.Chakraborthy, Avijit Mazumder (2011). "Pharmacological and Phytochemical Findings of *Tecoma stans*- A Review." JAPHR **1**(3): 75-81.

Vivek, R., R. Thangam, *et al.* (2012). "Green biosynthesis of silver nanoparticles from Annona squamosa leaf extract and its in vitro cytotoxic effect on MCF-7 cells." Process Biochemistry **47**(12): 2405-2410.

Zaki, S., M. F. El Kady, *et al.* (2011). "Biosynthesis and structural characterization of silver nanoparticles from bacterial isolates." Materials Research Bulletin **46**(10): 1571-1576.

CHAPTER FOUR

SIZE-DEPENDENT CELLULAR TOXICITY AND UPTAKE OF COMMERICAL COLLOIDAL GOLD NANOPARTICLES

Introduction

The estimated risk of prostate cancer is 21% and the lifetime risk of death is 2-5%. Even though it can be diagnosed early and therapy can be started immediately, patients developing metastatic conditions die. Apart from the potent issue of cancer, recurrent UTI is one of the most prevalent symptoms (Klein and Thompson 2012). *Escherichia coli* ORN178 are the most common cause of UTI in humans. Various urovirulence factors of *E. coli* ORN178 have been identified such as molecular biology of surface receptors of the urothelial cells of the urinary tract, the adhesin specificity and primarily and the Type 1 fimbriae associated with the organism (Sokurenko, Chesnokova *et al.* 1997). Detection, diagnosis and treatment of UTI play an important role in prostate cancer. One of the novel tools currently vastly studied are nanoparticles. Nanoparticles continue to be used as carriers for localized drug diffusion to treat and detect infections and diseases like cancer (P.C. Chen, S.C. Mwakwari *et al.* 2008).

Owing to their nano size it is easy for these particles to diffuse into the cells and effect desired responses in treatment of diseases. However, the size, type and surface charges of the particles play a vital role. Of the different kind of nanoparticles GNPs have been extensively studied in this regard. It has been shown that *E. coli* ORN178 binds

specifically to D-mannose, which is an integral part of the glycoproteins that are a part of the adhesive domain on host cells (Sharon 2006). The fimbriae of the uropathogenic *E. coli* ORN178 bind to the urolapkins on the surface of urothelial cells of the human bladder. We have demonstrated D-mannose functionalized 200 nm GNPs bind specifically to *E. coli* ORN178 (Vedantam, Tzeng *et al.* 2012). Continuing in the same direction in this study we attempt to see if GNPs can be used as specific biodiagnostic tool to detect and treat UTI in prostate cancer cells (DU-145).

This study investigates the binding time of *E. coli* ORN178 and *E. coli* ORN208 to DU-145 cells. It has been shown that *E. coli* ORN178 binds specifically to D-mannose only and *E. coli* ORN208 serves as a negative control as it has Type 1 pili that fail to bind to D-mannose. In order to study the cytotoxicity of plain and functionalized 20 nm and 200 nm GNPs to DU-145, growth curve of the prostate cancer cells was done. Cytotoxicity tests in log and lag phase were performed to study their biocompatibility in vitro. Cellular uptake of GNPs was estimated and protein corona of GNPs was studied.

Materials and methods

Cells and strains

The strains *E. coli* ORN178 and 208 were provided by Dr. Chu-Cheng Lin, Department of Zoology, National Taiwan Normal University and were transformed with plasmid pGREEN by electroporation (Sambrook and Russell 2001). Two different GNPs: 20 nm and 200 nm were purchased from Ted Pella Inc., USA. The concentration of 20

nm GNPs was 7×10^{11} particles/ml and 200 nm GNPs was 7×10^{8} particles/ml. The sugar D-mannan (Mn) was purchased from VWR (USA). The sugar was dissolved in 0.3 M sodium phosphate buffer. Surface functionalization of GNPs with the Mn was carried out by a modified multistep procedure (Aslan, Lakowicz *et al.* 2004; Vedantam, Tzeng *et al.* 2012). All chemicals required for functionalizing GNPs were purchased from VWR, USA.

Human prostate carcinoma cell line DU-145 was graciously given by Dr Arun Sreekumar, Baylor College of Medicine, Houston, Texas. Dulbecco's modified Eagle's medium (DMEM) was modified to contain Earles Balanced Salt Solution, non-essential amino acids, 2 mM L-glutamine, 1 mM sodium pyruvate, and 1500 mg/L sodium bicarbonate. It was supplemented with fetal bovine serum to a final concentration of 10%, 100 UI/ml penicillin G, and 100 µg/ml streptomycin in a humidified incubator with 5% at 37°C. All the media components were purchased from Promega, USA.

Binding of *E. coli* to DU-145 cells

An eight-well chamber slide was used to perform the bacterial cell adhesion assay to DU-145 cells. A total of 0.5 ml of DU-145 cells (1.5×10^{6}cells/ml) was seeded in each well. The chamber slide was incubated at 37°C overnight for attachment and fresh media was added. Fresh cultures of *E. coli* ORN178 (EC-178) and *E. coli* ORN208 (EC-208) were cultured overnight in Tryptic Soy Broth with ampicillin (50 µg/ml). The cultures were washed and resuspended in sterile PBS. A 100 µl (3×10^{8} cells/ml) aliquot of EC-178 and 208 was added to two wells each in the chamber slide. The slide was incubated

for 1, 2 and 3 h. At the 3 different time periods wells were washed with PBS and images were taken by a fluorescent scope (Zeiss LSM-510).

DU-145 Cell growth curve

Cells were plated 96-well microtiter plates at initial densities of 1000, 2000, 4000 and 8000 cells per well. The cell culture medium was changed every 3 days. Cell growth was tested by the CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) purchased from Promega, USA. It is a colorimetric method for determining the percentage of viable cells that are proliferating. Briefly, the MTS tetrazolium is bioreduced by the viable cells into a colored formazan product which is stable and can be measured at an absorbance of 490 nm. The amount of formazan produced is directly proportional to the number of living cells in the well. MTS assays were performed every day after seeding until day 8. All the experiments were carried out in triplicates.

Cytotoxicity of GNPs to DU-145 cells

The cytotoxicity of plain GNPs was tested by CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) purchased from Promega, USA. Cytotoxicity was measured in both the logarithmic and stationary phase of cell growth. For cytotoxicity measurements in the logarithmic phase, each cell line was incubated for 72 h in 96 well plate before adding the GNPs. Fresh medium containing increasing three different concentrations of the GNPs (10, 50 and 100 µl) was added to each well and the cells were incubated for 6, 24, 48 and 72 h time periods. The same was done for testing

cytotoxicity in the stationary phase. MTS assay was performed and the percentage of cell viability was determined.

Cell uptake assay of plain and Mn-GNPs

DU-145 cells (10 ml) were seeded in a cell culture dish containing 2 x 10^6 cells and cultured overnight. Once the cells were 70% confluent they were treated with plain and mannose functionalized 20 and 200 nm GNPs (50 µl). After three hours of incubation, the unbound GNPs in the cell culture treatments were removed by washing the cells with PBS buffer twice. The cells were trypsinized with Trypsin-EDTA and centrifuged. After removal of the supernatant, the cells were resuspended in PBS to a final volume of 5 ml. At this stage the total number of cells was quantified with a hemocytometer. Five ml of 50% nitric acid (HNO_3) was added to each sample to lyse the cells. Inductively Coupled Plasma Mass Spectrometry (ICP-MS) was performed to measure the gold mass in the various samples. The number of GNPs was calculated via the gold mass. The total number of GNPs in the solution was divided by the number of cells to determine the number of GNPs taken up by the cells (Connor, Mwamuka *et al.* 2005).

Cytotoxicity of functionalized GNPs to DU-145 cells and *E.coli* ORN178

The cytotoxicity of functionalized Mn-GNP of 200 nm particles was carried out by adding 200 µl of DU-145 cells (1.5 x 10^6 cell per well) into a 96 well plate. After incubation at 37°C for 24 h, the cells were treated with 200 nm Mn-GNPs, 200 nm Mn-

GNPs with bacteria EC-178 and 208. A total of volume 50µl of 200 nm Mn-GNPs was added. A preincubated mix, 1ml of the 200 nm Mn-GNPs and 1 ml of the microorganisms was incubated at 37°C with shaking for 1 h. The solution was centrifuged and washed with sterile PBS and resuspended in sterile PBS before adding to the 96 well plates. Different plates were set up for 6, 12, 24, 48 and 72 h time periods. The MTS assay was performed and the cell viability was determined.

Protein corona of commercial 20 nm and 200 nm GNPs

We wanted to study the protein adsorption of the commercial 20 nm and 200 nm GNPs to Fetal Bovine Serum (FBS) in DU-145 cell culture media and DMEM. The 20 nm and 200 nm GNPs were incubated with DMEM and cell culture medium for 1 h at 37°C. The 20 nm GNPs samples were spinned down at 10000 x g for 10 mins. The soups from FBS and DMEM samples were saved. The pellet containing the particles was washed thrice with nanopure water at 10000 x g. The particles were finally resuspended in nanopure water. The 200 nm GNPs were spinned down at 2655 x g for 10 mins.

The soups from FBS and DMEM samples were saved. The pellet containing the particles was washed thrice with nanopure water at 2655 x g. The particles were finally resuspended in nanopure water. Samples of pure nanoparticles of both 20 and 200 nm GNPs, DMEM and cell culture medium were also included to compare the binding of the FBS proteins to the nanoparticle surface. SDS-PAGE was performed for all the samples using a 4-20% gel (Biorad, USA). The gel was stained by Coomassie blue stain. A Smart Protein standard (Genscript, USA) was used to identify and analyze the presence of BSA

bound to the samples. The gel bands were cut up and image taken accordingly. The hydrodynamic size of the plain GNPs and protein-bound GNP samples were measured using dynamic light scattering (DLS) using Malvern Zetasizer.

Statistical analysis

All experiments were carried out in triplicates with results expressed as mean ±standard error (SE). Statistically significant differences were calculated using the two-tailed unpaired t-test or one-way analysis of variance (ANOVA) with a p value of ≤ 0.05, $p<0.01$ and $p<0.001$ considered significant using Prism 5.0 (GraphPad Software, CA).

Results

Binding of *E. coli* to DU-145 cells

The binding of EC-178 and 208 to DU-145 was studied for 1, 2 and 3 h. Phase contrast images were taken as shown in Figure 4.1. It was seen that the EC-208 cells did not bind to DU-145 cells at all during all the three time periods which is expected as it serves as a negative control (Figure 4.1b). On the contrary the EC-178 cells bind to the DU-145 cells at 2 h time period (Figure 4.1d). After 3 h time period they were found to be associated with the DU-145 cells (Figure 4.1e). Due to the wash step after every time period it was seen that only few cells managed to bind to the DU-145 cells between 2-3 hours.

DU-145 Cell growth curve

In order to quantify the toxicity of the 20 nm and 200 nm GNPs in DU-145 cells a growth was carried out. Since cells that actively grow and divide during the logarithmic growth should be more vulnerable to toxic metallic particles than cells that are nearing or at the stationary phase of cell culture. Thus, the growth curve was determined to estimate the logarithmic and stationary growth phases in relation to the number of cells seeded into each well of a 96-well culture plate (Figure 4.2). It was observed that the cells reach the logarithmic phase by 3-4th day of incubation. By the eighth day the DU-145 cells are in stationary.

Cytotoxicity of GNPs to DU-145 cells

The cytotoxicity of 20 nm and 200 nm GNPs at the different time points in log phase is shown in Figure 4.3. It is seen that the 20 nm GNPs seem to have a significant effect on the DU-145 cells only after 48 and 72 h of incubation period in all the three concentrations. Not much difference in viability is observed by 200 nm GNPs in the log phase when compared to 20 nm GNPs. It is seen that the size did not cause much decrease or increase in cell viability. In case of the stationary phase (Figure 4.4), the cytotoxicity of 20 nm and 200 nm GNPs is significant as they show 24-31 % reduction in cell viability at mid-range of concentration (50 µl) compared to other low and high concentrations. Hence, it was observed that the mid-range of 50 µl volume of GNPs significantly affected the percent cell viability in both log and stationary phase.

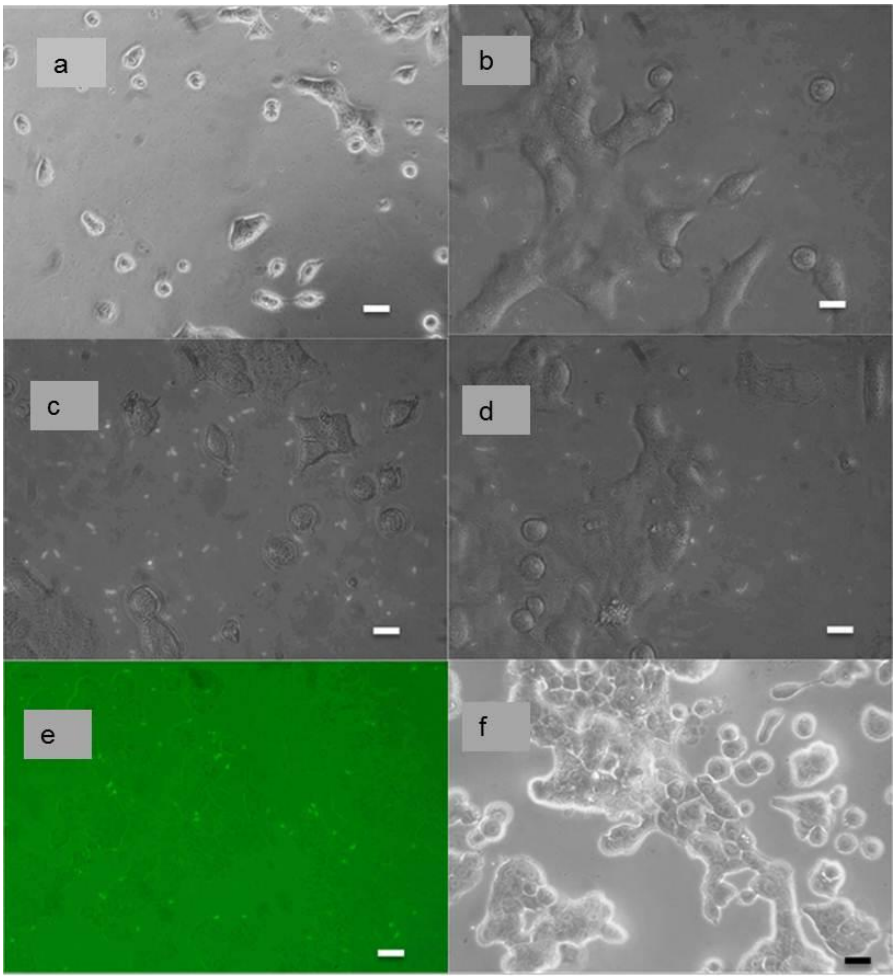

Figure 4.1. a) DU-145 cells with EC-178 at 0 min, b) DU-145 cells with EC-208, c) DU-145 cells with EC-178 at 1h, d) Bright field flourescent image of DU-145 cells with EC-178 at 2h, e) DU-145 cells with EC-178 at 3h, f) Control DU-145 cells. 200X magnification. Scare bar – 10 um

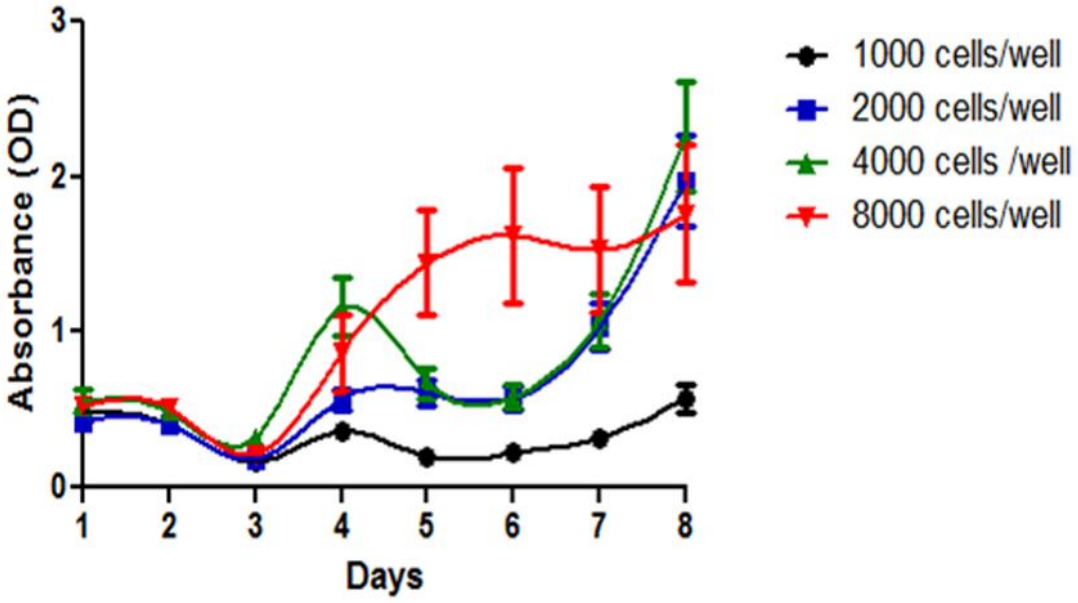

Figure 4.2. Cell growth curve of DU-145 using different cell-plating numbers

Cell uptake assay of plain and Mn-GNPs

After exposure to 20 and 200 nm plain and Mn-GNPs to DU-145 cells for three hours, the average number of GNPs per cell associated with each DU-145 cell was estimated as shown in Figure 4.5. The number of GNPs per cell in DU-145 cells was 3.4 x 10^4 for plain 20 nm GNP where as it was 1.7 x 10^4 for 200 nm plain GNPs. In contrast, exposure to 20 nm Mn-GNPs results in as much twice the increase of nanoparticle uptake compared to the plain 20 nm plain GNPs. There was a significant increase in the uptake of the 200 nm Mn-GNPs, more than double the number of GNPs when compared to the plain 200 nm GNPs. There does not seem to be a significant difference between uptake levels of Mn-GNPs (both 20 and 200 nm).

Cytotoxicity and competitive binding of functionalized GNPs to DU-145 cells and EC-178

Figure 4.6 shows the cytotoxicity of functionalized GNPs to the DU-145 cells at 1-6 h time points. A mid-range concentration of 200 nm Mn-GNPs (50 µl) was used in this assay. After various binding assays, it was seen that 50 µl is the minimum concentration 200 nm Mn-GNPs required to effect binding. It is seen that mannose functionalized 200 nm GNPs when bound to EC-178 relatively show the same percent viability when compared to the control DU-145 cells. On the other hand, the EC-178 bacterial cells bring about cell death in about 6 h. When the 200 nm Mn-GNPs and EC178 are added together at once to the DU-145 cells, 64% viability of the cell line is

observed. But, when the 200 nm Mn-GNPs are premixed with EC-178 and then added to the DU-145 cell 73% viability is observed.

This shows that when they are premixed the functionalized GNPs bind to EC-178 and prevent the binding of the latter to DU-145, thereby increasing the cell viability. Even though the increase is not significant, it shows that there could be competitive binding between EC-178 and 200 nm Mn-GNPs to bind to DU-145 cells. This also suggests that the Mn-GNPs could competitively bind to the cell surface that has mannose residues and there by block the attachment of bacteria to those mannose residues on the cell surface receptors (Miura, Nishimura *et al.* 2001).

Protein corona of GNPs

The hydrodynamic sizes of the plain and protein coated GNPs did increase when incubated with FBS (Figure 4.7). The DLS measurements indicate that the protein in FBS did adsorb and hence the increase in diameter (Table 4.1).

The 20 nm GNPs were found to be of 66.61±2.3 nm size and the 200 nm showed increase in size were of 261.9±5.3 nm. This confirms the presence of protein corona which remained coated even after multiple washes. The zeta values do show good stable protein coated 20 and 200 nm GNPs. The SDS-PAGE gel was done to confirm the same (Figure 4.8). The 20 nm and 200 nm GNPs (arrow marks) do confirm the presence of protein corona with a clear thick band. The samples incubated with plain DMEM did not show any bands at all. Hence, it is clear that there was protein bound to the GNPs.

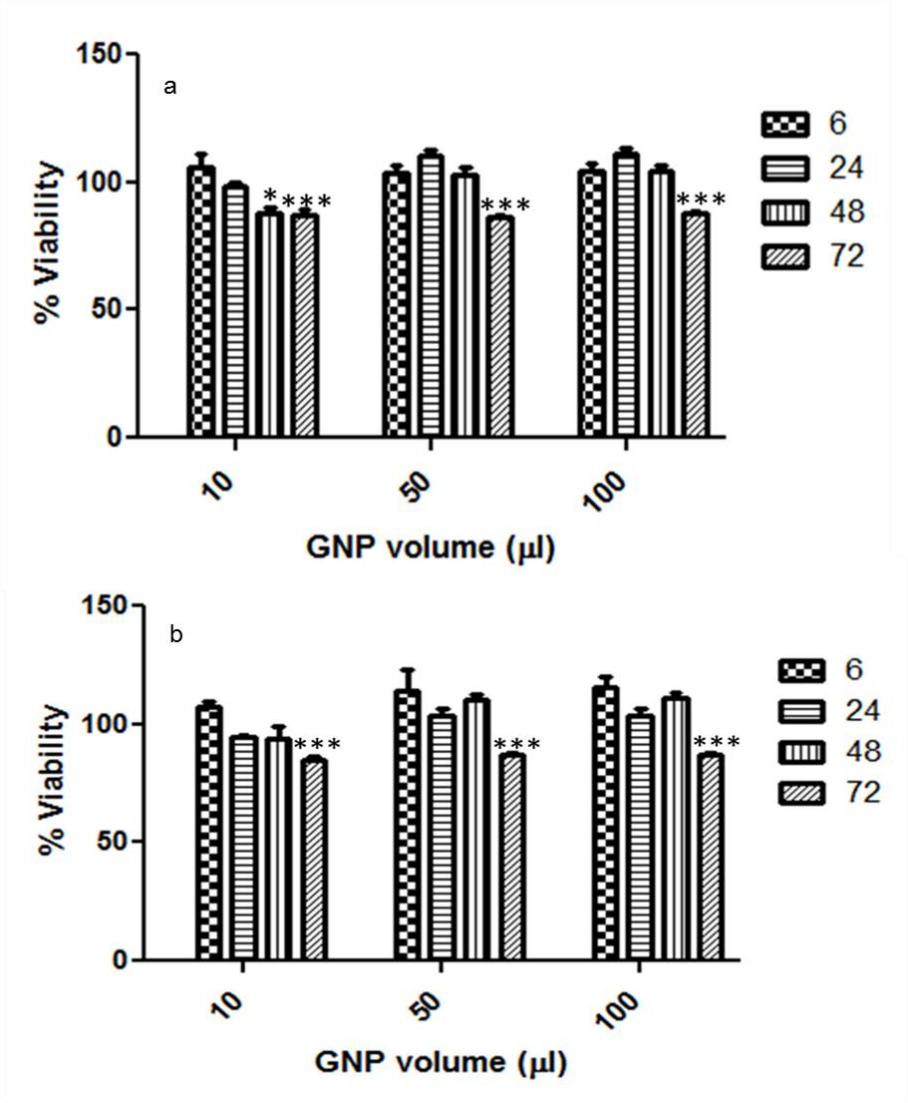

Figure 4.3. Cell cytotoxicity profiles of DU-145 cells in log phase. a) Cell viability of DU-145 in log phase when treated with plain 20 nm GNPs and b) Cell viability of DU-145 in log phase when treated with 200 nm GNPs. *$p<0.05$, ***$p<0.001$

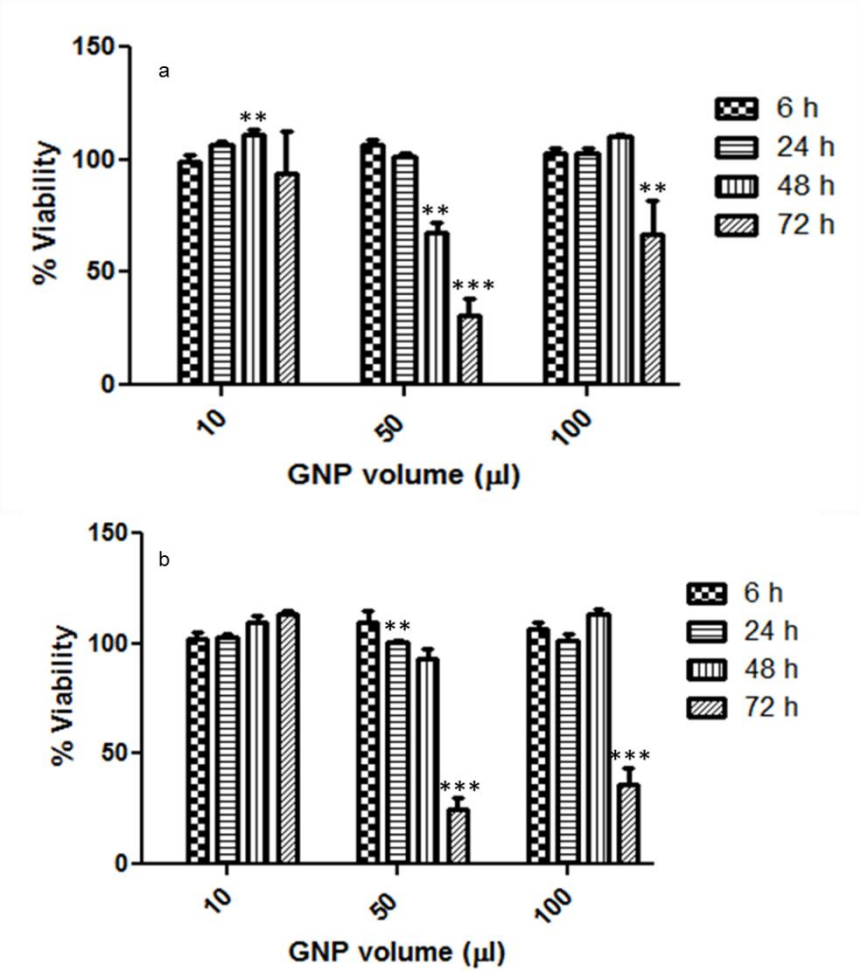

Figure 4.4 Cell cytotoxicity profiles of DU-145 cells in lag phase. a) Cell viability of DU-145 in lag phase when treated with plain 20 nm GNPs and b) Cell viability of DU-145 in lag phase when treated with 200 nm GNPs **p<0.01, ***p<0.001

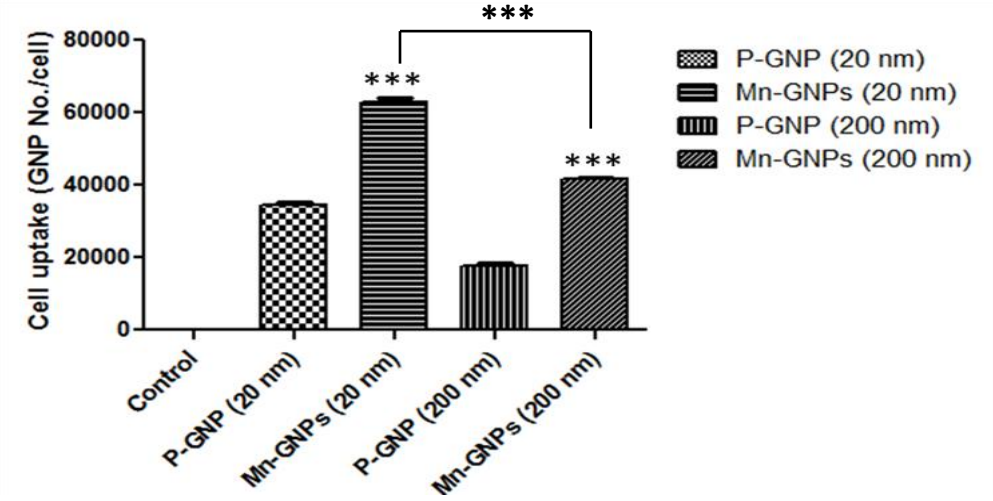

Figure 4.5. DU-145 cell uptake of plain GNPs (P-GNP) and mannose functionalized GNPs (Mn-GNPs) ***$p<0.001$

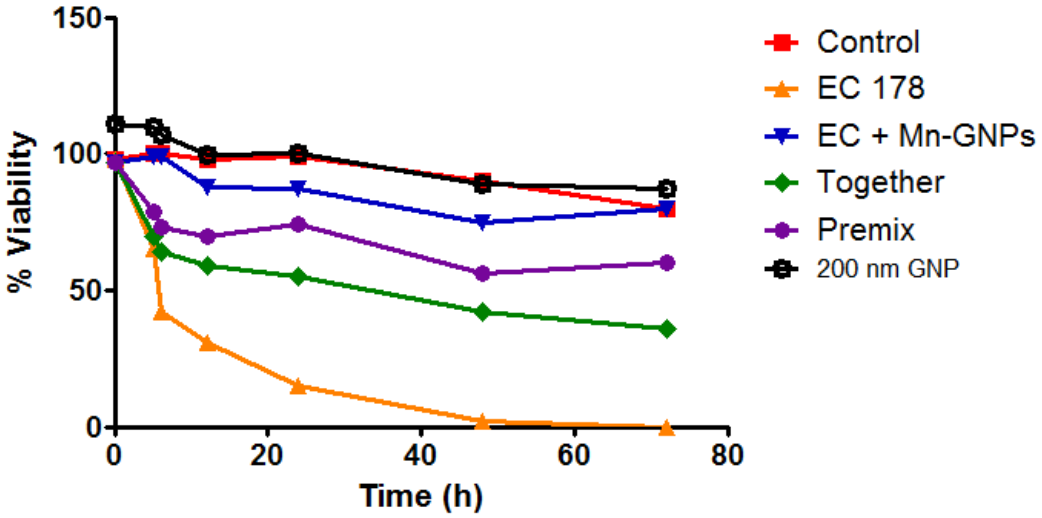

Figure 4.6. DU-145 cell growth pattern with different treatments at 6, 12, 24, 48 and 72 h time points

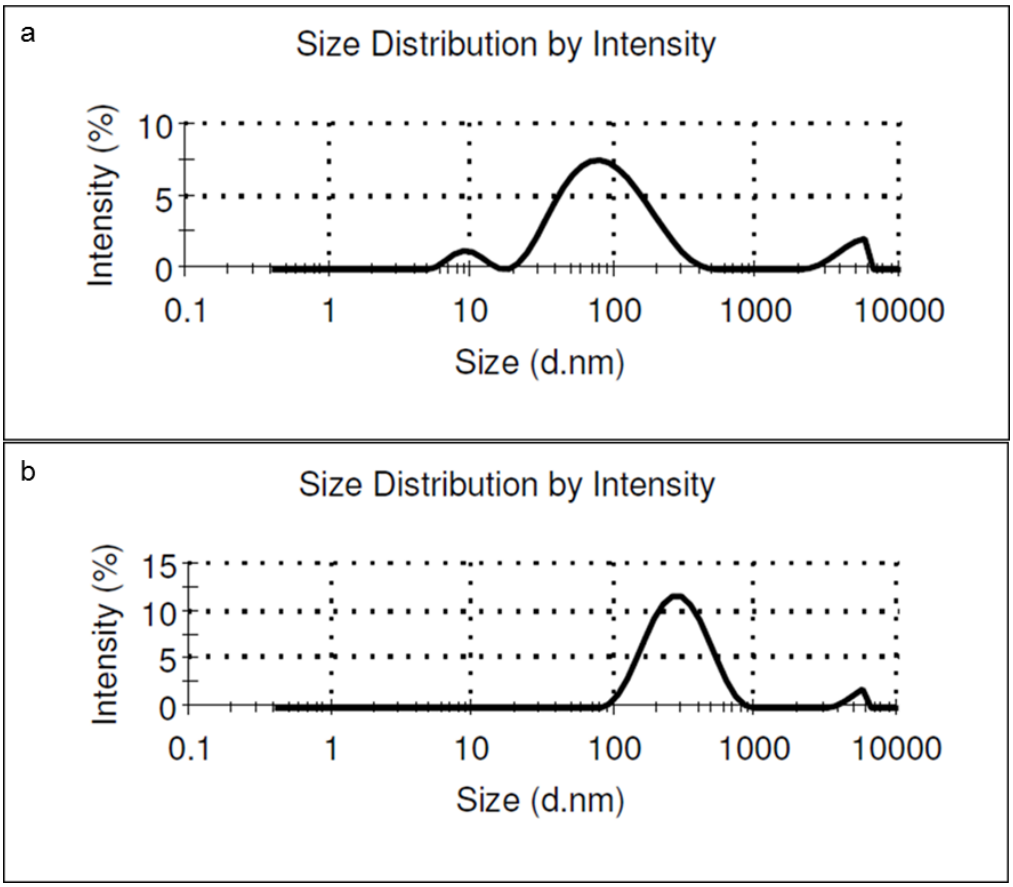

Figure 4.7. Hydrodynamic size distribution of a) protein coated 20 nm GNP and b) protein coated 200 nm GNP

Table 4.1. Hydrodynamic size distribution of GNPs

Sample	Size (nm)	Zeta potential (mV)
20 nm GNP	24±2.1	-54.4
Protein coated 20 nm GNP	66.61±2.3	-18.2
200 nm GNP	213.5±2.9	-49.3
Protein coated 200 nm GNP	261.9±5.3	-13.3

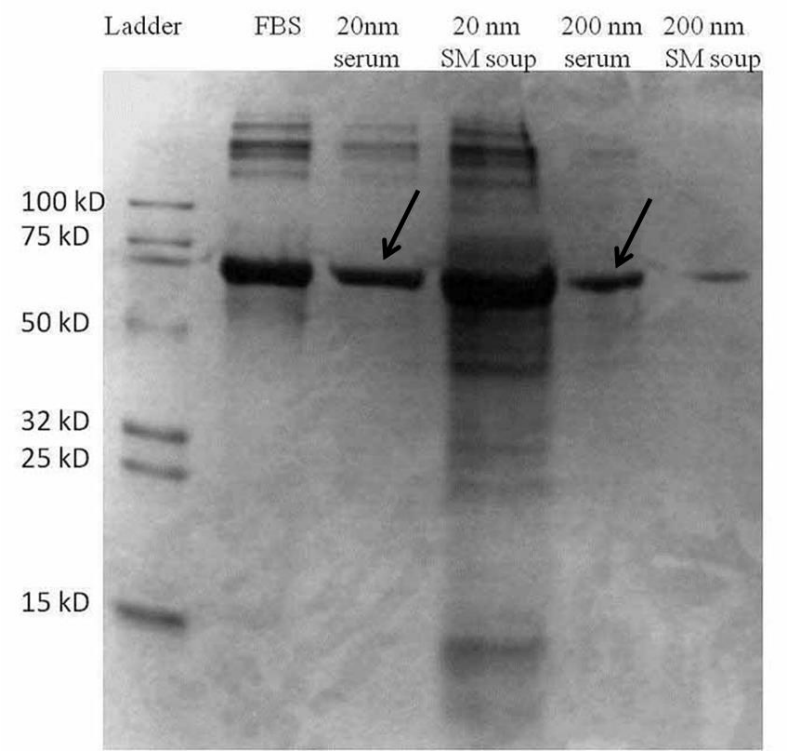

Figure 4.8. Coomassie-stained SDS-PAGE gel lanes for protein coated 20 and 200 nm GNPs. FBS is a combination of various proteins. The lanes '20 nm serum and 200 nm serum' show a band for protein bound to the GNPs suggesting the presence of protein corona. The samples incubated with plain DMEM did not show any protein bands (data not shown)

Application of chemotherapy with GNPs is a new treatment approach in cancer therapy (Lehmann, Natarajan *et al.* 2008). There has been extensive research done and many underlying reasons have been identified for the invasiveness of prostate cancer. Cell-cell adhesion molecules that are involved in cell aggregation are calcium-dependent moieties known as cadherins. When there is a dysfunction in the cadherin pathway cancer tumor tends to become invasive. E-cadherin is a marker of carcinogenesis in prostate cancer. The cadherin family proteins have mannose residues on their cell surface receptors (Paul, Ewing *et al.* 1997). These mannose residues also are detected by the uropathogenic bacteria *E. coli*. Keeping this in mind, the 200 nm GNPs were only functionalized with mannose to test adhesion and cytotoxicity. The bigger size GNPs were used to functionalize with mannose than 20 nm GNPs because due to their increased size. If they bind to the DU-145 cells and competitively block the mannose residues from becoming available for uropathogenic bacteria. Also, if the Mn-GNPs do bind to the prostate cancer cells, then the uropathogenic bacteria can detect, chemotactically and specifically adhere to the mannose bound to GNPs.

Once, many bacteria are bound to the GNPs then, due to their bigger size and the peristaltic flow near the tissue, the GNP-bacterial complex could be dislodged and can be detached from the prostate cancer cells. If this happens then the severity of the UTI can also be diagnosed, treated and prevented. As it was seen that the DU-145 cells are actively reproducing in log phase by 48 h of incubation, the cytotoxicity profiles indicated that there is no significant toxicity at all in 6 and 24 h incubated periods. Hence, Mn-GNPs can be safely used for detection or treatment of prostate cancer or UTI. The

viability profiles of DU-145 cells also indicate that the plain and Mn-GNPs do significantly decrease the viable cells by 48-72 h of incubation. Hence, if Mn-GNPs are used as a drug carrier can bring about synergistic killing of the prostate cancer and such a treatment is could be localized treatment and site directed when introduced locally.

Compared to the 20 nm plain and Mn-GNPs were taken up by the DU-145 cells more than the 200 nm plain and Mn-GNPs. It has been documented that 15 nm glucose functionalized GNPs have been taken up more than the plain or chemically functionalized GNPs (Zhang, Xing *et al.* 2008). They have also demonstrated there is enhanced radiation sensitivity in prostate cancer by GNPs. Hence, if GNPs are functionalized with mannose sugar, they can be used as a vehicle to treat UTI. Gold nanoparticles are heavy metals with increased f-factor that enhance radiosensitivity have been studied in mice (Hainfeld, Slatkin *et al.* 2004; Wilson, Xiaojing *et al.* 2009). They attributed the sensitivity to the high-Z radio-enhancement by GNPs. Since the uptake studies of GNPs in this study indicate that 20 nm Mn-GNPs are taken up more than 200 nm Mn-GNPs, they could be used for killing the DU-145 cells in promising clinical applications if used as drug carrier. The 200 nm Mn-GNPs can be used for antiadhesion of uropathogenic bacteria in prostate cancer cells. This approach can be used for future treatment of UTI cases as there is an increase in antibiotic resistance of microorganisms.

For this the uptake of the GNPs and their interaction with cellular proteins is essential. Hence, the protein adsorption of FBS on the 20 and 200 nm GNPs was studied. The DLS measurements and the SDS-PAGE gel indicate a strong band confirming the presence of the protein corona around the GNPs. It is crucial to know how the

nanoparticles behave in blood plasma and in vivo in general. Hence, there have been many studies where the GNPs have been studied in depth to understand the protein-nanoparticle complexes in vitro to make the nanoparticles more robust and practical for various clinical applications. The chemical functionalization, size of the nanoparticle, type of proteins and surface charges do decide the biological fate of the nanoparticle when administered for therapeutic purposes (Pan, Neuss *et al.* 2007; Nativo, Prior *et al.* 2008; Arnida, Malugin *et al.* 2010).

This protein corona is important as it decides the particle's longevity in the blood stream which is very important for therapeutic efficacy. It has been shown that 30 and 50 nm citrate-stabilized gold colloids were bound to 69 different proteins in plasma (Dobrovolskaia, Patri *et al.* 2009). It has been also demonstrated that the kinetic and equilibrium binding properties depend on protein identity as well as particle surface characteristics and size (Cedervall, Lynch *et al.* 2007). In order to understand the transport pathways utilized by nanoparticles, it is necessary to know how long lived they are in the living system, which will determine its biological fate (Lundqvist, Stigler *et al.* 2011). Researches have demonstrated that 15 nm GNPs form proteins/NP complexes in RPMI are more abundantly internalized in cells as compared to DMEM, overall exerting higher cytotoxic effects in HeLa cells (Maiorano, Sabella *et al.* 2010). It is important to understand what happens to the fate of the nanoparticles once they have been utilized for biological applications.

In conclusion It has been shown previously in our previous investigation that GNPs when functionalized with D-mannose bind specifically to only EC-178 (Vedantam, Tzeng *et al.* 2012). Based on this fact our investigation attempted to carry out cytotoxicity assays with plain and Mn-GNPs it is seen that 20 and 200 nm GNPs bring out significant decrease in cell viability by 48-72 h only both in log as well as lag phase of DU-145 cells. Also, it has been shown that there might be a competitive binding between Mn-functionalized and GNPs when present together with DU-145 cells. The plain 20 nm GNPs were more inside the cell when compared to the plain 200 nm GNPs. The Mn-GNPs of 20 nm size were also taken up more compared to the 200 nm Mn-GNPs.

It was also demonstrated that both 20 and 200 nm GNPs do bind to protein and form a stable protein corona. Since it has been shown that GNPs of different sizes between 20-100 nm have shown to alter signaling pathways in cells and mediate biological processes (Jiang, KimBetty *et al.* 2008), based on which they can be used for targeted drug delivery as well as for detection purposes. Lastly, the binding of EC-178 shows that competitive binding to Mn-GNP can be done to avoid binding of the bacteria to the DU-145 cells and hence, this mechanism can be further developed to prevent/detect recurrent UTI in prostate cancer cells.

Literature Cited

Arnida, Malugin, A., & Ghandehari, H. (2010). Cellular uptake and toxicity of gold nanoparticles in prostate cancer cells: a comparative study of rods and spheres. *Journal of Applied Toxicology, 30*(3), 212-217. doi: 10.1002/jat.1486

Aslan, K., Lakowicz, J. R., & Geddes, C. D. (2004). Tunable plasmonic glucose sensing based on the dissociation of Con A-aggregated dextran-coated gold colloids. *Analytica Chimica Acta, 517*(1–2), 139-144. doi: http://dx.doi.org/10.1016/j.aca.2004.04.060

Cedervall, T., Lynch, I., Lindman, S., Berggård, T., Thulin, E., Nilsson, H., . . . Linse, S. (2007). Understanding the nanoparticle–protein corona using methods to quantify exchange rates and affinities of proteins for nanoparticles. *Proceedings of the National Academy of Sciences, 104*(7), 2050-2055. doi: 10.1073/pnas.0608582104

Connor, E. E., Mwamuka, J., Gole, A., Murphy, C. J., & Wyatt, M. D. (2005). Gold nanoparticles are taken up by human cells but do not cause acute cytotoxicity. [Research Support, U S Gov't, Non-P H S]. *Small, 1*(3), 325-327.

Dobrovolskaia, M. A., Patri, A. K., Zheng, J., Clogston, J. D., Ayub, N., Aggarwal, P., . . . McNeil, S. E. (2009). Interaction of colloidal gold nanoparticles with human blood: effects on particle size and analysis of plasma protein binding profiles. *Nanomedicine: Nanotechnology, Biology and Medicine, 5*(2), 106-117. doi: http://dx.doi.org/10.1016/j.nano.2008.08.001

Hainfeld, J. F., Slatkin, D. N., & Smilowitz, H. M. (2004). The use of gold nanoparticles to enhance radiotherapy in mice. *Physics in Medicine and Biology, 49*(18), N309.

Jiang, W., KimBetty, Y. S., Rutka, J. T., & ChanWarren, C. W. (2008). Nanoparticle-mediated cellular response is size-dependent. [10.1038/nnano.2008.30]. *Nat Nano, 3*(3), 145-150. doi: http://www.nature.com/nnano/journal/v3/n3/suppinfo/nnano.2008.30_S1.html

Klein, E. A., & Thompson, I. M. (2012). Chemoprevention of prostate cancer: an updated view. *World J Urol, 30*(2), 189-194. doi: 10.1007/s00345-011-0822-9

Lehmann, J., Natarajan, A., Denardo, G. L., Ivkov, R., Foreman, A. R., Catapano, C., . . . Denardo, S. J. (2008). Short communication: nanoparticle thermotherapy and external beam radiation therapy for human prostate cancer cells. [Research Support, N I H , Extramural

Research Support, U S Gov't, Non-P H S]. *Cancer Biother Radiopharm, 23*(2), 265-271.

Lundqvist, M., Stigler, J., Cedervall, T., Berggård, T., Flanagan, M. B., Lynch, I., . . . Dawson, K. (2011). The Evolution of the Protein Corona around Nanoparticles: A Test Study. *ACS Nano, 5*(9), 7503-7509. doi: 10.1021/nn202458g

Maiorano, G., Sabella, S., Sorce, B., Brunetti, V., Malvindi, M. A., Cingolani, R., & Pompa, P. P. (2010). Effects of Cell Culture Media on the Dynamic Formation of Protein−Nanoparticle Complexes and Influence on the Cellular Response. *ACS Nano, 4*(12), 7481-7491. doi: 10.1021/nn101557e

Miura, H., Nishimura, K., Tsujimura, A., Matsumiya, K., Matsumoto, K., Nakamura, T., & Okuyama, A. (2001). Effects of hepatocyte growth factor on E-cadherin-mediated cell-cell adhesion in DU145 prostate cancer cells. [Research Support, Non-U S Gov't

Research Support, U S Gov't, P H S]. *Urology, 58*(6), 1064-1069.

Nativo, P., Prior, I. A., & Brust, M. (2008). Uptake and Intracellular Fate of Surface-Modified Gold Nanoparticles. *ACS Nano, 2*(8), 1639-1644. doi: 10.1021/nn800330a

P.C. Chen, S.C. Mwakwari, & Oyelere, A. K. (2008). Gold nanoparticles: from nanomedicine to nanosensing. *Nanotech. Sci. Appl., 1*, 45–66.

Pan, Y., Neuss, S., Leifert, A., Fischler, M., Wen, F., Simon, U., . . . Jahnen-Dechent, W. (2007). Size-dependent cytotoxicity of gold nanoparticles. [Research Support, Non-U S Gov't]. *Small, 3*(11), 1941-1949.

Paul, R., Ewing, C. M., Jarrard, D. F., & Isaacs, W. B. (1997). The cadherin cell-cell adhesion pathway in prostate cancer progression. [Review]. *Br J Urol, 1*, 37-43.

Sambrook, J., & Russell, D. (2001). *Molecular Cloning: A Laboratory Manual*: Cold Spring Harbor Laboratory Press.

Sharon, N. (2006). Carbohydrates as future anti-adhesion drugs for infectious diseases. [doi: DOI: 10.1016/j.bbagen.2005.12.008]. *Biochimica et Biophysica Acta (BBA) - General Subjects, 1760*(4), 527-537.

Sokurenko, E. V., Chesnokova, V., Doyle, R. J., & Hasty, D. L. (1997). Diversity of the Escherichia coli type 1 fimbrial lectin. Differential binding to mannosides and uroepithelial cells. [Research Support, Non-U S Gov't

Research Support, U S Gov't, Non-P H S

Research Support, U S Gov't, P H S]. *J Biol Chem, 272*(28), 17880-17886.

Vedantam, P., Tzeng, T.-R., Brown, A., Podila, R., Rao, A., & Staley, K. (2012). Binding of Escherichia coli to Functionalized Gold Nanoparticles. *Plasmonics, 7*(2), 301-308. doi: 10.1007/s11468-011-9307-3

Wilson, R., Xiaojing, Z., Linghong, G., Andrew, S., Xiuying, H., Yeping, X., . . . James, Z. X. (2009). Gold nanoparticle sensitize radiotherapy of prostate cancer cells by regulation of the cell cycle. *Nanotechnology, 20*(37), 375101.

Zhang, X., Xing, J. Z., Chen, J., Ko, L., Amanie, J., Gulavita, S., . . . Roa, W. (2008). Enhanced radiation sensitivity in prostate cancer by gold-nanoparticles. [Research Support, Non-U S Gov't]. *Clin Invest Med, 31*(3), E160-167.

CHAPTER FIVE

BINDING OF *ESCHERICHIA COLI* TO FUNCTIONALIZED GOLD NANOPARTICLES

Introduction

Tools for the study of bimolecular interactions have been developed based on the surface plasmon resonance of functionalized nanoparticles. Surface functionalization has proved to impart biofunctionality and this property has been exploited in developing different kinds of biosensors (Wijaya, Lenaerts *et al.* 2011). Although the application of colloidal metal nanoparticles in biology is not new, the optical observation of single metal particle labels and aggregated nanoparticles is of interest as a component of ultrasensitive detection systems. The surface plasmon resonance (SPR) is indicative of the light scattering by nanometer-sized colloidal metal particles. The function of the size, shape, particle properties can be inferred from the color of the light scattered. Colloidal gold nanoparticles (Au NPs) are brilliant red in color. Hence, biosensors, identification and detection systems of biomolecues can be developed based on the shift in SPR (David A 2003). Haynes *et al.* developed a biosensor that works by measuring a shift in the plasmon resonance peak induced by the local dielectric environment (Haynes and Van Duyne 2001).

Haes *et al.* measured a shift in the PR peak caused by a change in the local dielectric environment in silver nanoparticle arrays that could function as sensitive and selective nanoscale affinity biosensors (Haes and Van Duyne 2002).

Colloidal Au NPs tend to settled down after few hours. By adjusting the distance between discrete nanoparticles or layers of nanoparticles, the SPR can be controlled (Schmitt, Decher *et al.* 1997). The peak of extinction will reduce, as the aggregate size increases shifting and broadenign the red plasmon band. Storhoff *et al.* have reported that extinction spectra for DNA-linked gold nanoparticle aggregates composed of equal number of particles but with smaller separations between particles and have red shift in the plasmon peak without causing significant loss in peak extinction (Storhoff, Lazarides *et al.* 2000).

The optical response of a particular nanoparticle depends on its size and shape that defines the intrinsic modes as well as the dielectric environment that typically define the energy shifts. Taking advantage of the optical and electronic properties, Au NPs have been used in bioassays such as detection of DNA hybridization, probing binding to citrate-coated GNPs (Aizpurua, Hanarp *et al.* 2003; Brewer, Glomm *et al.* 2005).

Another application of biodetection is use of nanoparticles extensively in pathogen detection (Jain 2005; Rosi and Mirkin 2005). Pathogens bind to eukaryotic cell membranes by crucial interactions with biomolecules on the surface of the cells. There is a lot of evidence that the complex oligosaccharides in the glycocalyx are key players in controlling the normal and pathological processes (Varki 1993; Dwek 1996) in mammalian cells. Glyconanoparticles could hence mimic a cellular model with carbohydrate presented on the cell surface, which can be used as excellent tools in biomedicine and glycobiology studies. Firstly, their size is in range to many common biomolecules; secondly, carbohydrate-modified nanoparticles can mimic biomolecules;

thirdly, they provide glycocalyx-like surface properties and lastly, they have unique physical properties due to the quantum size effect (Murray, Kagan *et al.* 2000; López-Cartes, Rojas *et al.* 2005).

Many groups have functionalized Au NPs with mono-, di-, tri- or oligo-saccharides (Lin, Yeh *et al.* 2003; Aslan, Lakowicz *et al.* 2004; Zhang, Geddes *et al.* 2004; Svarovsky, Szekely *et al.* 2005; Takae, Akiyama *et al.* 2005) with applications in biosensing, drug delivery, vaccine development and in vivo cell imaging. For instance, our previous research has demonstrated that, galactosylated polymeric polystyrene nanoparticles have shown significant aggregation when incubated with *E. coli* (Qu, Gu *et al.* 2005). UV-vis extinction properties of DNA-linked nanoparticle aggregates have been determined (Lazarides and Schatz 1999) and it is seen that depending on the particle volume fraction the aggregate size increases, the surface plasmon absorption peak shifts to longer wavelengths throughout the UV-vis range. This suggests that the denser and larger the aggregates, broader the plasmon feature. The shift in the SPR can be manipulated in designing biosensors that involve pathogen detection systems.

By the use of glycan coated GNPs, the attachment/detachment mechanism of potential pathogens to Au NPs could be assessed and the optical properties displayed by the colloidal Au NPs can be measured. The role of sugar functionalized 200 nm (GNPs) in determining primary and fine sugar specificity in *E. coli* ORN178 and *E. coli* 13762 was investigated. *E. coli* ORN178, which is a wild-type that causes urinary tract infection. It has type 1 fimbrial protein that confers the unique quality of binding to D-mannose only (Harris, Spears *et al.* 2001). *E. coli* ORN208 was used as a negative

control. It has abnormal type 1 pili that fail to bind to D-mannose (Krogfelt, Bergmans *et al.* 1990). The other one is *E. coli* ATCC 13762 which causes enterotoxigenic diarrhea. *E. coli* 13762 is known as a K99+ strain that expresses K99 antigen that enters the humans as a foodborne pathogen via meat products especially in contaminated porcine meat. It has S type fimbrial proteins and binds specifically to Neuαc(α2-3)-Gal-(β1-4)Glc (Sharon 2006).

Materials and methods

Strains and Plasmid

The strains *E. coli* ORN178 and ORN208 were provided by Dr. Chu-Cheng Lin, Department of Zoology, National Taiwan Normal University, Taiwan. *E. coli* 13762 and yeast strain *Saccharomyces cerevisiae* were obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA). Transformed strains of *E. coli* ORN178 and ORN208 were procured from our lab. GFP-expressing bacterial cells of the three select strains were harvested from a TSA medium supplemented with ampicillin (50µg/ml). Yeast cells were cultivated at 30ºC on Potato Dextrose Agar (VWR, USA).

Transformation of the bacteria

Escherichia coli 13762 were transformed with plasmid pGREEN by electroporation (Sambrook and Russell 2001). The electro-competent cells were prepared by growing them in SOB medium (VWR, USA) with shaking at 37ºC to an optical density 0.6 (OD_{600}). The cells were harvested and centrifuged. They were washed with

ice-cold wash buffer (10% v/v glycerol in water). The cells were then resuspended in 3 ml of wash buffer and stored in eppendorf tubes containing100 µl quantity. Electrocompetent cells were stored in -80ºC. An aliquot of 100 µl competent cells was mixed with 100 ng of plasmid DNA of pGREEN. Transformation was carried out via electroporation using an ECM 399 Electroporation System (BTX, San Diego, CA) was achieved in a 0.1 cm gap cuvette at 1800 V, 50 µF, and 150 W. The mixture was pulsed and transferred to an eppendorf tube containing 1 ml of SOC (SOB plus 20 mM Mg^{2+} and 20 mM glucose) recovery medium. After allowing the cells to recover for two hours the cells were plated on Tryptic soy agar (TSA) with Ampicillin (50 µg/ml). Antibiotic resistance was checked by exposing the bacterial cells to blue light (488 nm).

Yeast Agglutination Assay

Yeast binding assay was done in order to verify the mannose-binding characteristics of *E. coli* 13762 (Ofek I and R.J. Doyle 1994). It has been established that *E. coli* 13762 (K99) has receptors for galactose and not mannose moieties (Sharon and Ofek 2000). Hence, this assay will confirm the binding specificity of the K99 strain and make sure if the transformation altered the binding profile of the organism.

Functionalization of GNP

Colloidal Au NPs of 200 nm size of 7 x 10^8 particles/ml concentration were purchased from, Ted Pella Inc., (USA). The Neuαc(α2-3)-Gal-(β1-4)Glc–Paa (Sg) was procured from Glyco Tech Corporation (USA) and D-Mannan (Mn) was purchased from

VWR (USA). The sugars were dissolved in 0.3 M sodium phosphate buffer. Absolute ethanol, 2-(2-aminoethoxy)ethanol (AEE) and N-hydroxy-2,5-pyrrolidinedione (NHS), 16-Mercaptohexadecanoic acid (16-MHDA), epichlorohydrin, 2-methoxyethyl ether (diglyme), N-3-(Dimethylaminopropyl)-N-ethyl-carbodiimide (EDC) were obtained from VWR (USA).

Sodium phosphate monobasic buffer solution (10mM) concentration at pH 7 was used. PBS of pH 7.4 in deionized water was used. All buffers were prepared in deionized water (>18M/cm). Glassware was washed with "piranha solution" (3:7, 30% H_2O_2/H_2SO4). Degassed ethanol was used to prepare 0.50mM 16-MHDA.

Preparation of functionalized Au NPs

The surface modification of the gold colloids was carried out by a modified multistep procedure as represented in Figure 5.1(Aslan, Lakowicz *et al.* 2004). Tween 20 was not used for physisorption as the nanoparticles' surfaces were plain with no charges. Briefly, the Au NPs were degassed with Argon gas. Equal volumes (500 µl) of 200 nm Au NP dispersions and 0.50mM 16-MHDA was then added and the final mixture was allowed to stand for 3 h for the chemisorption of 16-MHDA to gold colloids. Excess 16-MHDA was removed by centrifuging the mixture three times for 5 min at 1699 × g; the supernatants were discarded after each cycle and resuspended in phosphate buffer. 16-MHDA-modified gold colloids that remained in the centrifugate were then reacted with a mixture of freshly prepared 50mM NHS and 200mM EDC solution for 5 min. The resulting nanoparticle dispersion was centrifuged at 5 min, 1699 × g. The supernatant was

discarded, the remaining NHS ester-alkane thiol-modified Au NPs were reacted with a freshly prepared solution of AEE (2%, v/v) for 10 min. Excess AEE was removed by centrifugation for 5 min at 1699 × g at least three times. The pellet that contained AEE-modified Au NPs was centrifuged (5 min, 1699 × g). The hydroxyl groups on the AEE-modified Au NPs were activated with 0.6M epicholorohydrin solution in a 1:1 mixture of 0.4M NaOH and diglyme for 4 h at room temperature. The nanoparticle dispersion was then centrifuged for 10 min at 1699 × g and resuspended in diglyme and centrifuged again to remove the excess epicholorohydrin. The centrifugate, containing AEE-modified Au NPs now with active epoxide groups, were incubated in two glycan solutions namely, Sg and Mn solutions (0.1M NaOH) for 20 h. Finally, Sg and Mn-modified Au NPs were centrifuged for 5 min at 1699 × g and resuspended in 0.1M NaOH and centrifuged four more times to remove the excess sugar. The different sugar modified GNPs: Mn-Au NPs and Sg-Au NPs were stored in eppendorf tubes wrapped with aluminum foil in order to avoid aggregation induced by light.

The UV-Vis measurement for four selected functionalized Au NP samples: pristine Au NP, MHDA-Au NP, AEE-Au NP and *E. coli* ORN178 bound to Mn-Au NPs was carried out using a LAMBDA 950 UV/Vis/NIR Spectrophotometer (Perkin-Elmer, USA). Hydrodynamic diameter (Dz) and size distributions for the four GNP samples in aqueous solutions were determined by dynamic light scattering (DLS). The DLS instrumentation consisted of Malvern Zetasizer Nano ZS (Worcestershire, U.K.) system, ZEN3600 model. Measurements of diameter and ζ potential were made at 25 ± 1 °C. The

ζ average diameter reported herein was obtained as the average of three measurements performed on each sample. The morphology of the samples was imaged using a Hitachi S3400 scanning electron microscopy (SEM), model. A drop of the samples was put on poly-L-Lysine coated Si substrates, was air dried for 2 hrs and observed under SEM.

Confocal Imaging of Bacterial Agglutination by Fluorescence Microscopy

The *E. coli* ORN178, ORN 208 and K99 were grown on TSA plates containing ampicillin (50 µg/ml). Cells were washed with PBS and standardized to a concentration of 3 x 10^5 cells/ml. GNP suspension (3.5% wt/wt) was mixed with an equal volume of bacterial cell suspensions in eppendorf tubes. The mixture was inverted gently for few times and incubated for 15 minutes at room temperature on an orbital shaker with mild rotation. Wet mounts of the different samples were made and degree of aggregation was qualitatively assessed under a fluorescence microscope (Zeiss LSM-510). Different controls were set up for the three different types of *E. coli*. Negative control consisting of the particles only was also set up.

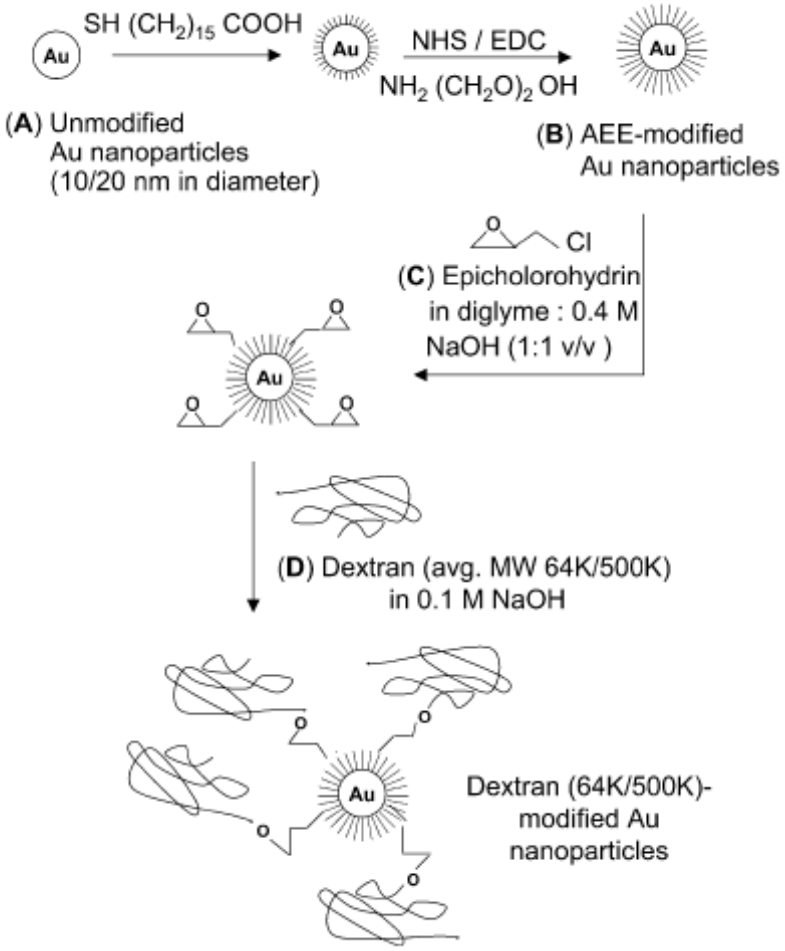

Figure 5.1. Scheme of functionalization of Mn and Sg coated 200 nm Au NPs (Aslan, Lakowicz *et al.* 2004)

Results

Transformation of the *E. coli* K99 strain

The *E. coli* 13762 were successfully transformed (Figure 5.2). The transformants fluoresced when viewed under the fluorescence microscope (Zeiss LSM-510) under the green fluorescent protein (GFP) filter. *E. coli* ORN178 (Figure 5.2a) and ORN208 (Figure 5.2b) appeared to be considerably longer rods when compared to the short rods of *E. coli* 13762 (Figure 5.2c).

Yeast agglutination Assay

The mannose binding characteristics of the transformants were verified using a yeast agglutination assay (Ofek, Mirelman *et al.* 1977) to document that the transformation had not altered the previously determined mannose-binding characteristics of *E. coli* ORN178 and ORN208. The binding specificity of *E. coli* 13762 to mannose was also determined. Figure 5.3a shows the transformant *E. coli* ORN178, not the ORN208 (Figure 5.3b), mediated the agglutination of yeast cells. Both strains were found to retain the same characteristics as their parent strains regarding their mannose-binding activities. *E. coli* 13762 (Figure 5.3c) did not show any visible binding to yeast cells both in the fluorescent and brightfield images indicating that they lack type 1 binding fimbria.

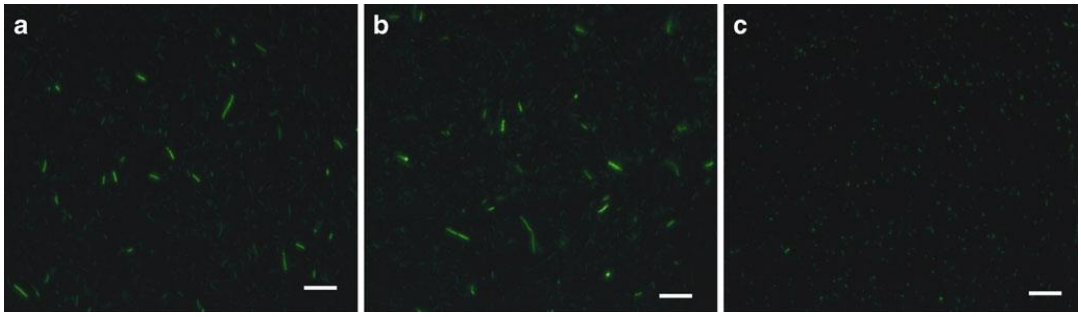

Figure 5.2. Fluorescent images of transformed a *E. coli* ORN178, b *E. coli* ORN208, and c *E. coli* 13762 with pGREEN plasmid as seen at 400× magnification. Scale bar 10 μm

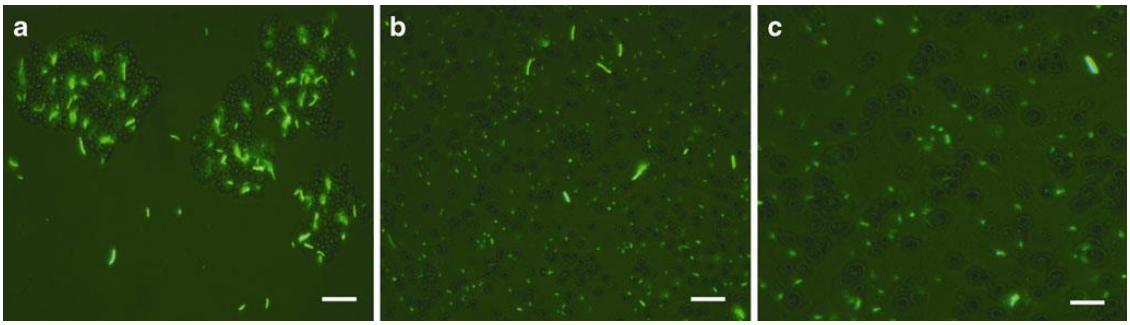

Figure 5.3. Yeast agglutination assay. a *E. coli* ORN178 binds to yeast cells and bring about clumping (400×), b no binding is seen in case of *E. coli* ORN208 (400×), and c shows no binding of *E. coli* 13762 with yeast cells. Scale bar 10 μm

Characterization of functionalized GNPs

The functionalized GNPs were characterized by UV-visible absorption spectroscopy, dynamic light scattering (DLS) and scanning electron microscopy (SEM) on pristine, MHDA, AEE- Au NPs and binding of E.coli ORN178 to Mn functionalized Au NPs. The SPR absorbance was found to be similar in the case of Sg-GNPs bound to *E. coli* 13762; hence, only the absorbance of Mn-Au NPs was studied. The plasmon band observed for the pristine Au NPs was at 570 nm which is characteristic of 200 nm GNPs (Figure 5.4).

The initial characterization of MHDA and AEE-GNPs also showed absorption maximum (max) of the surface plasmon band at 570 nm indicating the presence of functionalized GNPs in the functionalized samples. The Mn-GNPs when bound to *E. coli* ORN178 also displayed a SPR band of 570 nm. It is clearly seen that Au NPs keep their nano form even after chemical modifications. Although MHDA-Au NPs and AEE-Au NPs do aggregate as seen in Figure 5.5b and 5.5c, their size remains the same according to the DLS data obtained (Table 5.1). Figure 5.5d shows the binding of E.coli ORN178 to the Mn-Au NPs.

The pristine Au NPs as seen in the SEM image (Figure 5.5a) remain suspended, but the other samples do aggregate. The diameters of the samples measured in dispersion are listed in Table 5.1. The DLS data shows that that the mean diameter of pristine Au NPs is 213.5 nm. The diameter range of the pristine Au NPs is slightly larger than the quoted by the manufacturer (200 nm). This is not unexpected, as the DLS system is measuring the hydrodynamic size of the hydrated particles. The mean size of the MHDA-

Au NP, AEE-Au NP and *E.coli* ORN178 bound to Mn-Au NPs increase due to aggregation. All the four samples indicated a negative charge as determined by ζ potential measurements. The ζ-potential of the four samples ranged from -49.3 mV to -9.82 mV as the size of the sample increases. Mean ζ potential value of pristine Au NP colloids has been reported to be -42.5 (Diegoli, Manciulea *et al.* 2008), the values listed in Table 5.1 are consistent. As the Au NPs get functionalized they tend to aggregate and hence, could tend to be more unstable as a result of aggregation. This could cause the drop in ζ potential -49.3 mV of pristine Au NPs to -9.82 mV of the Mn-Au NPs bound to *E. coli* ORN178.

Binding of *E. coli* to GNPs

Binding specificities were examined as depicted in Figure 5.6. After incubation of Mn-GNPs with the *E. coli* ORN178 cells formed bacterial clumps, which emitted a bright fluorescence signal. These bacterial clumps consisted of hundreds of aggregated bacterial cells. The Mn-GNPs were found to aggregate in the presence of *E. coli* ORN178 only. Binding was in accordance to the yeast agglutination assay results showing that *E. coli* ORN178 has specific binding to mannan (Figure 5.6b&c). *E. coli* 13762 did not show any binding to Mn-GNPs, but consistently binds specifically to Sg-GNPs (Figure 5.6e&f). Bacterial clumps were formed by agglutination of Sg-GNPs and *E. coli* 13762 (Figure 5.6g&h). This proves that the two different *E. coli* have different binding specificities. The *E. coli* ORN178 did not show binding to Sg-GNPs. Specific binding of

Mn-GNPs to *E. coli* ORN178 and binding of Sg-GNPs to *E. coli* 13762 have been demonstrated.

This phenomenon is not a result of adsorption because no binding has been observed with bare GNPs lacking the Mannan or the Sg moieties (Figure 5.6a). *E. coli* ORN208 was used as a negative control in our experiments. It was not surprising that the abnormal type 1 pili expressing ORN208 clearly exhibited no binding to GNPs, Mn-GNPs or Sg-GNPs.

An example of non-attachment of *E. coli* ORN208 with Mn-GNPs (Figure 5.6d) is presented to show no binding. Samples at various stages of functionalization were kept aside to test binding of all the three bacterial strains. The intention was to see if any of the 16-MHDA/NHS/AEE/ECH-modified-GNPs could mediate aggregation by the means of surface absorption. Except for the sugar-modified-GNPs, no binding occurred during the course of the multi-step reaction.

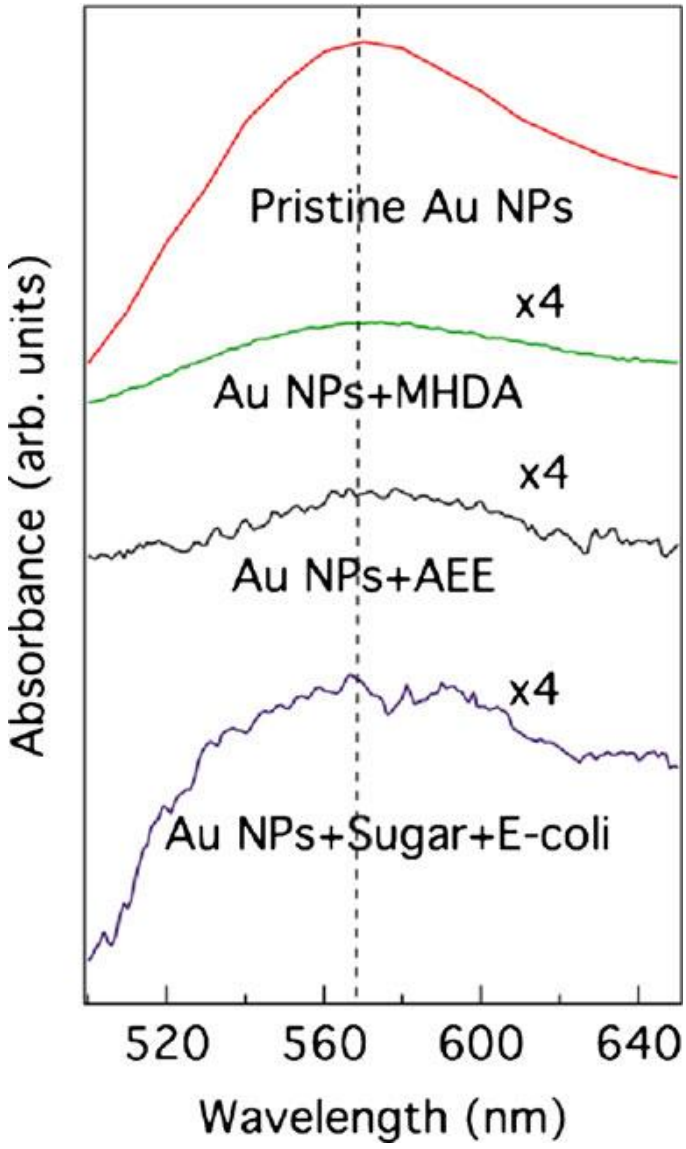

Figure 5.4. UV–vis absorbance of plain, 16-MHDA modified Au NP and Mn–Au NPs bound to *E. coli* ORN178

Table 5.1. DLS mean diameters and zeta potentials of functionalized Au NPs

Sample	*z* (mV)	Mean size (nm)
Pristine GNPs	-49.3	213.5
MHDA-GNPs	-36.6	226.4
AEE-GNPs	-14.0	462.7
Mn-GNPs	-9.82	5076

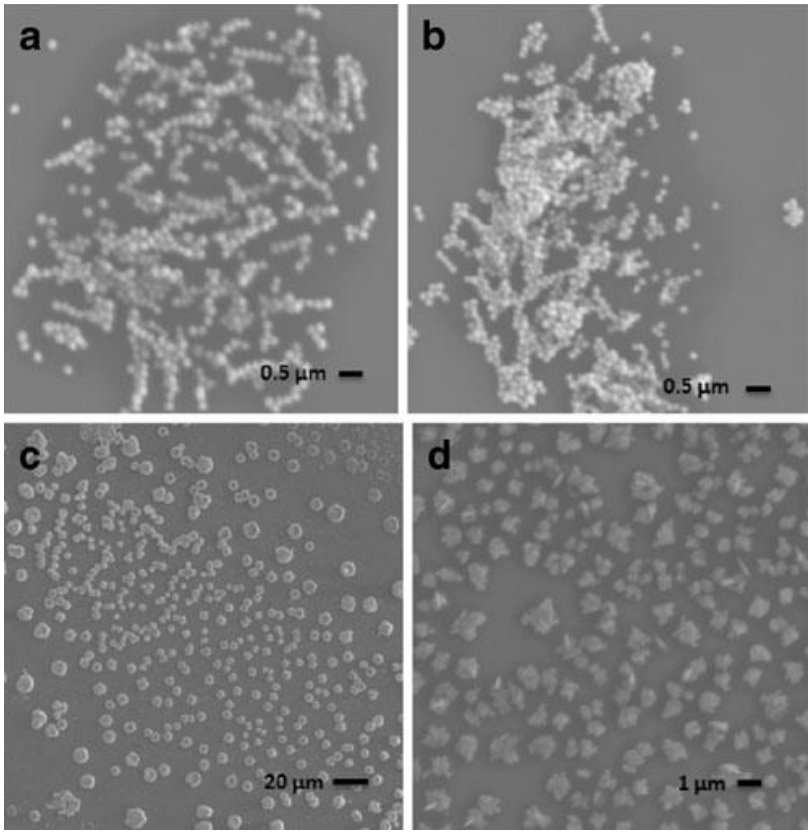

Figure 5.5. SEM images of a pristine Au NPs, b MHDA Au NPs, c AEE–Au NPs, and d aggregate of Mn–Au NPs *and E. coli* ORN178

Discussion

The optical spectrum of colloidal gold suspensions is dominated by SPR band, which is due to the collective dipole oscillation in the conduction band of gold. The position, intensity and the shape of the plasmon resonance band is influenced by many factors such as particle size and shape, dispersity and degree of aggregation (Diegoli, Manciulea *et al.* 2008). Dielectric constant and other stabilizing agents play an important role in conduction of Au band (David A 2003). The longitudinal plasmon resonance band has also been found indicative of Au NPs coming in close proximity during aggregation.

Diegoli *et al.* have also shown that the most there is a distinctive region in the plots of ζ-potential versus particle concentration, in which the ζ potential value is independent of nanoparticle concentration (Diegoli, Manciulea *et al.* 2008). It is referred to as the "stable region" of the plot. The results showed sample stability was indicated by their large negative ζ potential values, with the average mean ranging from -43 to -56 mV. Moreover, DLS and SEM results in this study have corroborated the results obtained by UV-visible spectroscopy.

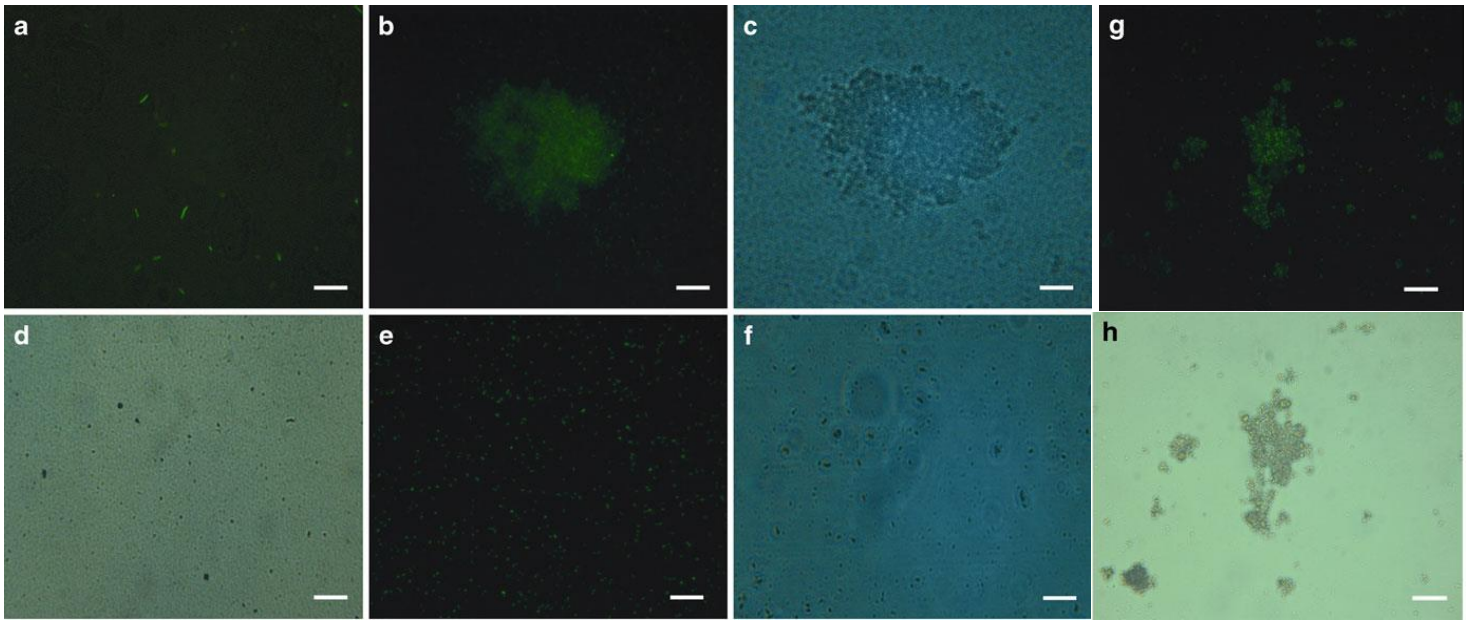

Figure 5.6. Bacterial agglutination by sugar functionalized GNPs. All images are in 400×. Scale bar 10 μm

a Control: plain GNPs with *E. coli* ORN178

b fluorescent image of Mn– Au NPs and *E. coli* ORN178

c brightfield image of Mn–Au NPs and *E. coli* ORN178

d negative control: bright filed image of *E. coli* ORN208 and Mn–Au NPs

e fluorescent image of *E. coli* 13762 and Mn–Au NPs

f bright field image of *E. coli* 13762 and plain Au NPs

g fluorescent image of *E. coli* 13762 and Sg–Au NPs

h brightfield image of *E. coli* 13762 and Sg–Au NPs.

Thus by exploiting the sensitivity of the optical parameters of particles, Au NPs can be used as a novel tool for pathogen detection which in this case is based on microbiological aspect of the *E. coli* strains binding to different sugars is simply based on their structural characteristics.

The most important life style of *E. coli* is its mechanism of attachment to host tissue. The myriad of adhesins discovered in *E. coli* to demonstrate the fact that it is an important determinant in establishment of bacterial attachment followed by colonization and infection. Pathogenic *E. coli* expresses many different kinds of adhesins which can be grouped according to their affinity to specific receptor structures. The functional organization of fimbrial lectin is different with respect to the position of the receptor binding sites. Primary sugar specificity or the fine sugar specificity by and large defines the specificity of the fimbrial lectin. The former refers to a simple carbohydrate structure, for example a monosaccharide that can inhibit the lectin mediated adhesion; whereas the latter refers to differences in binding to different oligosaccharides in lectins demonstrating the same primary sugar specificity (Sharon and Ofek 2000). Many enterobacteria express D-mannose lectins, which conform to the primary sugar specificity for type 1 fimbriae. Enterobacterial fimbrail adhesins that have been characterized according to their sugar specificities (Sharon and Ofek 2000). They are α-D-mannosides (type 1 fimbrial adhesin), α-D-Gal-(1-4)-β-D-Gal (P fimbrial adhesin) and NeuAcα2,3-galactose (S fimbrial adhesin) (Sokurenko, Chesnokova *et al.* 1997; Lin, Yeh *et al.* 2002).

The type 1 fimbria of *E. coli* ORN178 consists of mannose binding FimH lectin that aids in binding to glycoproteins that are a part of the adhesive domain on host cells.

The globoside-binding P fimbria of uropathogenic *E. coli* has shown to bind to the urolapkins on the surface of urothelial cells of the human bladder (Wu, Sun *et al.* 1996; Westerlund-Wikström and Korhonen 2005). Not only the type 1 fimbrial proteins have demonstrated a vital role in colonization of *E. coli* on host intestinal surfaces, they also play a role in causing urinary tract infections (Hung, Bouckaert *et al.* 2002). The enterotoxigenic (ETEC) *E. coli* (K99) causes diarrhea in animals such as pigs, calves and lambs (Simons, Willemsen *et al.* 1990).

A ganglioside glycolipid in FanC is involved in fimbrial adhesion, which consists of tetrasaccharide GalNAcβ-3Galα1-4Galβ1-4Glc in the binding pocket of K99. This ceramide when involved in lectin-binding pocket interaction brings about considerable conformational change in the binding protein that causes adhesion to host cells (Simons, Willemsen *et al.* 1991). Based on these factors two different types of *E. coli* strains were selected in order to study their binding specificity and from Figure 5.6 we can clearly concur that the primary and fine sugar specificities do play an important role in pathogenesis.

The yeast agglutination assay clearly indicated primary specificity by type 1 mediated binding of *E. coli* ORN178 to mannan residues on the surface of yeast cells. On the contrary, fine sugar specificity brought about no agglutination in *E. coli* 13762 (aka *E. coli* K99) with yeast cells as it exhibits type S fimbrial adhesins. It recognizes only a specific oligosaccharide sequence, i.e., NeuAc(α2-3)Gal(β1-4), which is not a part of yeast cell surface receptors. Due to the presence of abnormal pili, *E. coli* ORN208 failed to bind to yeast cells. A detailed study where mannose GNPs have been used to observe

the specific binding to FimH adhesion of bacterial type 1 pili by TEM has been demonstrated (Lin, Yeh *et al.* 2002). This establishes that aggregation of *E. coli* ORN178 was induced specifically by mannan recognition on the surface of the yeast cells.

In conclusion the UV-Vis measurements indicate the presence of Au SPR band confirming the nanoform of the particles despite functionalizing them. The mean sizes of the four select samples increased due to aggregation. The ζ potential dropped as the particles changed from a suspended to an aggregated state. We also found that mannose functionalized Au NPs (Mn-GNPs) specifically bind with *E. coli* ORN178 only but not with *E. coli* 13762. Fine sugar specificity was observed when Neuαc(α2-3)-Gal-(β1-4)Glc –Paa functionalized GNPs (Sg-GNPs) specifically showed binding with *E. coli* 13762 but not with *E. coli* ORN178. These results indicate that the binding induced by sugar-modified GNPs is specific to the characteristic sugar that bacteria recognize on binding sites located around the cell surface and, that such glyconanoparticles have the potential use in the identification of pathogens and also competitively prevent the binding of microorganisms to the eukaryotic cell surface receptors.

Biofunctionalized nanoparticles can serve as excellent carrier systems. With multiple receptors immobilized on carrier surface, each particle could interact with multiple bacteria resulting in the formation of aggregates. Merging of multiple aggregates mediated by these carrier particles could further prevent the bacteria from attaching to the host cells, especially in the urinary and the intestinal tracts owing to the high peristaltic pressures. The in vivo effects of the surface chemistry of the functionalized nanoparticles and their behavior in the UTI/intestinal tract warrant further investigation.

It is seen that UTI causing *E. coli* ORN178 evidently binds only to Mn-GNPs and not to Sn-GNPs. And ETEC 13762 exhibited specificity in binding to only Sn-GNPs and not to mannan. This indicates that there is high specificity in adhesin mediated binding exhibited by different microorganisms. From the above discussion it is clear that type 1 specificity is exhibited by many microorganisms that can easily bind to D-mannose and mediate generic adhesion, whereas certain microorganisms also recognize specific carbohydrate sequence which defines their fine sugar specificity. In the latter case, there is adhesin-specific adhesion that is not generic in nature. This means that binding of microorganisms can be altered with the use of carbohydrate functionalized nanoparticles to competitively bind to the cell surface receptors and block the adhesion of pathogens.

Theoretically, it can be envisioned that in such a scenario, binding can be reversed by aggregation of large amounts of functionalized GNPs clumping together and detaching the bacteria from the cell surface. Such nanoparticles could lead to the development of diagnostic tools that not only identify generic binding but also, can detect presence of specific species in a given genus. With the fast emerging antibiotic/drug resistant strains, nanoparticles may be employed to reduce the microbial load in diagnosis/treatment of medical diseases, contamination of food products, treatment of meat and poultry products, as carriers to deliver a particular drug for a localized effect, bioimaging of tissues and lastly as models for repairing cellular functions and alter pathogenesis. Our results there by indicate that blockage of bacterium-host interaction by specific sugar-modified GNPs can be an effective method for removal of bacteria from

hosts and detection of specific optical properties of the nanoparticles used can further assess the extent of binding.

Literature Cited

Aizpurua, J., Hanarp, P., Sutherland, D. S., Käll, M., Bryant, G. W., & García de Abajo, F. J. (2003). Optical Properties of Gold Nanorings. *Physical Review Letters, 90*(5), 057401.

Aslan, K., Lakowicz, J. R., & Geddes, C. D. (2004). Nanogold-plasmon-resonance-based glucose sensing. *Anal Biochem, 330*(1), 145-155. doi: 10.1016/j.ab.2004.03.032 S0003269704002647 [pii]

Aslan, K., Lakowicz, J. R., & Geddes, C. D. (2004). Tunable plasmonic glucose sensing based on the dissociation of Con A-aggregated dextran-coated gold colloids. *Analytica Chimica Acta, 517*(1–2), 139-144. doi: http://dx.doi.org/10.1016/j.aca.2004.04.060

Brewer, S. H., Glomm, W. R., Johnson, M. C., Knag, M. K., & Franzen, S. (2005). Probing BSA Binding to Citrate-Coated Gold Nanoparticles and Surfaces. [doi: 10.1021/la050588t]. *Langmuir, 21*(20), 9303-9307. doi: 10.1021/la050588t

David A, S. (2003). Plasmon resonant particles for biological detection. [doi: 10.1016/S0958-1669(02)00015-0]. *Current Opinion in Biotechnology, 14*(1), 13-22.

Diegoli, S., Manciulea, A. L., Begum, S., Jones, I. P., Lead, J. R., & Preece, J. A. (2008). Interaction between manufactured gold nanoparticles and naturally occurring organic macromolecules. [doi: 10.1016/j.scitotenv.2008.04.023]. *Science of The Total Environment, 402*(1), 51-61.

Dwek, R. A. (1996). Glycobiology: Toward Understanding the Function of Sugars. *Chem Rev, 96*(2), 683-720. doi: cr940283b [pii]

Haes, A. J., & Van Duyne, R. P. (2002). A Nanoscale Optical Biosensor: Sensitivity and Selectivity of an Approach Based on the Localized Surface Plasmon Resonance Spectroscopy of Triangular Silver Nanoparticles. [doi: 10.1021/ja020393x]. *Journal of the American Chemical Society, 124*(35), 10596-10604. doi: 10.1021/ja020393x

Harris, S. L., Spears, P. A., Havell, E. A., Hamrick, T. S., Horton, J. R., & Orndorff, P. E. (2001). Characterization of Escherichia coli Type 1 Pilus Mutants with Altered Binding Specificities. *J. Bacteriol., 183*(13), 4099-4102. doi: 10.1128/jb.183.13.4099-4102.2001

Haynes, C. L., & Van Duyne, R. P. (2001). Nanosphere Lithography: A Versatile Nanofabrication Tool for Studies of Size-Dependent Nanoparticle Optics. [doi: 10.1021/jp010657m]. *The Journal of Physical Chemistry B, 105*(24), 5599-5611. doi: 10.1021/jp010657m

Hung, C.-S., Bouckaert, J., Hung, D., Pinkner, J., Widberg, C., DeFusco, A., . . . Hultgren, S. J. (2002). Structural basis of tropism of Escherichia coli to the bladder during urinary tract infection. *Molecular Microbiology, 44*(4), 903-915. doi: 10.1046/j.1365-2958.2002.02915.x

Jain, K. K. (2005). Nanotechnology in clinical laboratory diagnostics. *Clin Chim Acta, 358*(1-2), 37-54. doi: S0009-8981(05)00145-2 [pii] 10.1016/j.cccn.2005.03.014

Krogfelt, K. A., Bergmans, H., & Klemm, P. (1990). Direct evidence that the FimH protein is the mannose-specific adhesin of Escherichia coli type 1 fimbriae. *Infect. Immun., 58*(6), 1995-1998.

Lazarides, A. A., & Schatz, G. C. (1999). DNA-Linked Metal Nanosphere Materials: Structural Basis for the Optical Properties. [doi: 10.1021/jp992179+]. *The Journal of Physical Chemistry B, 104*(3), 460-467. doi: 10.1021/jp992179+

Lin, C. C., Yeh, Y. C., Yang, C. Y., Chen, C. L., Chen, G. F., Chen, C. C., & Wu, Y. C. (2002). Selective binding of mannose-encapsulated gold nanoparticles to type 1 pili in Escherichia coli. *J Am Chem Soc, 124*(14), 3508-3509. doi: ja0200903 [pii]

Lin, C. C., Yeh, Y. C., Yang, C. Y., Chen, G. F., Chen, Y. C., Wu, Y. C., & Chen, C. C. (2003). Quantitative analysis of multivalent interactions of carbohydrate-encapsulated gold nanoparticles with concanavalin A. *Chem Commun (Camb)*(23), 2920-2921.

López-Cartes, C., Rojas, T. C., Litrán, R., Martínez-Martínez, D., de la Fuente, J. M., Penadés, S., & Fernández, A. (2005). Gold Nanoparticles with Different Capping Systems: An Electronic and Structural XAS Analysis. [doi: 10.1021/jp050184+]. *The Journal of Physical Chemistry B, 109*(18), 8761-8766. doi: 10.1021/jp050184+

Murray, C. B., Kagan, C. R., & Bawendi, M. G. (2000). Synthesis and characterization of Monodisperse Nanocrystals and close-packed Nanocrystal and assemblies. Annual Review of Materials Science, 30(1), 545-610 doi:10.1146/annurev.matsci.30.1.545

Ofek I, & R., D. (1994). *Bacterial adhesion to cells and tissues*: ondon: Chapman & Hall.

Ofek, I., Mirelman, D., & Sharon, N. (1977). Adherence of Escherichia coli to human mucosal cells mediated by mannose receptors. *Nature, 265*(5595), 623-625.

Qu, L., Gu, L., Li, H., Taylor, S., Elkin, T., Luo, P. G., . . . Sun, Y.-P. (2005). Galactosylated Polymeric Nanoparticles: Synthesis and Adhesion Interactions with Escherichia coli. [doi:10.1166/jbn.2005.009]. *Journal of Biomedical Nanotechnology, 1*, 61-67.

Rosi, N. L., & Mirkin, C. A. (2005). Nanostructures in biodiagnostics. *Chem Rev, 105*(4), 1547-1562. doi: 10.1021/cr030067f

Sambrook, J., & Russell, D. (2001). *Molecular Cloning: A Laboratory Manual*: Cold Spring Harbor Laboratory Press.

Schmitt, J., Decher, G., Dressick, W. J., Brandow, S. L., Geer, R. E., Shashidhar, R., & Calvert, J. M. (1997). Meta

l nanoparticle/polymer superlattice films: Fabrication and control of layer structure. *Advanced Materials, 9*(1), 61-65. doi: 10.1002/adma.19970090114

Sharon, N. (2006). Carbohydrates as future anti-adhesion drugs for infectious diseases. [doi: DOI: 10.1016/j.bbagen.2005.12.008]. *Biochimica et Biophysica Acta (BBA) - General Subjects, 1760*(4), 527-537.

Sharon, N., & Ofek, I. (2000). Safe as mother's milk: Carbohydrates as future anti-adhesion drugs for bacterial diseases. *Glycoconjugate Journal, 17*(7), 659-664. doi: 10.1023/a:1011091029973

Simons, B. L., Willemsen, P. T. J., Bakker, D., Roosendaal, B., De Graaf, F. K., & Oudega, B. (1990). Structure, localization and function of FanF, a minor component of K99 fibrillae of enterotoxigenic Escherichia coii. *Molecular Microbiology, 4*(12), 2041-2050. doi: 10.1111/j.1365-2958.1990.tb00564.x

Simons, L. H., Willemsen, P. T. J., Bakker, D., de Graaf, F. K., & Oudega, B. (1991). Localization and function of FanH and FanG, minor components of K99 fimbriae of enterotoxigenic Escherichia coli. [doi: DOI: 10.1016/0882-4010(91)90018-6]. *Microbial Pathogenesis, 11*(5), 325-336.

Sokurenko, E. V., Chesnokova, V., Doyle, R. J., & Hasty, D. L. (1997). Diversity of the Escherichia coli type 1 fimbrial lectin. Differential binding to mannosides and uroepithelial cells. [Research Support, Non-U S Gov't

Storhoff, J. J., Lazarides, A. A., Mucic, R. C., Mirkin, C. A., Letsinger, R. L., & Schatz, G. C. (2000). What Controls the Optical Properties of DNA-Linked Gold Nanoparticle Assemblies? [doi: 10.1021/ja993825l]. *Journal of the American Chemical Society, 122*(19), 4640-4650. doi: 10.1021/ja993825l

Svarovsky, S. A., Szekely, Z., & Barchi, J. J. (2005). Synthesis of gold nanoparticles bearing the Thomsen-Friedenreich disaccharide: a new multivalent presentation of an important tumor antigen. [doi: DOI: 10.1016/j.tetasy.2004.12.003]. *Tetrahedron: Asymmetry, 16*(2), 587-598.

Takae, S., Akiyama, Y., Otsuka, H., Nakamura, T., Nagasaki, Y., & Kataoka, K. (2005). Ligand Density Effect on Biorecognition by PEGylated Gold Nanoparticles: Regulated Interaction of RCA120 Lectin with Lactose Installed to the Distal End of Tethered PEG Strands on Gold Surface. [doi: 10.1021/bm049427e]. *Biomacromolecules, 6*(2), 818-824. doi: 10.1021/bm049427e

Varki, A. (1993). Biological roles of oligosaccharides: all of the theories are correct. *Glycobiology, 3*, 1297 - 1340.

Westerlund-Wikström, B., & Korhonen, T. K. (2005). Molecular structure of adhesin domains in Escherichia coli fimbriae. [doi: DOI: 10.1016/j.ijmm.2005.06.010]. *International Journal of Medical Microbiology, 295*(6-7), 479-486.

Wijaya, E., Lenaerts, C., Maricot, S., Hastanin, J., Habraken, S., Vilcot, J.-P., . . . Szunerits, S. (2011). Surface plasmon resonance-based biosensors: From the development of different SPR structures to novel surface functionalization strategies. [doi: 10.1016/j.cossms.2011.05.001]. *Current Opinion in Solid State and Materials Science, 15*(5), 208-224.

Wu, X. R., Sun, T. T., & Medina, J. J. (1996). In vitro binding of type 1-fimbriated Escherichia coli to uroplakins Ia and Ib: relation to urinary tract infections. *Proceedings of the National Academy of Sciences of the United States of America, 93*(18), 9630-9635.

Zhang, J., Geddes, C. D., & Lakowicz, J. R. (2004). Complexation of polysaccharide and monosaccharide with thiolate boronic acid capped on silver nanoparticle. [doi: DOI: 10.1016/j.ab.2004.05.051]. *Analytical Biochemistry, 332*(2), 253-260.

CHAPTER SIX

CONCLUSION

Various strategies have been employed in synthesis of nanoparticles in order to make them biocompatible. Based on the type of synthesis the nanoparticle's behavior in vitro varies. The initial studies of this dissertation focused on the cytotoxicity profiles of commercial, laser ablation and green synthetic particles. Extend of DNA damage caused in term of apoptosis indicated that not only apoptosis was size dependent but was also significantly different when compared to the commercial and laser ablation nanoparticles. Due to their chemically active surface charges the laser ablation particles show potential use in cancer therapy. Whereas there is also a great thrust in the green nanotechnology for development of cost effective methods of production of good quality nanostructures with practical commercial viability. But due the fact that green synthesis method leads to the formation of mono and poly-disperse nanoparticles, the stability and size control of the nanoparticles needs to be further investigated in order to the used as functional nanoparticles for biomedical applications.

The gold and silver nanoparticles used in this study showed promising in antibacterial properties due the partial influence of the properties of the extracts used. The antimicrobial properties of the extracts used enhanced synergistically enhanced the antibiotic activity against Staphylococcus aureus, Escherichia coli and Enterococcus faecalis. One limitation in the green synthesis was to obtain a pure mono-dispersed sample of nano structures. Also, methods to quantify the amount of nanoparticles per ppm/ml need to be established to quantify the minimal dose of nanoparticle to bring

about the desired antimicrobial effect. The biogenic NP-drug composite could open new avenues for the development of drugs for multi-drug resistant bacteria.

The fate of the nanoparticles in the cell is determined importantly based on its surface coatings. It was evident that bare nanoparticles elicit a different response compared to nanoparticles with different protein coatings of ALB and FBS. Nanoparticles with protein corona were less susceptible to cause cytotoxic effects in cells when compared to bare nanoparticles. Hence, protein coated nanoparticles could act as a good vector for drug therapy in cancer.

Based on the investigations on the cytotoxicity and size of the nanoparticles the last part of this dissertation dealt with the development of sugar functionalized nanoparticles that can be developed for biodiagnostic use. Urinary tract infection (UTI) is a predominant condition in prostate cancer patients. Escherichia coli ORN178 (EC-178) is the uropathogen that causes recurrent infection by binding specifically to adhesins of prostate cancer cells (DU-145 cells). In this study, the binding time of EC-178 to DU-145 cells, the cytotoxicity and uptake of plain and mannose functionalized and 20 and 200 nm GNPs (d-mannan (Mn)-GNPs) was investigated. It was seen that EC-178 binds and is inside the DU-145 cells by 3 h of incubation period. Plain 20 nm GNPs decrease the percentage of viable cells in 48 and 72 h in log and lag phase of DU-145 cells. It was also observed that the Mn-GNPs were taken up by the DU-145 cells significantly more than the plain GNPs.

Also, gold nanoparticles of 200 nm size were functionalized with two distinct glycoconjugates mannose (Mn–Au NPs) and Neuαc(α2-3)-Gal-(β1-4)Glc–Paa (Sg–Au

NPs) in order to investigate primary and fine sugar specificity of uropathogenic *E. coli* ORN178 and enterotoxigenic *E. coli* 13762, respectively. Fine sugar specificity was observed when Neuαc(α2-3)-Gal-(β1-4)Glc–Paa functionalized Au NPs (Sg–Au NPs) specifically showed binding with *E. coli* 13762 but not with *E. coli* ORN178. This specificity of *E. coli* strains to identify and bind to characteristic sugar moieties can be used in the development of biodiagnostic tools with Au NPs as carriers for diagnosis/treatment of human and veterinary diseases. In regards to the growing antibiotic resistance of microorganisms, gold nanoparticles can also be functionalized specifically to reverse adhesion of *E. coli* to host tissue and can be detected by their optical properties. There is an ocean of information coming forth at a rapid rate; nanotechnology definitely gives scope to much more biomedical applications in cancer therapy.

APPENDIX - RIGHTS AND PERMISSIONS

7/24/13 Rightslink Printable License
https://s100.copyright.com/AppDispatchServlet 1/4

SPRINGER LICENSE

TERMS AND CONDITIONS
Jul 24, 2013
This is a License Agreement between Pallavi Vedantam ("You") and Springer ("Springer") provided by Copyright Clearance Center ("CCC"). The license consists of your order details, the terms and conditions provided by Springer, and the payment terms and conditions.

License Number 3195361311018
License date Jul 24, 2013
Licensed content publisher Springer
Licensed content Publication Plasmonics
Licensed content title Binding of Escherichia coli to Functionalized Gold Nanoparticles
Licensed content author Pallavi Vedantam
Licensed content date Jan 1, 2012
Volume number 7
Issue number 2
Type of Use Thesis/Dissertation
Portion Full text
Number of copies 1
Author of this Springer Article Yes and you are the sole author of the new work
Title of your thesis / dissertation Functionalized nanomaterials and their biological applications
Expected completion date Aug 2013
Estimated size(pages) 138

License Number 3195361037120
License date Jul 24, 2013
Licensed content publisher Springer
Licensed content publication Cancer Nanotechnology
Licensed content title Size-dependent cellular toxicity and uptake of commercial colloidal gold nanoparticles in DU-145 cells
Licensed content author Pallavi Vedantam
Licensed content date Jan 1, 2013
Volume number 4
Issue number 1
Type of Use Thesis/Dissertation

Portion Full text
Number of copies 1
Author of this Springer article Yes and you are the sole author of the new work
Order reference number
Title of your thesis /dissertation Functionalized nanomaterials and their biological applications
Expected completion date Aug 2013

Terms and Conditions
With reference to your request to reprint in your thesis material on which Springer Science and Business Media control the copyright, permission is granted, free of charge, for the use indicated in your enquiry. Licenses are for one-time use only with a maximum distribution equal to the number that you identified in the licensing process. This License includes use in an electronic form, provided its password protected or on the university's intranet or repository, including UMI (according to the definition at the Sherpa website:http://www.sherpa.ac.uk/romeo/). For any other electronic use, please contact Springer at (permissions.dordrecht@springer.com or permissions.heidelberg@springer.com). The material can only be used for the purpose of defending your thesis, and with a maximum of 100 extra copies in paper. Although Springer holds copyright to the material and is entitled to negotiate on rights, this license is only valid, subject to a courtesy information to the author (address is given with the article/chapter) and provided it concerns original material which does not carry references to other sources (if material in question appears with credit to another source, authorization from that source is required as well).
Permission free of charge on this occasion does not prejudice any rights we might have to charge for reproduction of our copyrighted material in the future.

Altering/Modifying Material
Not Permitted You may not alter or modify the material in any manner. Abbreviations, additions, deletions and/or any other alterations shall be made only with prior written authorization of the author(s) and/or Springer Science + Business Media. (Please contact Springer at (permissions.dordrecht@springer.com or permissions.heidelberg@springer.com)

Reservation of Rights
Springer Science + Business Media reserves all rights not specifically granted in the combination of (i) the license details provided by you and accepted in the course of this licensing transaction, (ii) these terms and conditions and (iii) CCC's Billing and Payment terms and conditions.

Copyright Notice:Disclaimer
You must include the following copyright and permission notice in connection with any reproduction of the licensed material: "Springer and the original publisher /journal title, volume, year of publication, page, chapter/article title, name(s) of author(s), figure

number(s), original copyright notice) is given to the publication in which the material was originally published, by adding; with kind permission from Springer Science and Business Media"

No Transfer of License
This license is personal to you and may not be sublicensed, assigned, or transferred by you to any other person without Springer Science + Business Media's written permission.

No Amendment Except in Writing
This license may not be amended except in a writing signed by both parties (or, in the case of Springer Science + Business Media, by CCC on Springer Science + Business Media's behalf).

Objection to Contrary Terms
Springer Science + Business Media hereby objects to any terms contained in any purchase order, acknowledgment, check endorsement or other writing prepared by you, which terms are inconsistent with these terms and conditions or CCC's Billing and Payment terms and conditions. These terms and conditions, together with CCC's Billing and Payment terms and conditions (which are incorporated herein), comprise the entire agreement between you and Springer Science +Business Media (and CCC) concerning this licensing transaction. In the event of any conflict between your obligations established by these terms and conditions and those established by CCC's Billing and Payment terms and conditions, these terms and conditions shall control. Jurisdiction
All disputes that may arise in connection with this present License, or the breach thereof, shall be settled exclusively by arbitration, to be held in The Netherlands, in accordance with Dutch law, and to be conducted under the Rules of the 'Netherlands Arbitrage Instituut' (Netherlands Institute of Arbitration).*OR:*All disputes that may arise in connection with this present License, or the breach thereof, shall be settled exclusively by arbitration, to be held in the Federal Republic of Germany, in accordance with German law.