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LOMA LINDA UNIVERSITY
School of Medicine
Department of Earth and Biological Sciences
in conjunction with the
Faculty of Graduate Studies

Synthesis and Characterization of Hemocompatible Antibiotic Nanoparticles

by

Elvin Muhindo Walemba

A Dissertation submitted in partial satisfaction of
the requirements for the degree
Doctor of Philosophy in Biology

March 2020

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Each person whose signature appears below certifies that this thesis/dissertation in his/her opinion is adequate, in scope and quality, as a thesis/dissertation for the degree Doctor of Philosophy.

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ABBREVIATIONS

ADP	Adenosine diphosphate
AFM	Atomic Force Microscopy
Ag	Silver
$AgNO_3$	Silver nitrate
APTES	(3-aminopropyl) triethoxysilane
Au	Gold
BHI	Brain Heart Infusion
BHT	Butylated hydroxytoluene
BM1	Bimetallic silver-gold nanoparticle with 3% gold
BM2	Bimetallic silver-gold nanoparticle with 16% gold
BSA	Bovine serum albumin
CD	Cluster of differentiation
CDC	Centers for Disease Control
CFU	Colony forming unit
DLS	Dynamic Light Scattering
EDL	Electric Double Layer
EDS	Energy Dispersive X-ray Spectroscopy
EPA	Environmental Protection Agency
FDA	Food and Drug Administration
FSC	Forward scatter
GSH	Glutathione
H_2O_2	Hydrogen Peroxide

$HAuCl_4$	Chloroauric acid
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
hMSC	Human mesenchymal stem cells
IEP	Isoelectric Point
KCl	Potassium chloride
LPS	Lipopolysaccharide
MFI	Mean fluorescent intensity
MP-AES	Microwave Plasma-Atomic Emission Spectroscopy
NASA	National Aeronautics and Space Administration
NP	Nanoparticle
NTA	Nanoparticle Tracking Analysis
OD	Optical density
PBS	Phosphate buffered saline
PE	Phycoerythrin
PerCP	Peridim-chlorophyll proteins
PFA	Paraformaldehyde
PI	Propidium iodide
PPA	Percent platelet activation
PZC	Point of Zero Charge
rcf	Relative centrifugal force
SEM	Scanning electron microscope
SSC	Side scatter
TEM	Transmission Electron Microscopy

TLR	Toll-like receptor
UV-vis	Ultraviolet visible
W83	*Porphyromonas gingivalis* strain *W83*
WHO	World Health Organisation

ABSTRACT OF THE DISSERTATION

Synthesis and Characterization of Hemocompatible Antimicrobial Nanoparticles

by

Elvin Muhindo Walemba

Doctor of Philosophy, Graduate Program in Biology
Loma Linda University, March 2020
Dr. Danilo Boskovic, Chairperson

Porphyromonas gingivalis is a causative agent of periodontal disease and a risk factor for cardiovascular disease. Novel treatment of periodontal disease using nanomaterials can supplement or replace the use of antibiotics and mechanical debridement. Nanomaterials can be optimized for effectiveness and specificity and minimum toxicity. Synthesized Ag nanoparticles stabilized in Pluronic F127 were used to produce bimetallic core/shell Ag/Au nanoparticles with optimized Ag^{+} release and reduced toxicity. We tested the F127-stabilized nanoparticles for their antimicrobial activity and hemocompatibility in planktonic bacteria and citrated whole blood, respectively. Both Ag and Ag/Au bimetallic nanoparticles inhibited *P. gingivalis W83* growth. We then evaluated the hemocompatibility of the nanoparticles and the stabilizer F127 by probing their impact on platelet activation. Pluronic F127, over a range from 1 – 10^{-11} % w/v, on its own activated platelets above baseline (*p*-value 0.05). In contrast, Pluronic 127-stabilized Ag and Ag/Au bimetallic nanoparticles did not induce platelet activation above baseline (*p*-value 0.05). Our results suggest the Ag and Ag/Au bimetallic nanoparticles are effective inhibitors of bacterial growth, and stabilizer Pluronic F127 can activate platelets. More careful characterization of the role of Pluronic F127 in the context of thrombosis/hemostasis is needed.

CHAPTER ONE

INTRODUCTION TO NANOTECHNOLOGY

1.0.1 *A Short History*

The study of nanomaterials, which were termed "colloids" by Michael Faraday, can be traced back to his interest in understanding the color of colloidal gold. Faraday studied the interaction of light with gold particles in solution, focusing on how the particles were made, and the nature and properties of ruby gold. On February 5, 1857, Michael Faraday presented the findings in a paper entitled "Experimental relations of gold (and other metals) to light" to the Royal Society in London (Thompson, 2007). This may be considered the birth of modern colloidal chemistry and an essential step in the development of nanoscience. Still, others consider the speech given by Richard Feynman in 1959 entitled, "There's Plenty of Room at the Bottom," to be the first systematic presentation of nanotechnology. Nevertheless, the term "nanotechnology" was first used by Norio Taniguchi in a paper at the 1974 International Conference of Production Engineering entitled, "On the Basic Concept of 'Nano-Technology" (History of Nanotechnology, 2016).

1.0.2 *Terms and Definitions*

Since then, intense interest in materials at the nanometer scale has driven developments in nanoscience and nanotechnology. Nanoscience is the study of objects and systems where at least one dimension is 1–100 nm. The term nanotechnology, as defined by National Aeronautics and Space Administration (NASA), refers to the "creation of functional materials, devices, and systems through control of matter on the

nanometer scale (1-100 nm), and exploitation of novel phenomena and properties (physical, chemical, biological)" (NASA - NASA Ames Research Center Public Affairs Office, 2008). The European Commission's definition of nanoparticles is given below and is the definition followed in the following discussions.

> A natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50% or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm – 100 nm. In specific cases and where warranted by concerns for the environment, health, safety or competitiveness the number size distribution threshold of 50% may be replaced by a threshold between 1 and 50% (European Commission, 2011)

Various terms are used to describe nanomaterials. The terms are defined below to remove ambiguity. They include nanoparticle, nanocluster, nanopowder, colloid, nanocrystal, and quantum dots. Amorphous/semi-crystalline nanostructures smaller in size (i.e., 1–10 nm), with narrow size distribution, are termed nanoclusters. In contrast, colloidal materials are more polydisperse and less well characterized. The agglomeration of non-crystalline nanostructure subunits should best be termed a nanopowder. Any crystalline nanomaterial is referred to as a nanocrystal, and nanocrystals composed of semiconductor material are called quantum dots (Fahlman, 2011). Nanoparticles are the focus of this dissertation and the material referred to in the definition by the European Commission.

1.0.3 ***Source and Origin***

Nanoparticles form naturally or can be manufactured. They are present in the soil, water, air, and food (Griffin et al., 2017; Rogers, 2016). Natural nanoparticles include organic substances such as liposomes in living organisms (Bozzuto & Molinari, 2015;

Gao et al., 2013) and inorganics, such as silicates (Park et al., 2016), aluminosilicates (Heiligtag & Niederberger, 2013), and clay and other soil components (Dybowska et al., 2015).

1.0.4 *Applications and Uses*

Biochemistry, organic chemistry, molecular biology, physics, and surface science all employ nanotechnology. It is currently one of the fastest-growing fields in technology and continues to impact the development and design of a host of novel products, some of which may prove revolutionary or paradigm-shifting in their applications. According to StatNano, there are over 8,000 products and about 200 policy documents on nanotechnology ("STATNANO : Nano Science, Technology and Industry Information," 2017). Some manufactured products which have nanoparticles include clothing (Rivero et al., 2015) and topical applications (Gupta et al., 2013). Nanomaterials are also used in medicine and pharmacy for tissue engineering, drug delivery systems, cancer treatments and diagnoses, biodiagnostics, and imaging (Pelaz et al., 2017; Sutariya & Pathak, 2015). Thus, consistent guidelines for users are needed to classify nanomaterials according to properties, fate, and transport.

1.0.5 *Regulation of Nanotechnology*

The Environmental Protection Agency (EPA) lists four main types of synthetic nanomaterials. These are (1) carbon-based materials, (2) metal-based materials, (3) dendrimers, and (4) composites (U.S. Environmental Protection Agency, 2007). Nanomaterials are manufactured by controlled nucleation, self-assembly, templating, molecular assembly, and engineering, among other methods (Dhand et al., 2015; Ealias & Saravanakumar, 2017). Manufactured products that have nanoparticles include clothing

(Rivero et al., 2015) and topical applications (Gupta et al., 2013). Nanomaterials are also used in medicine and pharmacy for tissue engineering, diagnoses, drug delivery systems, treatments, and imaging (Pelaz et al., 2017; Sutariya & Pathak, 2015).

The EPA regulates nanomaterial-containing products under Toxic Substances Control Act (94th United States Congress, 1976) and Federal Insecticide, Fungicide, and Rodenticide Act (61st United States Congress, 1910). Food, drugs, or cosmetics containing nanomaterials are regulated under the Federal Food, Drug, and Cosmetic Act (75th United States Congress, 1938). Even with all these regulatory bodies, nanotechnology regulation in the United States is an area of concern because manufacturers have not been required to label products containing nanomaterials (Kessler, 2011). As of August 14, 2017, the EPA provides guidance for all nanomaterials under the Toxic Substances Control Act section 8(a). In doing this, the government hopes to manage the proliferation of nanoproducts and understand how this will affect health and the environment.

1.0.6 ***Nanopollution***

Nanopollution, defined as "all waste generated by nanodevices or during the nanomaterials manufacturing process" (Prasad, 2008), will only increase as new nanoproducts are developed and released to the market (Nanotechnology Workgroup, 2007). Flora and fauna have no natural mechanisms to process environmentally released inorganic nanomaterials. With development of nanotechnology and the accumulation of nanomaterials in nature, it is crucial to understand the effects of nanomaterials on flora and fauna. Any adverse effects must be reported so that regulatory agencies may make proper decisions about the use and application of nanotechnology.

1.1 Characteristics of Nanomaterials

1.1.1 *Scaling*

Nanotechnology is based on properties that scale with size (Guo et al., 2014). For example, the high surface-to-volume ratio of nanomaterials imparts high reactivity potential for catalysis or sensor applications. The volume of an object $V \propto L^3$, (where L is the characteristic atomic length) decreases with scale more rapidly than does the surface area, $S \propto L^2$, so that $S/V \propto L^{-1}$. This scaling dependence is reflected in changes in material properties, like melting point (Roduner, 2006; Singh et al., 2017). As a result, changes in material properties (e.g., magnetization, optical properties (color), melting point, hardness, etc.) relative to bulk characteristics can be modified without a change in chemical composition (Chiradze et al., 2016; Hasan, 2016; Roduner, 2006).

1.1.2 *Chemical and Physical Properties of Nanoparticles*

Physicochemical properties are defined as "physical properties, solvation properties related to interactions with different media, and properties or molecular attributes that define intrinsic chemical reactivity" (National Research Council, 2014). There are four main characteristics of nanoparticles that determine their effect on biological systems. These are material composition, size, shape, and surface chemistry (Albanese et al., 2012; Beddoes et al., 2015; Benetti et al., 2013; Janát-Amsbury et al., 2011; Lundqvist et al., 2008; Tiedemann et al., 2014). An in-depth analysis of different ways each characteristic of the metal can affect the function and use of the nanoparticle is beyond the scope of this dissertation. A brief explanation is given instead.

Nanoparticle physicochemical properties of composition, size, shape, and surface chemistry potentially affect nanoparticle-cell interactions, including interactions with the

immune system. These physicochemical properties also determine the efficiency of nanoparticle elimination from the body (Longmire et al., 2008). Biocompatibility, therefore, can be an essential consideration when synthesizing nanoparticles.

1.1.2.1 **Material**

The material from which a nanoparticle is synthesized determines its compatibility with living systems. For example, metals used to synthesize nanoparticles are chosen for their tunable mechanical, electrical, magnetic, optical, and chemical properties. Organic nanomaterials are synthesized and occur naturally (Sytar et al., 2017). Some natural organic nanoparticles include the viral capsid, horny materials on animals, and foraminifera. The chosen metal and its corresponding metal oxide can have varying reactivity. Figure 1 shows the reactivity of some metals that are commonly used to synthesize nanoparticles.

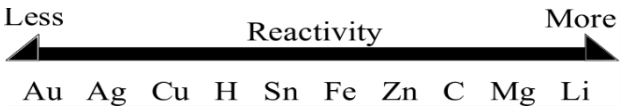

Figure 1. Reactivity of some metals commonly used in the synthesis of nanomaterials, from least to most reactive, with Hydrogen (H) and Carbon (C) included for reference. Redox potential is an essential characteristic of nanoproducts.

The desired use for the nanomaterial significantly affects the choice of the metal. For example gold and silver, silver-gold alloy nanoparticles are used in diagnostic imaging and drug delivery (Chen & Schluesener, 2008; Liu et al., 2012); carbon nanomaterials are used in electronics and optics (Jariwala et al., 2013); titanium dioxide is used in photocatalysis (Mayer et al., 2014); gold in cancer treatment (Cai et al., 2008); and silver as an antibiotic (Holden et al., 2016; Kundu, 2017; Yan et al., 2012).

1.1.2.2 **Surface Charging Effects**

Currently, the approaches to model charge, potential, and the force of the interface between the aqueous medium and nanoparticle surface, range from density functional theory and molecular dynamics calculations to empirical surface complexation models. The central concept that links these approaches is the electric double layer (EDL) model. When a surface is immersed in an electrolyte solution, there will be an equilibrium distribution of ions, with a decreasing population of ions of opposite charge away from the interface. A surface charge will give rise to an EDL that consists of an inner (compact) part, characterized by the chemical properties of the surface material, and a diffuse part built up of the mobile ions in the solution. These two parts, the compact (called Stern layer) and the diffuse layer, are typically described by Helmholtz, Gouy-Chapman, and Stern models. The interface between the compact and diffuse layers where the solvated ions move the nanoparticle is called the shear plane. The electrical potential at this point (1/e) relative to the surface is called the zeta potential (ζ) and is defined as the potential difference between the dispersion medium and the stationary layer of fluid attached to the particle.

An extension of the EDL model is the Derjaguin, Landau, Verwey, Overbeek (DLVO) theory, which models the aggregation of aqueous dispersions and describes the interaction energy, W_T, between charged surfaces interacting through a liquid medium. This model has three contributing parts, the van der Waals (VDW) attraction, and repulsion from the EDL, and steric repulsion due to bulky surface groups. The EDL size around the particle is pH, temperature, electrolytic type, and concentration-dependent.

(1) $$WT = W_{vdw} + W_{edl} + W_s$$

W_{vdw} is the VDW attractive energy

(2) $W_{vdw} = -A/(12 \pi D^2)$,

where A is the Hamaker constant, and D is the particle separation.

$W_{edl} = 2 \pi \varepsilon a \zeta^2 \exp(-\kappa D)$, where a is the particle radius, π is the solvent permeability, κ is a function of the ionic composition and ζ is the zeta potential. Note κ^{-1} is the double layer thickness or Debye length, where $\kappa^{-1} = \frac{0.304\text{nm}}{\sqrt{\text{I(mol/L)}}}$ at 25°C for monovalent ions, and *I* is the ionic charge (concentration of ions in solution). The Debye length is the distance where the EDL potential is 1/e of its initial value. Thus, increasing the ionic strength of the solution from 10^{-3} to 10^{-2} M will reduce the screening length from 10 to 3 nm. The EDL is proportional to both size and zeta potential. The steric repulsion W_s energy is short-acting and will be dependent on temperature, length, and branching of the surface polymeric coating. According to this model, repulsive EDL or steric forces dominate attractive VDW interactions in colloidally stable suspensions.

The zeta potential can be measured experimentally against pH. The pH point where the zeta potential is zero is the parameter pH_0. In electrokinetic measurements (light scattering), pH_0 is called the isoelectric point (IEP). At this pH, there is zero velocity for particles subjected to an external electric field. Analogous to proteins, the positive and negative charges around the nanoparticles are balanced. For metal oxides, if there are no other electrolytes apart from H^+/OH^-, zero velocity usually means zero net charge. In such a context, the term *point of zero charge* (*PZC*) is more appropriate. However, it is crucial to note IEP does not provide information about the location of charges. A nanoparticle is charged positive below and negative above its IEP. Nanoparticle charge impacts adsorption, is inversely related to size (Bakshi et al., 2015),

and is material-specific (Wagner et al., 2014). Moreover, nanoparticle reactivity and interactions, such as the formation of corona in human plasma, are significantly reduced, at IEP. Non-coated nanoparticles also show greater aggregation close to the IEP (Wagner et al., 2014; Zhang et al., 2016).

1.1.2.3 **Size and Shape**

Chemical reactivity of nanoparticles is inversely proportional to their size. Smaller nanoparticles have greater reactivity because of their higher surface area to volume ratios (Gatoo et al., 2014; Luo et al., 2015; Shameli et al., 2012). Smaller nanoparticles are also more likely to enter cells (Barar, 2015; Gatoo et al., 2014) and disrupt internal biochemical pathways, as well as cell-surface events (Durán et al., 2016a). Smaller nanoparticles can accumulate in the liver, lungs, kidneys, and even the brain (Recordati et al., 2016), leading to adverse health effects.

In addition to size, the shape of nanoparticles can directly impact its characteristics, because very different shapes can lead to similar volumes but different surface areas (Table 1). By altering the shape, it is possible to place nearly all the atoms on the surface, making nanoparticle shape a fundamental determinant of the reactivity of the nanoparticle (Bastús et al., 2012; Gilbertson et al., 2016). Atoms on a flat plane are less reactive because they have higher coordination than those at vertices (Bastús et al., 2012). The shape also determines how easily and readily cells take up the nanoparticles (Albanese et al., 2012; Buchman et al., 2016; Ahmed et al., 2016). For example, endocytosis occurs much faster for anisotropically-shaped nanomaterials than spherical nanomaterials (Gatoo et al., 2014). Nanoparticle shape also determines solvation (Jin et al., 2012), transport, and diffusion rate around the body (Uhl et al., 2018). Nanoparticle

shape determines the physical interactions within biological media and with cells and tissues, with some preferential interaction between the nanomaterial and specific protein (Albanese et al., 2012b; Ankamwar, 2012; Buchman et al., 2016; Toy et al., 2014). Sharp edges may cause cell and tissue injury, such as in blood vessels, and prevent cellular uptake by endocytosis (Vácha et al., 2011).

Noble metal nanoparticles (Au, Ag, Cu) exhibit localized surface plasmon resonance (LSPR) because of the oscillations of conduction electrons induced by light (Langer et al., 2017). Gold nanoparticles are used in sensors, probes, catalysis, and diagnostics because of their LSPR (Murphy, 2010). LSPR is shape-dependent (Rycenga et al., 2011), where corners induce more LSPR on a broader energy range than flat regions (Noguez, 2007). This would allow specific applications of nanoparticles based on their shape.

Table 1. Possible physicochemical characteristics of nanoparticles (adapted from Sun et al., 2014).

Nanoparticle Characteristic	Variation
Material	Protein-drug conjugate
	Liposome
	Solid lipid hybrid particle
	Hydrogel particle
	Polymer Particle
	Dendrimer
	Carbon nanotube
	Metal nanoparticle
Size	1 – 100 nm
Shape	Sphere
	Rod
	Cube
	Plate
	Star
Surface	Targeting ligand
	Surface charge
	Surface functional group
	PEGylation or other coating

1.1.2.4 **Surface Chemistry**

Surface atoms have higher energy than interior atoms, implying that surface modifications may be used to tune nanoparticles (Bastús et al., 2012). Moreover, coating nanoparticles, or addition of specialized functional groups, can alter their reactive properties Table 1. Surface modifications include: alloying (Alissawi et al., 2013; Navarro & Werts, 2013; Tao et al., 2014), covalent protein/drug conjugation (Di Pasqua et al., 2009; Huang et al., 2016; Shiang et al., 2010), polymer coating (Sahdev et al., 2014; Stevenson et al., 2012) or addition of surface functional group(s) (Di Pasqua et al., 2009; Giljohann et al., 2010; X. Li et al., 2014). Surface modifications may reduce toxicity (Sasidharan et al., 2016), enhance nanoparticle stabilization (Sahdev et al., 2014;

Stevenson et al., 2012) or bring about specific ligand targeting (Banerjee et al., 2016; Ding et al., 2012; Shiang et al., 2010).

1.2 Silver, Gold and Silver-Gold Bimetallic Nanoparticles

1.2.1 *Silver Nanoparticles*

Silver nanoparticles are the most widely used metallic nanomaterial in medicine; at least 75% of medical nanoparticle products contain silver (StatNano, 2017). Biomedical uses of silver nanoparticles include drug delivery (dos Santos et al., 2014; Mahl et al., 2012), diagnostics (Heera & Shanmugam, 2015; Wei et al., 2015), antiviral therapy (Lara et al., 2011; Slavin, 2006), anticancer therapy (Azam et al., 2012; Buttacavoli et al., 2018; Venugopal et al., 2017; Wei et al., 2015), as well asphotosensitizing and radiosensitizing agents (Wei et al., 2015).

The antibacterial activity of silver nanoparticles is well documented (Assar & Hamouda, 2010; Durán et al., 2016; Gurunathan et al., 2014; Helmlinger et al., 2016; Panáček et al., 2006; Rajeshkumar & Malarkodi, 2014; Rawashdeh & Haik, 2009; Smekalova et al., 2016; Swathy et al., 2014; Theivasanthi & Alagar, 2011; Thirumurugan & Dhanaraju, 2010; Vimbela et al., 2017; Xinping et al., 2011). Antibacterial silver is synthesized by both chemical and physical methods (Prabhu & Poulose, 2012; Wei et al., 2015). New “green synthesis” methods utilizing plant extracts have been used to produce silver nanoparticles with enhanced antibacterial and biocompatible characteristics (Gurunathan, 2015; Kuppurangan et al., 2015; Okafor et al., 2013; Rafique et al., 2017; Shameli et al., 2012; Siemieniec, 2013; Vadlapudi & Kaladhar, 2014).

1.2.2 *Gold Nanoparticles*

Gold nanoparticles have a number of well-established uses. Non-biomedical applications include optics (Carrillo-Cazares et al., 2017; Huang & El-Sayed, 2010; Huang et al., 2007), electronics (Lee et al., 2002; Homberger & Simon, 2010; Sousa et al., 2017), and in water purification systems (Kamala et al., 2015). The use of gold nanoparticles in biomedicine is also widespread (Alaqad & Saleh, 2016; Panahi et al., 2016) and includes photodynamic therapy, delivery of therapeutic agents (Dobrovolskaia et al., 2009), and in antibacterial treatments (Cui et al., 2012; Zhang et al., 2015; Zhao & Jiang, 2013; Zhao et al., 2010).

1.2.3 *Silver-Gold Bimetallic Nanoparticles*

Bimetallic nanoparticles are synthesized to harness the benefits of both incorporated metals. They can be synthesized as either core-shell or alloyed particles. For silver-gold bimetallic nanoparticles, the ratio of gold to silver and/or diameter of core vs. the thickness of the shell determines the activity of the nanoparticles. The additive or complementary effects of bimetallic nanomaterials broaden the applications compared to single metal nanoparticles.

1.2.3.1 **Uses of Silver-Gold Nanoparticles**

Pluronic-stabilized silver-gold bimetallic nanoparticles were reported to have higher catalytic activity in the reduction of 4-nitrophenol than either silver or gold nanoparticles individually (Holden et al., 2014), enhanced aluminum ion sensing capability (Zhou et al., 2013) and improved optical mercury detection (Tao et al., 2014). These enhancements are useful in the field of environmental monitoring. Moreover, gold alloying was found to lower nanoparticle toxicity towards oocytes (Tiedemann et al.,

2014), probably due to lower levels of free silver ions (Li et al., 2010). This is important for nanoparticles that contaminate water bodies because reduced nanoparticle toxicity reduces the environmental impact on fauna. In the context of health improvement, it was also reported that core-shell (Ag core, Au shell) nanoparticles significantly inhibited Lewis lung carcinoma growth (Shmarakov et al., 2017). Also, it was found that silver-gold alloys have tunable localized surface plasmon resonances (Hubenthal et al., 2005), indicating they can be synthesized for use in sensors, probes, catalysis and diagnostic applications (Alkilany & Murphy, 2010).

1.2.3.1.1 *Antibacterial bimetallic nanoparticles*

Although there are numerous uses for bimetallic silver-gold nanoparticles, their use in infection control has present and immediate urgency. Ag nanoparticles have an established antibacterial function (Vimbela et al., 2017; Smekalova et al., 2016; Durán et al., 2016). Table 2 outlines numerous studies that report the antibacterial efficacy of bimetallic silver-gold nanoparticles. Ag nanoparticles have higher antibacterial activity than Au nanoparticles, which have very low antibacterial activity. Some studies have reported that Au nanoparticles on their own do not have antibacterial activity (Tao, 2018). Yet, complexes of Au (I and III) have shown some antibacterial activity, as reported by Zhang et al. (2015). In this context, silver-gold bimetallic nanoparticles have an intermediate activity that is not directly proportional to the amount of Ag in the nanoparticle or the ratio of Ag to Au. Rather, the Au in the alloy disproportionally affects the antibacterial activity and toxicity of the nanoparticle. This effect is proportional to the Au content in the nanoparticle (Grade et al., 2014; Padmos et al., 2015; Ristig et al., 2015).

Table 2. Antibacterial activity of bimetallic silver-gold nanoparticles

Nanoparticle Description	Action	Reference
Au-Ag (core-shell)	High efficacy against Gram-negative bacteria at low [Ag]. NP attach to bacterial cell and damage membrane	Banerjee et al., 2011
Ag-Au alloy	Antibacterial activity is proportional to Ag content and placement. Cytotoxicity is dependent on [Au] but activity was lower than for pure Ag	Padmos et al., 2015
Biosynthesized Au-Ag alloy	Highest antitubercular activity compared to AgNP	Singh et al., 2016
Dextran-coated Ag-Au alloy	Antibacterial activity against numerous bacteria	Bankura et al., 2014
Commelina nudiflora-synthesized Au-Ag alloy	High minimum inhibitory and bactericidal activity against oral pathogens	Kuppusamy et al., 2017
Ag-Au alloy	Toxicity to Daphinia reduces as [Au] increases	Li et al., 2010
Ag-Au alloy	Ag-Au NP had no significant effect on hMSC compared to Ag and Au NP	Mahl et al., 2012
Ag-Au alloy	NP more biocompatible; 20% Au reduced antibacterial activity. To achieve similar MIC as pure Ag, 2x [AgAu] was needed	Grade et al., 2014
P. zeylanica-synthesized Ag-Au alloy	Inhibited single-culture biofilms 93-98% compared to control	Press et al., 2014
Ag-Au alloy	Toxicity is not proportional to [Ag]; Ag:Au of 80:20 showed the highest toxicity towards hMSC and HeLa cells	Ristig et al., 2015

Ag = silver, Au = gold; NP = nanoparticle; hMSC = human mesenchymal stem cells;

1.2.4 *Nanomaterials in the Human Body*

There are several ways that nanomaterials can enter the body: through dermal, respiratory, or gastrointestinal interfaces, with different responses from each site. The circumstances may include unexpected workplace or environmental exposure. Therefore, we need to understand the physiological effects of nanomaterials in the body.

1.2.4.1 **Nanoparticles at the Epithelium**

The skin has a large surface area and can be used to deliver local and systemic drugs (Desai et al., 2010; Vogt et al., 2016). The 10 μm thick keratin layer on the skin can prevent the absorption and unintended entry of nanomaterials across the skin when there are no tears, cuts, or abrasions. Neither free nanoparticles nor microparticles can cross intact skin (Prow et al., 2011). Nanoparticles, such as zinc oxide and titanium dioxide used in topical skin products do not penetrate intact skin (Cross et al., 2007; Kimura et al., 2012; Zvyagin et al., 2008). In contrast, polymeric nanoparticles can penetrate deep into the dermis in photo-damaged skin (Hung et al., 2015).

Nanoparticles experience a different type of barrier at the mucosal membrane. For example, at the mucosal membrane of the lungs, some nanoparticles translocate across the epithelial layer into the circulatory system (Todoroff & Vanbever, 2011), where macrophages may remove them through phagocytosis (Geiser et al., 2013; Semmler-Behnke et al., 2007). Microparticles are removed by mucociliary clearance along the bronchi (Todoroff & Vanbever, 2011).

The oral intake of nanoparticles by an average person is $\approx 10^{12}$ to 10^{14} nano- and microparticles daily (Simkó et al., 2010). Nevertheless, there is no consensus on the fate of nanoparticles in the gastrointestinal tract (Bergin & Witzmann, 2013; Date et al.,

2016). The alimentary canal lining also functions as a barrier to nanoparticle entry into the body. This makes it difficult to predict a general fate to nanoparticles in the gastrointestinal tract. More studies are required in this area because of the presence of nanoparticles in food, air, and water.

1.2.4.2 **Nanoparticles in Tissue**

For any nanomaterial to enter tissue, it must cross an epithelium. Once in the body, nanomaterials are distributed widely by fluids of the vascular and lymphatic systems to places where they interact with tissue cells and organs (Anjum et al., 2016). Organic nanoparticles may undergo physicochemical modifications such as encapsulation or formation of corona; however, inorganic nanoparticles persist and accumulate in the body (Mahapatro & Singh, 2011; Schaumann et al., 2015) mainly in the spleen, kidneys, and liver (De Jong et al., 2008; Recordati et al., 2016) and less in the lungs and heart (Sutariya & Pathak, 2015). Previously it was believed that the blood-brain barrier provided a very capable physical barrier to most substances. However, nanoparticles may accumulate in the brain due to “leaky regions” (ventricular and circumventricular areas) in the brain, lacking the protective shield of the blood-brain barrier (Saraiva et al., 2016). Recent studies on drug delivery to the brain show that nanoparticles cross the blood-brain barrier to deliver drugs into the brain (Yiqun et al., 2018).

1.2.4.3 **Nanoparticles in Blood**

Regardless of the method of entry, many nanoparticles end up in the blood, where they may have toxic effects on cells and tissues. Nanoparticles may also adsorb proteins to form a corona with accompanying increase in the hydrodynamic size, thus influencing biocompatibility, distribution, and clearance from the body (Liu & Peng, 2017; Lynch &

Dawson, 2008; Sasidharan et al., 2016). Nanoparticle-protein coronas differ because different surfaces attract/adsorb specific plasma proteins (Ge et al., 2015; Lundqvist et al., 2008; Lynch & Dawson, 2008). The corona influences cellular uptake, the host inflammatory response, and nanoparticle accumulation, degradation and clearance (Ge et al., 2015; Saptarshi et al., 2013). Interactions with vascular proteins such as fibrinogen may lead to structural and/or functional changes that can impact hemostasis (De Paoli Lacerda et al., 2010; Deng et al., 2011). Nanoparticle-induced changes in other proteins in the blood can adversely affect the inflammatory reactions (Borm, 2005; Khanna et al., 2015), the immune responses (Dobrovolskaia et al., 2008; Dobrovolskaia & McNeil, 2007), or hemostasis, through activation or inhibition of platelet action (Deb et al., 2011; Ilinskaya & Dobrovolskaia, 2013b; Jun et al., 2011; Ragaseema et al., 2012; Stevens et al., 2009).

Post-synthesis surface modification of the nanoparticles with biocompatible substances, such as polyethylene glycol or other polymers, facilitates better distribution, more efficient targeting, and reduced immune cell uptake (Anselmo et al., 2015). Surface modifications make nanoparticles suitable for medical and/or dental applications.

1.3 *Nanotechnology Summary*

Nanotechnology is a recent innovation on products that have been around for centuries, and are found in all aspects of daily life. As the use of nanotechnology becomes more widespread, government regulation to protect the health of all flora and fauna will need to become more standardized. Nanosilver has antibacterial activity and can play a role in infection control. However, its toxicity may limit its usefulness in clinical practice. Because nanoparticle activity is determined by size, shape, composition,

and surface chemistry, silver nanoparticles may be modified to enhance infection control while limiting toxicity. Alloys of silver and gold have been shown to have reduced toxicity and enhanced biocompatibility. Combined with surface capping using polymers or other biocompatible substances can broaden the use of silver nanoparticles into many different applications.

1.4 Periodontal Disease

Periodontal disease comprises a chronic inflammatory condition characterized by destruction of the periodontal tissues and resulting in loss of connective tissue attachment and alveolar bone, and development of pathologic pockets around the diseased teeth (Loe, 1993). It is a ubiquitous disease but not commonly associated with adverse health outcomes. According to the World Health Organization (WHO), gingival bleeding and periodontal disease are common across all age groups worldwide (Table 3), and both are signs of poor oral health (Petersen & Ogawa, 2012). Although there are regional and geographical differences in oral health, there is an observable trend that prevalence of severe periodontitis increases with age for all regions represented. As of 2010, 3.9 billion people globally have had some form of oral disease or complications associated with untreated caries in their permanent teeth. As 35% of the world's population is affected, periodontal disease represents the most prevalent oral health problem worldwide (Marcenes et al., 2013). In the United States, the National Health and Nutrition Examination Survey of 2010 reported that of adults 30 years and older, more than 47% had some form of periodontitis, with severity ranging from mild (8.7%), and moderate (30.0%), to severe (8.5%) (Eke et al., 2015; Eke et al., 2012). These findings indicate the periodontal disease is a significant health burden nationally.

Disease severity is determined by physical examination of teeth and gums. Although gum disease is present in all populations, its severity is not uniform. The assessment involves measuring the pocket depth from six sites of all teeth except the molars (Eke et al., 2015). Pocket depths can range from ≤ 3 mm (i.e., healthy) through ≥ 6 (i.e., advanced periodontitis) (Sweeting et al., 2008) (Table 4). Additionally, the chronic inflammation associated with periodontitis may also cause bleeding on probing.

The 65–74-year-old group has the highest prevalence of periodontitis. Many adults have little or no dental insurance and do not have regular dental check-ups. As a result, oral disease can progress from gingivitis to advanced periodontitis before they seek treatment from a dentist.

Table 3. Prevalence of mild to severe forms of periodontal disease in different age groups around the world. Data in this table is adapted from Petersen & Ogawa (2012).

Age Group (years)	Percentage of Population Presenting with	
	Gingival Bleeding and Calculus	Severe Periodontitis
15–19	40–80	10–15
35–44	30–60	10–20
65–74	20–80	5–25

Table 4. Periodontal disease severity in relation to pocket depth. (Data compiled using Eke et al., 2015).

Pocket Depth (mm)	Disease Severity
3	Healthy
≤4	Gingivitis
4–5	Slight periodontitis
5–6	Moderate periodontitis
≥6	Advanced periodontitis

1.4.1 *Risk Factors*

Large epidemiological and clinical studies identified risk factors associated with development of periodontal disease. Various risk factors are listed in Table 5, showing that periodontal disease is a complex process.

Table 5. Risk factors for periodontal disease

Risk Factor	Effect on Periodontal Disease	Reference
Age	64% of people aged ≥65 have either moderate or severe periodontitis	Eke et al., 2012; Shiau & Reynolds, 2010; Genco & Borgnakke, 2013
Gender	Risk in men > risk in women. Men have poorer oral hygiene. Pregnancy is also a factor.	Shiau & Reynolds, 2010; Shulze & Busse, 2016; Genco & Borgnakke, 2013
Race	Order of prevalence is Hispanic > non-Hispanic blacks, non-Hispanic Asian Americans > non-Hispanic Whites.	Eke et al., 2012; Eke et al., 2015; Genco & Borgnakke, 2013
Socioeconomic Status	Higher prevalence in poorer, less educated than in higher earning more educated.	Eke et al., 2012; Eke et al., 2015; Genco & Borgnakke, 2013
Smoking	More prevalent in smokers; more aggressive infection; higher incidence of tooth loss; smoking-induced damage to the vasculature, and supportive periodontal structures	Eke et al., 2012; Eke et al., 2015; Arcavi & Bnowitz, 2004; Bagaitkar et al., 2008; Naderi et al., 2015; Mai et al., 2013
Alcohol	Consumption promotes development and progression	Lages et al., 2015; Shepherd, 2011; Tezal et al., 2001;
Diabetes	Diabetics are at higher risk. Diabetes disrupts glycemic control.	Keller et al., 2015; Preshaw et al., 2012
Metablic Syndrome	Positive correlation	Bharti & Khurana, 2009; Gurav, 2014; Kaye et al., 2016
Osteoporosis	More aggressive	Esfahanian et al., 2012; Koduganti et al., 2009; Garcia et al., 2000
Stress	General health risk	Goyal et al. 2013; Mannem & Chava, 2012; Warren et al., 2014
Genes	Polymorphisms in interleukins 1A, 1B, 6, 10, MMP-3, and MMP-9 are associated with significant risk	da Silva et al., 2017; Michalowicz, 1994
Diet	Foods rich in antioxidants are protective against periodontitis	Woelber et al., 2017
Immunodeficiency or immune-suppressed	Immunodeficient people are more likely to develop periodontitis with progresses faster	Gracia et al., 2000

Periodontal disease is due to a bacterial infection with a number of bacteria routinely recovered from periodontitis patients. The most prevalent are Gram-negative anaerobic bacteria including: *A. actinomycetemcomitans*, *P. gingivalis*, *P. intermedia*, *B. forsythus*, *C. rectus*, *E. nodatum*, *P. micros*, *S. intermedius* and *Treponema sp.* (Lovegrove, 2004). Of these, the "red complex" bacteria, *P. gingivalis*, *Treponema denticola*, and *Tannerella forsythia* are the most important pathogens in adult periodontal disease (Contreras et al., 2015; Suzuki et al., 2013).

1.4.2 *Porphyromonas gingivalis Infection*

P. gingivalis is important in the development of periodontal disease and is associated with various systemic diseases including atherosclerosis (Hayashi et al., 2011; Hussain et al., 2015; Lei et al., 2011; Li et al., 2002; Rodrigues et al., 2012), rheumatoid arthritis (Maresz et al., 2013; Zaric et al., 2010), oral and oro-digestive cancers (Atanasova & Yilmaz, 2014; Ha et al., 2015; Nagy et al., 1998) and cardiovascular disease (Olsen & Yilmaz, 2016; Velsko et al., 2014). Control and management of *P. gingivalis* infection and maintenance of good oral health is, therefore, helpful in preventing periodontitis and minimizing the development of systemic disorders.

1.4.2.1 *Porphyromonas gingivalis* Natural History and Virulence Factors

P. gingivalis is a Gram-negative, rod-shaped, asaccharolytic, anaerobic, pathogenic bacterium of the red complex, predominantly found in the oral cavity as part of a biofilm (Lanza et al., 2016; Rôças et al., 2001; Suzuki et al., 2013). In lab cultures, *P. gingivalis* forms black colonies on blood agar due to the accumulation of hemin on cell surface. As a "keystone pathogen", *P. gingivalis* affects the growth and development of the biofilm and disrupts the normally balanced host-microbial interaction, thus leading to

disease (Hajishengallis et al., 2012a). *P. gingivalis* can also evade or counter the host immune system establishing itself in the periodontal pocket (Hajishengallis, 2014; Hajishengallis et al., 2012; Olsen & Hajishengallis, 2016; Zenobia & Hajishengallis, 2015). *P. gingivalis* disrupts normal leukocyte migration and immune defense function, thereby causing inflammation, through which it can obtain nutrients from tissue (Bostanci & Belibasakis, 2012; Darveau et al., 1998; Maekawa et al., 2014; Zenobia & Hajishengallis, 2015). Immune system disruption contributes to tissue destruction.

Numerous *P. gingivalis* virulence factors are involved in tissue destruction and evasion of the host immune system. Holt et al. (1999) listed over 30 virulence factors that function to establish the bacterium in the host. However, the major virulence factors of *P. gingivalis* are a capsular polysaccharide, lipopolysaccharide (LPS), the major fimbriae, and gingipains (Bostanci & Belibasakis, 2012).

1.4.2.1.1 ***Capsule***

The capsule is a heterogeneous polysaccharide polymer exterior to the LPS that protects against phagocytosis and enzymatic destruction (Bostanci & Belibasakis, 2012; Singh et al., 2011). For example, dendritic cells and macrophages phagocytose non-encapsulated *P. gingivalis* at 4 and 30 times the rate of encapsulated *P. gingivalis*, respectively (Singh et al. 2011). Capsulated *P. gingivalis* survived phagocytosis by dendritic cells and macrophages in a study comparing a capsule-deficient mutant and a capsulated parental strain (Singh et al., 2011). Capsulated *P. gingivalis* was also shown to dampen the immune response and disperse from gingival tissue to infect and colonize distant areas of the body (Hajishengallis, 2014; Irshad et al., 2012; Iwai et al., 2011; Nakayama & Ohara, 2017). Further, encapsulated *P. gingivalis* disrupts the leukocyte

response (Brunner et al., 2010), invades human gingival fibroblasts and potentially survives even in the presence of antibiotics (Amornchat et al., 2003; Brunner et al., 2010; Irshad et al., 2012). In mouse models, encapsulated *P. gingivalis* produced a spreading type of infection with exudate, compared to a localized infection of non-encapsulated *P. gingivalis* (Laine & Winkelhoff, 1998), and enhanced cellular invasion associated with co-aggregation in mixed infection models (Polak et al., 2017). The capsule acts as a physical barrier shielding microbial surface components from the immune system (Lenz et al., 2016; Singh et al., 2011). It greatly enhances the virulence and survival of *P. gingivalis* (Sing et al., 2011). Similar processes may occur with *P. gingivalis* infections in humans, enhancing virulence and evading immune clearance.

1.4.2.1.2 ***Lipopolysaccharide***

P. gingivalis LPS is different from that of other Gram-negative bacteria (Laheij et al., 2015; Rangarajan et al., 2008). While LPS, in general, is a potent activator of host defense response and highly inflammatory (Pulendran et al., 2001), *P. gingivalis* LPS elicits only a weak immune response. Heterogeneous lipid A molecule of *P. gingivalis* LPS is responsible for reduced immune response (Jain & Darveau, 2010) and enhanced virulence and pathogenicity. The modified lipid A molecule also allows the bacterium to manipulate the Toll-like receptor (TLR) response and promote chronic inflammation (Sochalska & Potempa, 2017). High levels of LPS promote inflammation associated with *P. gingivalis* infection through the delay of neutrophil apoptosis, increased production of interleukin-1 beta, tumor necrosis factor-alpha, and interleukin-8, all of which are pro-inflammatory cytokines (Murray & Wilton, 2003). LPS also increases oxidative stress in the cell model of infection with ligament fibroblasts (Gölz et al., 2014). Additionally, *P.*

gingivalis produces outer membrane vesicles containing gingipains and LPS, which facilitate bacterial aggregation, colonization, and interaction with host tissues and cells (Nakayama & Ohara, 2017).

1.4.2.1.3 ***Fimbriae***

Enersen et al. (2013) provided an extensive review of *P. gingivalis* fimbriae, which are proteinaceous surface structures 3–25 μm in length present on most strains. Fimbriae are grouped into short and long,with long fimbriae further subdivided into six genotypes (I, Ib, II-V), withextensive heterogeneity (Lenz et al., 2016). Short fimbriae appear to play a role in the auto-aggregation of bacteria (Lin, Wu, & Xie, 2006), without which accumulation and subsequent colonization would prove more difficult.

In contrast, the long type II fimbriae are reported to enhance *P. gingivalis* virulence through improved adhesive and invasive interactions of bacteria with epithelial cells (Kuboniwa et al., 2009; Amano et al., 2004; Weinberg et al., 1997; Inaba et al., 2007; Nakagawa et al., 2006). In addition, type II fimbriae are also more efficient inducers of severe inflammatory response (Inaba et al., 2007). Moreover, type II fimbriae also inhibit migration and proliferation of host cells (Nakagawa et al., 2006).

Since most *P. gingivalis* possess both short and long fimbriae, these have a combined virulence promoting effect on bacterial aggregation and colonization, inflammation, and dysregulation of host cellular migration.

1.4.2.1.4 ***Gingipains***

Gingipains are cysteine proteases that are believed to be most significant and the most studied *P. gingivalis* virulence factors (Uehara et al., 2008). These proteases are found either on cell surface, in secreted vesicles or as soluble proteins. Gingipains are

encoded by genes *rgpA*, *rgpB,* and *kgp,* and are divided into two main groups, namely arginine gingipains (RgpA and RgpB) and lysine gingipains (Kgp). Rgp catalyzes the hydrolysis of proteins and small molecule substrates with a preference for Arg in position one. The Kgp gingipain has strict specificity for lysyl bonds.

Gingipains are likely responsible for almost all *P. gingivalis* mediated tissue destruction through their proteolytic activity (Sheets et al., 2006). They are central to periodontal disease progression and cardiovascular disease development (Kurita-Ochiai & Yamamoto, 2014; Marschall, 2016; Osbourne et al., 2012; Uehara et al., 2008). Gingipains modulate adherence to and colonization of host cells (Chen et al., 2001), neutralize host defenses (Guo et al., 2010), manipulate host inflammatory response (Hajishengallis, 2014), promote tissue destruction for nutrient acquisition (Suzuki et al., 2013), and lead to cell death during invasion and dissemination both in the periodontium and other tissues (Velsko et al., 2014; Yilmaz et al., 2006; Yousefi et al., 2008). Gingipains also have important "housekeeping" functions for *P. gingivalis*. In this context, they control enzyme processing for various cell surface proteins, and participate in the maturation of the hemoglobin-binding receptor protein domain, hemagglutinin (Imamura, 2003). Gingipains also modulate coinfection with other bacteria (Jung et al., 2017), which contribute to biofilm formation. Finaly, gingipains are crucial for the establishment, persistence, and progression of *P. gingivalis* infection and for periodontal disease.

1.4.3 *Summary of Periodontal Disease*

Periodontal disease is a significant health problem worldwide with the highest incidence rate of severe periodontitis in older age group (i.e., 65–74). A number of studies indicate a strong correlation between periodontal disease and systemic diseases, including atherosclerosis and other cardiovascular diseases. It appears that a small number of bacteria, the red complex, appear to be responsible for periodontal disease. Of these, the keystone pathogen, *P. gingivalis*, plays an important role in the establishment and progression of periodontitis, through the action of its various virulence factors, which affect growth and development of the oral biofilm and disrupt the normal host-microbial interaction. These virulence factors enhance the pathogenicity of *P. gingivalis* and make it an important target for control of periodontitis.

1.5 Scope of the Dissertation

There is a need for new types of effective antibiotics because of microbial development of resistance to well established antibiotics and because of new emerging infections. Nanotechnology represents a new approach to infection control and treatment. Nanoparticles made of silver were shown to have antibiotic activity against numerous bacteria. Properties of nanoparticles can be specifically designed by changing their size, shape, and surface chemistry, In order to enhance biocompatibility and reduce toxicity to host cells. A way to modifysilver nanoparticles is to alloy them with gold, reducing silver toxicity and enhancing the overall nanoparticle biocompatibility. Additional modifications using surface capping agents such as polymers can further improve biocompatibility. Biocompatible antimicrobial silver-gold bimetallic nanoparticles may prove to be an effective tool for control and/or elimination of *P. gingivalis* infection, and

thus provide an additional or alternative treatment for periodontal disease and elimination of the associated systemic complications.

In Chapter Two, a more detailed look at the use of nanoparticles for the management of *P. gingivalis* infection is presented. The synthesis and characterization of monodisperse nanoparticles are discussed with an emphasis on size determination.

In Chapter Three, synthesized nanoparticles are evaluated for their ability to inhibit the planktonic growth of *P. gingivalis*. By managing *P. gingivalis* infection and reducing or preventing the development of oral biofilm, and subsequent progression from gingivitis to periodontitis, antibacterial nanoparticles can be a contributing factor in the improvement of oral and systemic health.

In Chapter Four, synthesized nanoparticles are tested for their effect on hemostasis. The effects of synthesized nanoparticles on platelet activation are examined in a whole human blood model. The objective is to develop efficacious antimicrobial nanoparticles which are also biocompatible.

In Chapter Five, we present a summary of the main elements and results of the study, their implications, and also offer suggestions for future studies to enhance the understanding of the role of nanoparticles as hemocompatible antibiotics.

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CHAPTER TWO

SYNTHESIS OF SILVER AND SILVER-GOLD BIMETALLIC NANOPARTICLES

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2.1 Abstract

Silver nanoparticles, as broad-spectrum antimicrobial agents, also exhibit cellular toxicity. Surface modifications, however, can improve biocompatibility and reduce toxic side effects. Alloying of silver nanoparticles with gold, for example, modulates their reactivity and toxicity. Moreover, due to the predominant impact of surface effects, smaller nanoparticles tend to have higher cytotoxicity. Thus, in addition to surface modifications, control of nanoparticle size distribution is also needed for adequate toxicity control. Using a modified Tollens' reaction where the solution pH and silver nitrate to maltose ratios were varied, we synthesized ≈ 20 nm silver (Ag) and silver-gold bimetallic alloy nanoparticles with ≈3 or ≈16% gold (BM1 or BM2 respectively). The nanoparticle sizes produced, with the same chemical composition, was technique-dependent, and monitored using dynamic light scattering (DLS), atomic force microscopy (AFM), and transmission electron microscopy (TEM) ($p \leq 0.05$). With increasing gold content nanoparticle sizes tended to be smaller, so that Ag>BM1>BM2, independent of the sizing method ($p \leq 0.05$). These results reveal that size determination is sensitive to the characterization method, which in turn is a function of the hydration shell for DLS, surface coating for TEM, and indentation forces for AFM.

2.2 Introduction

Antibiotic resistance has greatly increased over the last 60 years (Brooks & Brooks, 2014). This is a global problem and covers widely used antibiotics against common infectious organisms such as *Staphylococcus*, which exhibited resistance to penicillin in 1940, methicillin in 1962, linezolid in 2001, vancomycin in 2002 and ceftaroline in 2011. Moreover, extensively drug-resistant organisms such as tuberculosis have emerged since 2000 (Ventola, 2015a; Brooks & Brooks, 2014). To combat antibiotic resistance in bacteria, the Centers for Disease Control (CDC) and others recommended improved diagnoses, antibiotic stewardship, tracking of antibiotics and their prescriptions, along with optimized therapeutic regimens and infection control (Ventola, 2015b; Brooks & Brooks, 2014). While antibiotic resistance is likely contributed to by overuse, inappropriate prescribing, and extensive agricultural use, an often overlooked but important cause is the relative lack of new effective antibiotics (Ventola, 2015a; Brooks & Brooks, 2014).

Important progress against antibiotic resistant infections includes development of new treatment agents. Some new antibiotics, based on conventional chemistry/biochemistry, have recently been approved and deployed. These include dalbavancin, oritavancin, tedizolid, and avibactam. Other next-generation aminoglycosides, beta-lactamase inhibitors, quinolones, ketolides, tetracyclines, and oxazolidinones, are also in development (Ventola, 2015b). Local drug delivery may also help to reduce the development of drug resistance (Brooks & Brooks, 2014). Compared to conventional drugs, nanoparticle delivery systems have enhanced or increased cellular penetration, and can be modified for targeted drug delivery and other functional

advantages (Palez et al., 2017). Silver nanoparticles have been used to control microbes for some time (Wang et al., 2017; Dhanalakshmi et al., 2013; Gurunathan et al., 2014). With developing nanotechnology new products with lower host toxicity and improved biocompatibility will likely become an important addition to infection control strategies.

2.2.1 **Nanoparticles**

Nanomaterials are characterized by sizes ranging from 1 to 100 nm in at least one dimension. Because of the heightened surface attributes, their physicochemical properties differ from the bulk. Nanoparticles are used to treat infections and as drug carriers in biomedicine (Faraji & Wipf, 2009; Pelaz et al., 2017). In dentistry, nanomaterials are used to treat caries, in tooth whitening and surface polishing, for dental fillings and implants, and as ingredients in toothpaste (Priyadarsini et al., 2018; Noronha et al., 2017). Some of the current research is aimed at developing nanomaterials suitable to replace conventional dental composites (Bapat et al., 2019; Van Landuyt et al., 2014).

2.2.2 ***Antibiotic Nanoparticles***

Antimicrobial nanoparticles are of particular interest because they can prevent bacterial infections when used in composites or as constituents of other biomaterials. Additionally, antimicrobial nanoparticles can be used to treat and control established bacterial infections (Wang et al., 2017; Pelaz et al., 2017)). This is important because oral infections are known to contain bacteria that can develop resistance to conventional treatments (Feres et al., 2002; Rams et al., 2014; van Winkelhoff et al., 2000). Silver nanoparticles possess known antibacterial properties, but they have overlapping therapeutic and toxicity windows. However, silver nanoparticles can be modified for

biocompatibility through surface functionalization that includes capping/coating and alloying with other metals (Grade et al., 2014; Irwin et al., 2010; Ravindran et al., 2013).

2.2.3 *Nanoparticle Synthesis*

Numerous methods used to synthesize nanoparticles can be categorized into "top-down" or "bottom-up" approaches. The "top-down" approach involves the breakdown of bulk materials to nanosized particles, while the "bottom-up" approach starts with atoms and molecules that are then assembled within the phase they are in (gas or liquid) to form nanomaterials (Horikoshi and Serpone, 2013). Figure 1 presents a schematic of the various methods used to synthesize nanoparticles. The "wet" method is popular because it uses inexpensive analytical equipment. A disadvantage of this method is that the reducing agent can add impurities to nanoparticle colloidal suspensions.

Impurities, known or unknown, are undesired substances present with the products of a reaction. For nanoparticle synthesis protocols considered here, impurities are largely unidentified substances. Generally, impurities tend to reduce the efficacy of the product. However, under certain circumstances impurities may also enhance the product efficacy, as is the case for the production of some phytonanoparticles (synthesized from plant extracts) (Tahir et al., 2016). The reducing agent, therefore, may enhance or reduce the efficacy of synthesized nanoparticles.

The chemical reduction of metal ions is a typical liquid/liquid method that allows the synthesis of particles of different shapes, including nanorods, nanowires, nanoplates, and hollow or solid nanoparticles. In addition to low-cost reagents and basic equipment, a further benefit of this method is that the nanoparticles can be optimized for shape and size with careful adjustment of the reducing agent, the dispersing agent, temperature and reaction time (Horikoshi & Serpone, 2013).

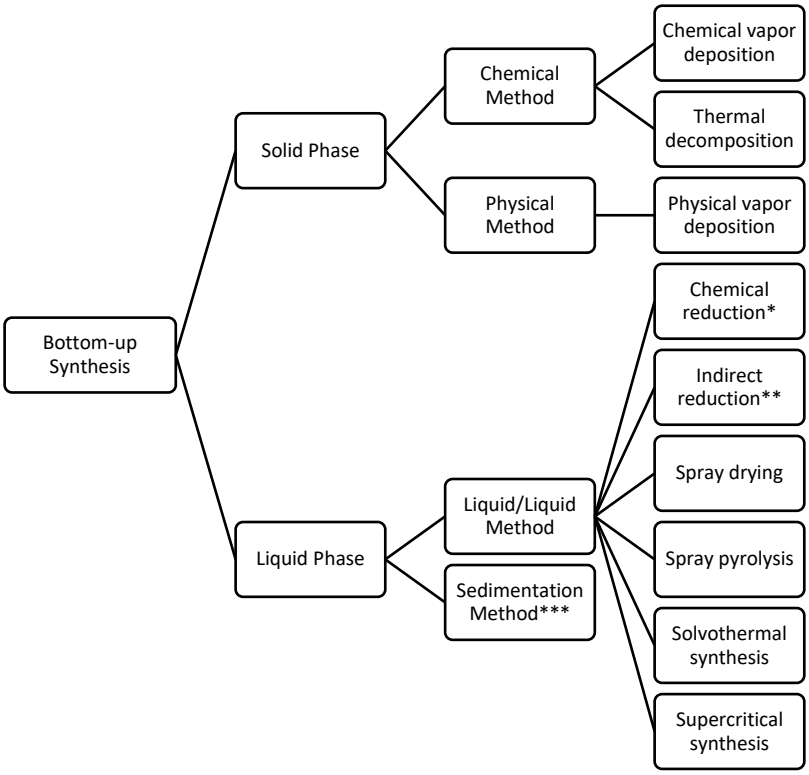


Figure 1. **Bottom-up synthesis of nanoparticles**. Chemical reduction method* includes the use of polyols, organic acids, sodium borohydride, and sugars. Indirect reduction** includes photoreaction, gamma ray, ultrasonic wave, and liquid plasma use. In the sedimentation method,*** the processes include co-precipitation, sol-gel/gel-sol, alkaline precipitation, and colloidal chemistry. Figure adapted from Horikoshi & Serpone, 2013.

In the chemical reduction method, for example with silver, metal ions are reduced.

$$ne^- + M^{n+} \rightarrow M^0 \quad (1a)$$

$$e^- + Ag^+ \rightarrow Ag^0 \quad (1b)$$

The Ag seeds can agglomerate into oligomer clusters which eventually form colloidal Ag nanoparticles (El-Nour, et al., 2010). Two common liquid/liquid methods of nanoparticle synthesis use organic acids (Turkevich method) and sugars (Tollens' reaction) as reducing agents.

In the Turkevich method, $AgNO_3$ is reduced in the presence of sodium citrate, where the citrate ions act as both a reducing agent and a stabilizer. While this helps limit the number of reagents required, it also makes it difficult to control the shape and size of the synthesized nanoparticles (Turkevich et al., 1951).

We used alkaline pH to open up the glucose ring in maltose and present a free aldehyde group, RCHO, to reduce aqueous silver. Silver, formed in Tollens' reaction, gives a clear solution of diamminesilver(I) complex ($[Ag(NH_3)_2]^+$) (Equation 2). The ratio of ammonia to silver nitrate controls the size and yield of silver nanoparticles (Panáček et al., 2006). An excess of ammonia leads to the synthesis of smaller nanoparticles because of the abundance of nuclei, whereas an excess of silver ions would lead to the reduction of the ions on already formed silver nuclei and an increase in nanoparticle size (Equation 3).

$$Ag^+ + 2NH_3 \rightarrow [Ag(NH_3)_2]^+ \quad (2)$$

$$2[\mathrm{Ag(NH_3)_2}]^+ + \mathrm{RCHO} + H_2O \rightarrow 2\mathrm{Ag(s)} + \mathrm{RCOOH} + 4NH_3 + 2H^+ \quad (3)$$

Because the ammonia traps all the free silver ions, the initial nucleation step determines both nanoparticle size and its distribution (Gorup, et al., 2011). The bimetallic nanoparticles were synthesized via the galvanic replacement reaction between $HAuCl_4$ and silver nanoparticle, so that silver nanoparticle is oxidized and $AuCl_4^-$ is reduced (Young, 2006; Holden et al., 2014; Park et al., 2014; Maschmeyer, 2017), as shown in equation (4).

$$3Ag + AuCl_4^- \rightarrow 3Ag^+ + 4Cl^- + Au \quad (4)$$

The amount of alloying depends on the amount of gold salt added where a molar excess results in the complete dissolution of silver (Purbia & Paria, 2015). The deposition of gold on the silver to form an alloy results from the difference in the standard reduction potential of $AuCl_4^-/Au$ (0.99 V, against standard hydrogen electrode) and that of Ag^+/Ag (0.80 V, against standard hydrogen electrode) (Young, 2006). We synthesized bimetallic nanoparticles with differing gold content by varying the final [$HAuCl_4$].

2.2.4 *Nanoparticle Characterization*

Following synthesis, the characterization of nanoparticles is necessary to determine their physicochemical characteristics. Unfortunately, contradictory reports of nanoparticle functional features, in spite of similar sizes, may be due to inadequate nanoparticle characterization. Some of the most commonly used methods to characterize nanoparticles include Ultraviolet-visible (UV-vis) spectrometry, Dynamic Light Scattering (DLS), Nanoparticle Tracking Analysis (NTA), Energy Dispersive X-ray Spectroscopy (EDS), Atomic Force Microscopy (AFM), and Transmission Electron

Microscopy (TEM). Based on these techniques structural benchmarks can be established to rationalize nanoparticle functional activity.

2.2.4.1 **UV-Vis Spectroscopy**

UV-vis spectroscopy is a technique based on light absorbance in the ultraviolet and visible spectral ranges by dissolved material in a suitable solvent. This technique is used for quantitative and qualitative analysis of solutions. Quantitative results are obtained from the application of Beer-Lambert law that states that the absorbance of a solution is directly proportional to the concentration of the absorbing species in the solution for a predetermined fixed path length of light. The proportionality constant is referred to as the molar extinction coefficient, calculated from a calibration curve, for the absorbing substance under specified pH and solvent conditions. Qualitative measurements are obtained by characterizing the observed absorbance peaks for a substance under specific pH and solvent conditions. The strongest absorption along the absorption spectrum, is termed the substances' lambda maximum (λ_{max}) and is characteristic of each substance. UV-vis spectroscopy can be used for determination of colloidal stability of metallic nanoparticles based on their physical changes in suspension (Ray et al., 2015). Thus, UV-vis spectroscopy is a quick, simple to use and economical tool for determining important characteristics of colloidal suspensions such as silver or gold nanoparticle solutions (Paramelle et al., 2014; Haiss et al., 2007).

2.2.4.2 **Dynamic light scattering**

Dynamic light scattering (DLS) is employed to determine the size distribution of particles in suspension. Laser light is directed through a solution containing dispersed particles. Particles in the solution scatter light proportionally to the sixth-power of

particle radius r, (Intensity $\propto r^6$). The intensity of the scattering fluctuates as the particles undergo Brownian motion, causing constructive and destructive interferences. For DLS, the intensity fluctuations of the scattered light are captured and correlated against short decay times to obtain an intensity autocorrelation function. The hydrodynamic radius of solid spherical particles is derived using the Stokes-Einstein equation.

$$D_t = \frac{k_B T}{6\pi\eta R_H} \quad (5)$$

Where k_B = Boltzmann constant (1.38064852 x 10^{-23} J/K), T = temperature, η = absolute viscosity (1 Pa s = 1 N s/m^2 = 1 kg/(m s)) and R_H = hydrodynamic radius (nm). The derivations for the autocorrelation function are beyond the scope of this work, but indicate that DLS measurement results depend on the viscosity of the solvent used, the temperature of the solution, the refractive index of the nanoparticle, and the instrument settings (Bhattacharjee, 2016). DLS analysis was done with a Nicomp ZLS3000 (Particle Sizing Systems, FL, USA) instrument. This system uses a unique algorithm to describe sizing results in either a Gaussian or a multi-modal distribution. It can separate close bimodal populations. The measured size is the hydrodynamic size of the nanoparticle including the hydration shell. Moreover, DLS can be used to evaluate the colloidal stability by tracking the size of the nanoparticles over time or by measuring the Zeta potential. An increase in the mean size of the nanoparticles over time indicates nanoparticle aggregation. The Zeta potential of nanoparticle solutions is used as an indicator of the electrostatic stability of nanoparticles (Battacharjee, 2016). Based on numerous studies, Zeta potential values of ± 0–10 mV, ± 10–20 mV, ± 20–30 mV, and ± 30 mV are electrostatically unstable, relatively stable, moderately stable, and highly stable, respectively (Salopek et al., 1992; Battacharjee, 2016). Overall, colloidal stability

of nanoparticles in solution is determined by the collective effects of Van der Waals' attractive forces, electrostatic repulsion and steric hindrance.

2.2.4.3 **Nanoparticle Tracking Analysis**

NTA, first commercialized in 2006, is used to size nanoparticles between 30–1000 nm. The lower sizing limit is dependent on the refractive index of nanoparticles (Filipe et al., 2010), and can measure down to 10 nm for some substances (Contado, 2015). For NTA, like DLS, the size is derived from the rate of movement through the solution (Brownian motion) using the Stokes-Einstein equation (Equation 5). Equation 6 measures the mean squared displacement, $\overline{(x,y)^2}$ of the nanoparticle in two dimensions (Filipe et al., 2010; Hole et al., 2013).

$$\overline{(x,y)^2} = \frac{2k_B T}{3R_H \pi \eta} \tag{6}$$

Rate of nanoparticle movement is dependent on the temperature of the solution *T*, solution viscosity η, and hydrodynamic radius, R_H. The NTA technique combines laser light scattering with a charge-coupled camera to capture the Brownian motion of individual nanoparticles. In contrast to the DLS technique, the hydrodynamic size is calculated and collected for optically and digitally captured nanoparticles, particle by particle. Therefore, the NanoSight NS300 can provide size and concentration information with clearer representation of varying nanoparticle sizes in polydisperse solutions (Filipe, Hawe, & Jiskoot, 2010). The advantage of the NTA technique is that it represents a form of particle census taking and so is not biased towards larger nanoparticles or aggregates (Hole et al., 2013). Additionally, because it measures a large number of particles simultaneously, this increases statistical confidence (Contado, 2015).

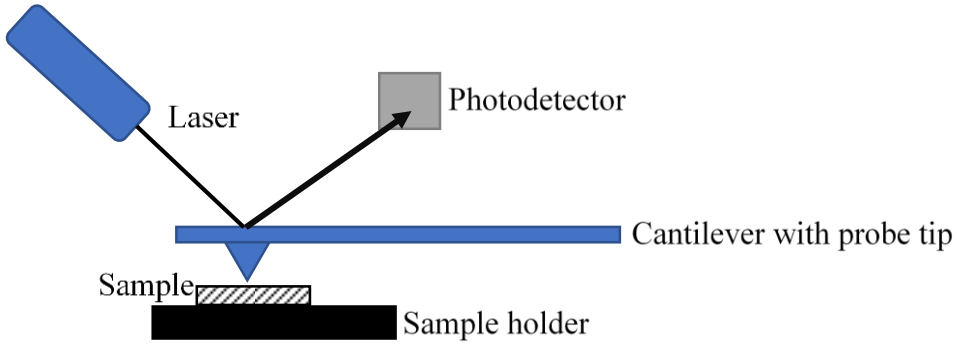


Figure 2. Schematic of AFM. The basic parts of the AFM include a laser, cantilever with a probe, photodetector, and sample holder. The photodetector is connected to electronic equipment that converts the cantilever deflection, measured in volts, to nanometers in motion.

2.2.4.4 **Energy dispersive X-ray spectroscopy**

Energy dispersive spectroscopy (EDS) is used for elemental analysis. In brief, a beam of high energy particles (electrons, protons) is directed onto the sample to excite electrons and eject them from affected elements. The displaced electrons leave behind unoccupied 'holes' that become filled by nearby electrons from higher energy shells. The energy difference between the two energy levels results in photon emission in the X-ray part of the spectrum. The wavelength of emitted X-ray is characteristic of the atomic structure of the sample and is used to determine sample composition (Slater, Lewis & Haigh, 2016).

2.2.4.5 **Atomic force microscopy**

Atomic force microscopy (AFM) can form images of objects that are fractions of a nanometer in size, without using either light from sample or focusing lenses. This reduces errors from diffraction and aberration during measurement.

The basic mechanism uses a probe that is attached to a cantilever (Figure 2). The probe moves over a sample via a raster scanning motion, either by constant contact or by tapping. As the contact changes due to the shape and size of the object, deflection in the cantilever is detected by the recording mechanism. The deflection of the cantilever is calibrated to relate voltage to nanometers of motion. The electrical signal can be converted to image outputs in both 2- and 3-D formats. AFM can be used to image most surfaces and for particles in ambient air, in liquids, or in their native tissue. AFM can resolve images down to 0.1 and 1 nm in the vertical and horizontal planes, respectively (Contador, 2015).

2.2.4.6 **Transmission electron microscopy**

Transmission electron microscopy (TEM) is a relatively fast high-resolution imaging tool that uses electrons transmitted through a specimen under high vacuum to form an image. In brief, electrons emitted from a cathode, and accelerated by high voltage, pass through a thin specimen. As electrons pass through the specimen, they are scattered elastically or inelastically. These electrons pass onto a fluorescent screen, thus generating an image that is seen as contrasts in electron densities. A 3-D image can be produced using TEM by taking multiple images of the sample from different angles. TEM imaging can resolve objects of less than 50 picometers (0.05 nanometers). Sample preparation for TEM can be quite complex, but nanoparticles are essentially electron transparent and are easily imaged using TEM. Although TEM is the preferred method for the determination of nanoparticle mean size, size distribution, and nanoparticle shape, the expense of TEM and the needed technical skills keeps it out of reach for many.

2.2.5 **Scope of this Study**

In this study, we contrast the Turkevich and modified Tollens' approaches to making monodisperse silver-containing nanoparticles. Modified Tollens reaction is used to make size-specific nanoparticle seeds of silver, by varying the pH of Tollens reagent and the ratio of 0.02 M silver nitrate to 0.01 M D-maltose. Then the nanoparticle seeds were used to synthesize silver-gold bimetallic nanoparticles of varying gold compositions (≈3% and ≈16% gold), by varying the volume of 0.1 M chloroauric acid. We report the use of Pluronic F127 as a stabilizer of silver-gold bimetallic alloy nanoparticles.

Pluronic F127, a Food and Drug Administration (FDA) approved polymer, was used as a stabilizing agent of silver nanoparticles. Gold is used to synthesize silver-gold bimetallic alloys because of a number of advantages such as: a) gold is resistant to oxidation, b) precursor Au(III) is water-soluble and c) reduced gold surfaces conjugate readily with other compounds to make versatile nanoparticles. Further, gold in bimetallic nanoparticles modulates the release of silver ions from nanoparticle surfaces (Alissawi et al., 2013; Sotiriou et al., 2014). As a result, bimetallic silver-gold nanoparticles retain antibacterial activity while reducing toxicity toward eukaryotic cells (Grade et al., 2014; Mahl et al., 2012; Padmos et al., 2015; Ristig et al., 2015).

All nanoparticle preparations were characterized with respect to particle sizes, shapes, and colloidal stabilities. Furthermore, the size measurements were validated by using three different sizing methods and comparing their observations. Size differences (if present) were noted among the synthesized species of nanoparticles.

2.3 Materials and Methods

2.3.1 *Materials*

Ammonium hydroxide (28-30%), sodium hydroxide (≥98%), D-maltose (≥99%), silver nitrate (≥99%), gold (III) chloride hydrate ($HAuCl_4.3H_2O$; 99.999% trace metals basis), Pluronic F-127 ($EO_{100}PO_{65}EO_{100}$, MW ≈ 12500; batch number 038K0113), phosphate buffered saline tablets, bovine serum albumin, and reduced glutathione reagents were used as received (Sigma-Aldrich, USA). Milli-Q water (Millipore) was used in all experiments.

2.3.2 *Maltose Method of Nanoparticle Synthesis*

Glutathione-capped nanoparticles were synthesized at room temperature using the reduction of $AgNO_3$ by maltose in an alkaline medium, as described previously (Holden et al., 2014). Then the solution of synthesized silver nanoparticles was adjusted to an absorbance of 10, measured at 400 nm, using ≈ 2% Pluronic F-127. Then a galvanic replacement reaction was employed between $HAuCl_4$ (0.1 M stock; 0 - 25μL) and the silver nanoparticle seeds (10 mL) to make 3% (BM1) or 16% gold (BM2) bimetallic nanoparticles. The stock 0.1 M $HAuCl_4$ was added to silver nanoparticles for a final concentration of 0.025 mM or 0.15 mM, to make BM1 or BM2, respectively, and incubated at 25 °C for 30 minutes. Then samples were washed by centrifugation at 10,000g for 10 minutes. Solutions of Ag, BM1 or BM2 nanoparticles, were all normalized to an absorbance of 10 at 400 nm using UV-vis spectrometry by adjustment with 2% Pluronic F127. Nanoparticles were stored in black vials at 25 °C until use.

2.3.3 *Sodium Citrate Method of Nanoparticle Synthesis*

Sodium citrate-capped silver nanoparticles were synthesized using a mixture of freshly prepared sodium citrate and tannic acid. Solution A (100 mL) contained 0.0075 mM tannic acid and 0.025 mM sodium citrate. Solution B (49.5 mL) contained 0.04 mM tannic acid and 0.10 mM sodium citrate. Solution B was refluxed at 80ºC when 500 µL of 25 mM $AgNO_3$ was injected into it. Then the temperature of solution B was reduced to 65 ºC, and the reaction allowed to run for 10 minutes. At that time, 10 mL of silver nanoparticle seeds were removed by syringe and injected into a 15 mL Falcon tube, which was placed on ice. Immediately, 10 mL solution A was injected into this solution containing silver nanoparticle seeds, followed by addition of 100 µL of 25 mM $AgNO_3$ to form solution R. The reaction was allowed to run for 30 minutes at 65 ºC. Then, 10 mL of solution R was collected by syringe and placed on ice. The removed volume was replaced with 10 mL of solution A and incubated for 30 min at 65 ºC. This 30–minute cycle was repeated seven times.

2.3.4 *Characterization of Nanoparticles*

UV-vis spectroscopy can be employed to determine both concentration and size of synthesized nanoparticles (Sikder et al., 2018; Haiss et al., 2007; Ray et al., 2015), and can also be used to monitor particle aggregation over time (Ray et al., 2015). UV-vis spectral measurements were taken at room temperature, using Varian Cary 300 spectrophotometer (Agilent), in a quartz cell with a path length of 1 cm. Silver nanoparticles ranging in size ≈ 10 – 20 nm have a maximum absorbance peak, λ_{max}, between 390–410 nm (Paramelle et al., 2014). Because λ_{max} is characteristic of nanoparticles with specific composition and size, it can also be used to identify the

approximate size and composition of the synthesized particles. Given nanoparticles of known size and composition, absorbance at λ_{max} is also a function of relative concentration. Absorbance is proportional to concentration (Beer-Lambert Law: $A = \varepsilon lc$, where A = absorbance (no units), ε = molar extinction coefficient (M^{-1} cm^{-1}), and l = path length of light through solution (cm), c = concentration (mol L^{-1})) (Sikder et al., 2018).

DLS was also used to assess particle size. In brief, DLS analysis was carried out with Nicomp ZLS3000 (Particle Sizing Systems, FL, USA) equipped with a He-Ne laser of wavelength 658 nm and having a maximum power output of 100 mW. DLS measures the changes incident light undergoes when it encounters dissolved or dispersed particles and is scattered by them. The hydrodynamic size is calculated from light scattering intensity based on autocorrelation function using equation (5).

Nanoparticles were imaged using AFM and TEM techniques. AFM was performed using a multimode–8 scanning probe microscope (Bruker, Santa Barbra CA) in the peak force tapping (k = 0.4 Nm^{-1}, f – 70 kHz) mode. The prepared sample for AFM imaging was placed on 18 mm mica AFM discs (Ted Pella, Redding CA), which was silanized using 0.5 mM (3-aminopropyl) triethoxysilane (APTES) (Sigma-Aldrich, USA) as follows. The mica surface was stripped with sellotape, then rinsed with 70% ethanol and allowed to air dry. Forty µL of 0.5 mM APTES made up in ethanol was pipetted onto a clean parafilm strip. The pre-cleaned mica was placed clean surface down on the APTES solution and left for 30 - 60 minutes. After silanization, the excess APTES was washed off with water. A diluted (at least 1:2 dilution) 20 µL nanoparticle sample was pipetted onto a clean parafilm surface, and the mica disk was placed silanized side down, on the sample for 45 – 60 min at 25 °C. Then, the mica was rinsed with filtered deionized

water and air-dried. The sample AFM images were processed using the Gwyddion analysis tool (http://gwyddion.net). For TEM, the sample was washed by centrifugation (8,000g, 5 minutes), the supernatant discarded, and the sample diluted 100-fold in water. A 5μL volume was pipetted onto a 200-mesh carbon-coated Cu grid (Ted Pella, Redding CA) and allowed to air dry. Images were taken on an FEI brand Tecnai-12 at 120 kV (UC Riverside). Images were processed using the Gwyddion analysis tool.

The synthesized nanoparticles need to be stable for storage and in a medium suitable for later use. Nanoparticle stability was assessed by the Zeta potential and UV-vis measurements. For Zeta potential determination, nanoparticles were diluted 1000-fold in 1 mM KCl solution and assessed using the Nicomp ZLS 3000. For UV-vis analysis, silver nanoparticle samples were diluted 1/20 in 2% Pluronic F127, 1.5% BSA, or water. The UV-vis spectrum was then obtained for a 360–800 nm range, with readings every 3 hours for 24 hours. The silver-gold bimetallic nanoparticles were diluted 1/5 with 2% Pluronic F127, 1.5% BSA, or with water followed by UV-vis spectral analysis as described above for silver. Silver nanoparticle solutions were diluted more because the yield of silver nanoparticles was higher. Readings were taken at 0, 3, 6, 18, and 24 hours.

The concentration of nanoparticles was determined using Microwave Plasma-Atomic Emission Spectrometer (MP-AES) following the size determination (Table 1). Samples (1 mL) for analysis by MP-AES were adjusted to OD 1.0, 0.75, 0.50, 0.25 in water. A volume of 100 μL of each sample concentration was dissolved in 1 mL of 5% Nitric Acid overnight. Then the samples were diluted to a final concentration of 3% nitric acid, and a final volume of 5 mL. Samples were loaded into 50 mL Agilent tubes and brought up to 50 mL by further addition of 3% nitric acid. These tubes were then placed

in the automatic sampling rack to be analyzed on Agilent MP-AES. Each nanoparticle solution was sampled three times, according to the settings. The original sample underwent a 50-fold dilution. The nanoparticle concentration was determined from the equation of a standard curve obtained by using a silver standard (Sigma Aldrich).

Table 1. Calculation of Ag solution (ppm) concentration from the UV-vis spectrum. One mL of OD = 10 of the ≈ 20 nm glutathione-capped Ag nanoparticles was washed and concentrated by centrifugation. The pellet was digested in 3 mL of 5% nitric acid at 25 °C overnight. The solution was diluted to 5 mL of 3% nitric acid. The sample was analyzed by MP-AES. Ag (Sigma Aldrich) was used for the standard curve. Sample concentration was calculated from equation y = 0.1225x + 0.0025 and corrected for 50-fold dilution.

	Nanoparticle Concentration	
OD	**ppm**	**mg/mL ($x10^{-4}$)**
0.1	0.738	7.38
0.5	3.188	31.9
1.0	6.250	62.5
2.5	15.438	154.0
5.0	30.750	308.0
10.0	61.375	614.0

The NanoSight NS300, using nanoparticle tracking, provided nanoparticle size and concentration information and distinguished between nanoparticle populations (Filipe, Hawe, & Jiskoot, 2010). Excess surfactant might potentially affect nanoparticle size determination. Because of this, prior to NTA, samples were washed by centrifugation (8,000 rcf, 10 minutes), to remove excess surfactant, and diluted in water

to yield 30–100 particles/frame under the green laser at 532 nm. A 1 mL aliquot of sample was taken up into a 1mL syringe, loaded onto syringe-pump, and injected into the viewing unit. Samples were analyzed using the following NanoSight NS300 machine settings: detection threshold was set to 12, sample pump speed was set to 20 on automatic, temperature was 26.8 to 27°C and was viscosity 0.888 to 0.892. The blur size and maximum jump distance were set to auto.

Samples (1 mL) for energy dispersive spectroscopy (EDS) analysis were washed twice by centrifugation at 8,000 rcf for 10 minutes, and the pellet suspended to the original volume in water. A 10 µL aliquot was pipetted onto carbon tape on the SEM grid. The sample was analyzed on the Thermo NNS Energy-dispersive X-ray analyzer (EDX) attached to a Vega LSH SEM to determine the Ag:Au ratio. In addition, the sample was again washed twice by centrifugation at 10,000g for 10 minutes and resuspended in water. A 10 µL sample was laid on the carbon tape on an 18 mm mica disk (Ted Pella, Redding CA) and allow to air dry. At least three different regions on each prepared nanoparticle sample were assessed for gold content. For each nanoparticle species, two different samples were probed to determine Ag:Au ratios. The mean gold content was determined from the elemental percentage calculated by the Vega LSH SEM software.

2.3.5 ***Statistical Analysis***

The measured size of the synthesized nanoparticles was presented as mean ± SE when comparing different measurement methods, or mean ± SD when determining the size of a nanoparticle species from measurements of many particles. SE can also be used when there is uncertainty about the sample used to measure the diameter of nanoparticles

representing the larger total population, or for samples with large variation (Altman & Bland, 2005). The least-squares mean (LS means) ± SE was computed for nanoparticle sizes determined by several methods using SAS software. The LS mean test was used because the number of samples between tests varied greatly. DLS size determinations were based on hundreds of thousands of particles, while using either AFM or TEM, several tens to a few hundred nanoparticles were examined. The least-squares means statistic is also less sensitive to missing data and offers a better estimate of the true population mean. Therefore, the best method to compare the mean sizes of nanoparticles determined by these methods was using the least squares means. Significance was set at p=0.05.

2.4 Results

UV-vis analysis of nanoparticles (Ag, BM1 and BM2), synthesized by maltose method or the sodium-citrate method, revealed that the yield of nanoparticles produced using the citrate method was apparently higher. The observed absorbance at λ_{max} was higher than that for nanoparticles produced by the maltose method at the same dilution. A decrease in the mean nanoparticle diameter was observed, as gold salt was added, resulting from the etching during galvanic replacement (Figure 3). Moreover, Figure 3 shows that there is a red-shift in the λ_{max} for glutathione (GSH)-capped and citrate-capped nanoparticles of 398–405 nm and 403–419 nm, respectively. Both TEM and AFM analysis of the GSH-capped nanoparticles revealed that nanoparticles had a uniform shape and a narrow size distribution (Figure 4).

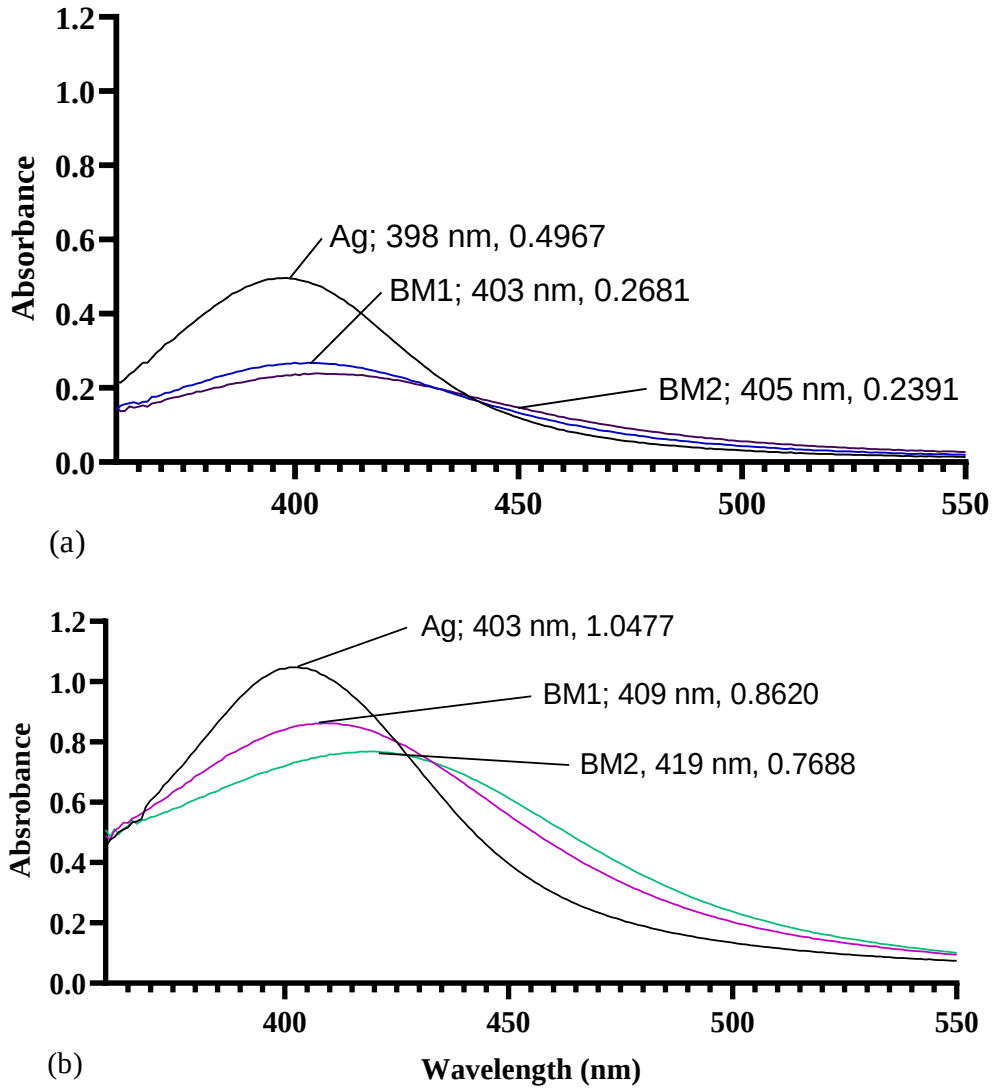


Figure 3. **UV-vis spectra for ≈ 20 nm (a) glutathione-capped and (b) citrate-capped nanoparticle**. Glutathione capped nanoparticles were synthesized by the maltose method. The silver nanoparticle seeds were used to make BM1 and BM2 (3% and 16% gold, respectively) bimetallic nanoparticles. The spectra show absorbance and λ_{max} for Ag, BM1, and BM2. The spectra present a 10-fold dilution of synthesized nanoparticles in 2% Pluronic F127. Citrate-capped nanoparticles were synthesized using the tannic acid/sodium citrate buffer method. The silver nanoparticle seed solution was used to synthesize bimetallic nanoparticles with 84% and 97% silver (BM2 and BM1, respectively). There is a redshift seen in nanoparticles from Ag to BM1 to BM2 indicative of gold incorporation.

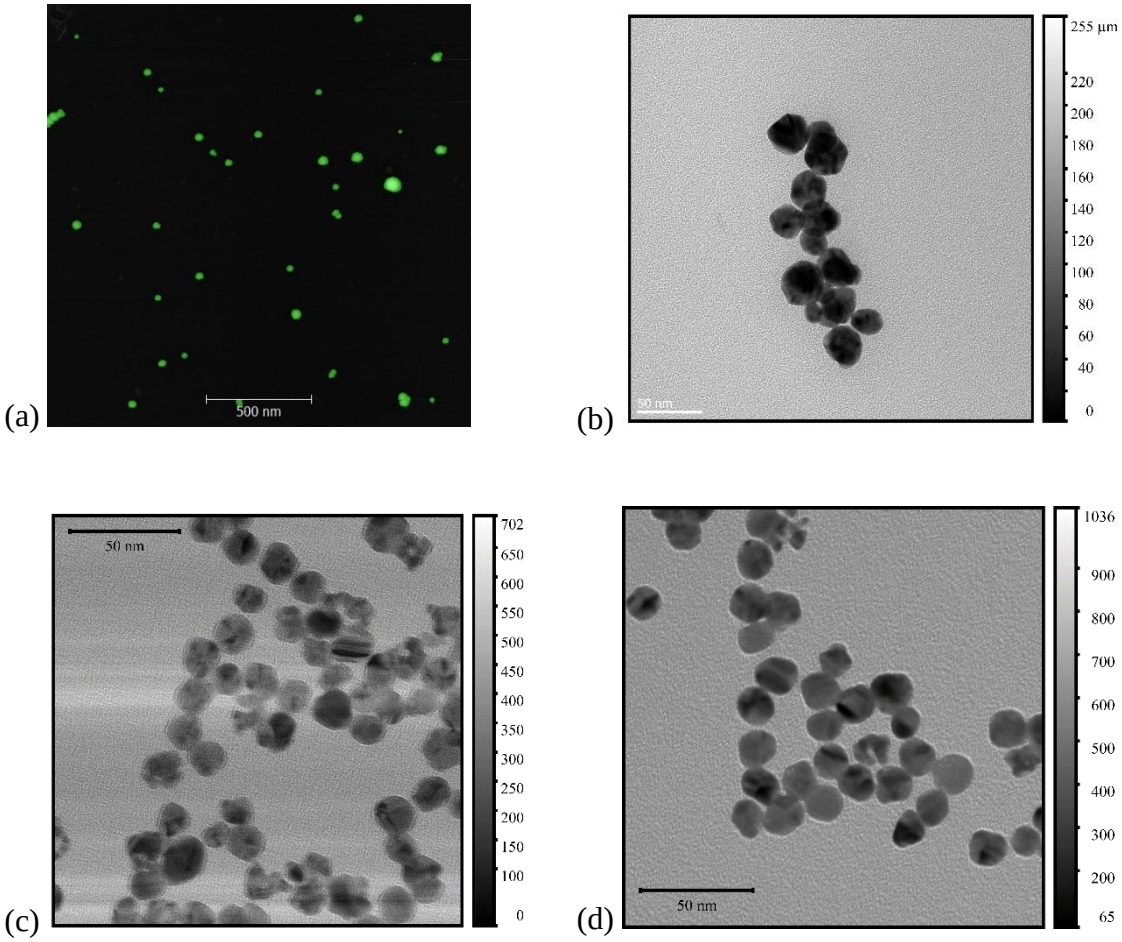


Figure 4. **AFM images of synthesized glutathione-capped nanoparticles**. (a) Representative AFM of synthesized bimetallic nanoparticles. The image shows a uniform distribution of size and shape. The nanoparticles represented are BM1; mean nanoparticle diameter = 18.9 ± 0.8 nm. Representative TEM of synthesized nanoparticles of (b) Ag, (c) BM1, and (d) BM2. The mean diameter (nm) ± standard deviation (SD) of Ag, BM1 and BM2 was 16.78 ± 2.18 (N = 52), 16.12 ± 0.33 (N = 13), and 15.79 ± 1.90 (N=51), respectively.

In Figure 5, the three measurement methods (DLS, AFM, and TEM) are compared for the three nanoparticle species: Ag, BM1, and BM2. For all nanoparticles, the mean sizes were lowest when measured by TEM. Interestingly, when AFM was used, the measured size of BM1 was larger than that obtained by DLS. This may be due to the sample preparation method used for AFM analysis, which may favor larger nanoparticles. As

expected, the mean sizes obtained by DLS for Ag and BM2 nanoparticles was larger than those observed by TEM. This may be because DLS size measurements are affected by hydrodynamic effects. All measurement methods demonstrated a progressive reduction in mean size with increasing gold content, so that Ag > BM1 > BM2.

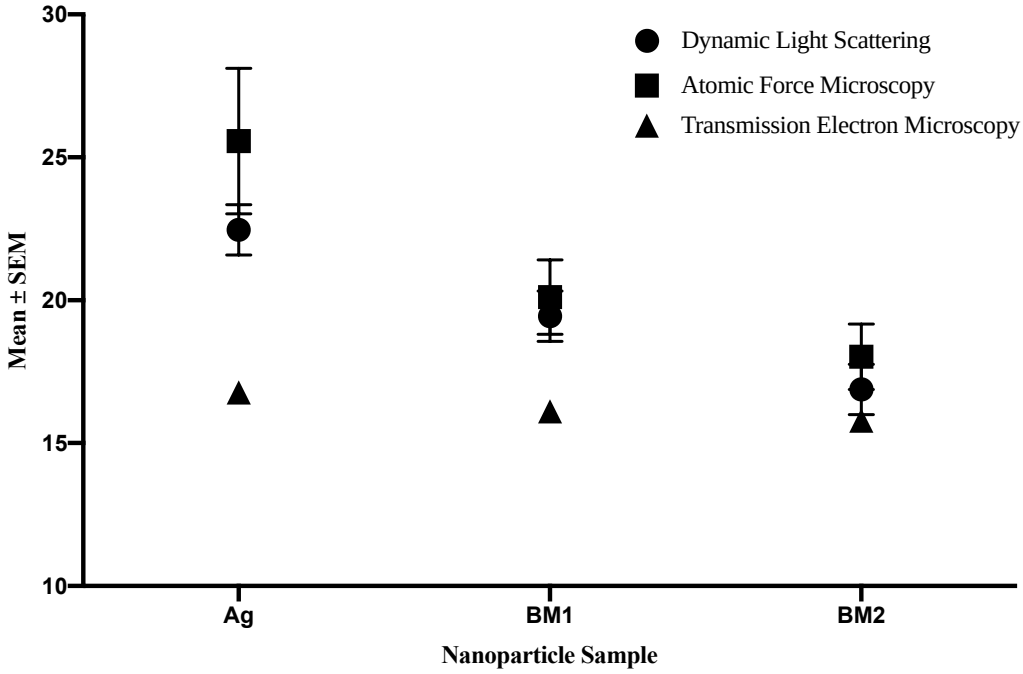


Figure 5. Sizing of maltose-synthesized nanoparticles using DLS, AFM, and TEM. The mean ± SEM diameter of the nanoparticles measured by different techniques presents a general trend Ag > BM1 > BM2. The mean sizes of Ag nanoparticles were 22.46±0.88, 25.57±2.55, 16.78±0.27 nm; BM1 19.40±0.88, 20.11±1.31, 16.12±0.33; and BM2 16.88±0.88, 18.03±1.15, 15.79±0.27 for DLS, AFM and TEM respectively.

The differences in sizes of Ag, BM1, and BM2 nanoparticles were significant for DLS (p=0.003), AFM (p=0.035), and TEM (p=0.027). In all cases (DLS, AFM, TEM) mean sizes of Ag nanoparticles were significantly larger than the mean sizes of BM2. However, mean sizes of BM1 nanoparticles were not significantly different to Ag or

BM2. The mean sizes for Ag measured by DLS and AFM were significantly larger than those measured by TEM.

Two investigations were undertaken regarding the sizes of synthesized nanoparticles. First, to examine whether there is a difference in measured sizes of the different nanoparticle species (Table 2). It was observed that there were significant differences between the three nanoparticle species ($p = 0.05$). Second, to examine the effects of different sizing methods on the measured sizes of particular nanoparticle species (Table 3). The LS mean statistic was employed because of the large variation in the number of nanoparticles used to determine the mean diameter for different measuring approaches. Determinations of mean sizes of Ag and BM1 were significantly different as measured by different methods ($p < 0.001$ and $p = 0.027$, respectively). In contrast, for BM2, the varying methodologies did not result in significantly different measured sizes.

Table 2. The mean size of synthesized nanoparticles Ag, BM1, and BM2

Sizing Technique	F-Value	p-Value
DLS	10.06	0.003
ARM	3.45	0.035
TEM	3.75	0.027

	DLS			AFM			TEM		
Nanoparticle composition	Mean	SE	95% C.I.	Mean	SE	95% C.I.	Mean	SE	95% C.I.
Ag	22.46	0.88	(20.02, 24.90)	25.57	2.55	(19.37, 31.77)	16.78	0.266	(16.13, 17.42)
BM1	19.44	0.88	(17.00, 21.88)	20.11	1.31	(16.93, 23.29)	16.12	0.334	(15.31, 16.93)
BM2	16.88	0.88	14.44, 19.32)	18.03	1.15	(15.23, 20.83)	15.79	0.274	(15.12, 16.45)

The top panel shows the difference in the sizes of the nanoparticles Ag, BM1, and BM2 measured by different sizing techniques DLS, AFM, and TEM. There were significant different in the measured sizes of the nanoparticles Ag, BM1 and BM2 as measured by DLS ($p = 0.003$), AFM ($p = 0.035$), and TEM ($p = 0.027$) where the measured mean±SE size for Ag was significantly higher than the BM2. The BM1 was not significantly different compared to Ag and BM2 for DLS, AFM, and TEM. The lower panel shows that mean±SE for Ag > BM1 > BM2. The range for DLS and AFM is large whereas the range for TEM is small.

Table 3. Nanoparticle species size is dependent on the measurement technique

Nanoparticle Composition	F-Value	p-Value
Ag	24.46	<0.001
BM1	3.81	0.027
BM2	2.11	0.126

	Ag			BM1			BM2		
Sizing Technique	LS Mean	SE	95% C.I.	LS Mean	SE	95% C.I.	LS Mean	SE	95% C.I.
DLS	22.46	1.73	(18.22, 26.70)	19.44	2.73	(12.71, 26.17)	16.88	2.41	(11.02, 22.74)
AFM	25.57	7.22	(22.57, 28.57)	20.11	1.00	(17.67, 22.55)	18.03	0.77	(16.16, 19.90)
TEM	16.78	0.54	(15.46, 18.09)	16.12	1.07	(13.50, 18.74)	15.79	0.77	(13.92, 17.66)

There were significant differences in the mean diameter of Ag nanoparticles when sizing methods DLS, AFM and TEM were compared ($p<0.001$). The mean measurement from TEM was significantly lower than the ones from DLS and AFM. There were significant differences between the means of BM1 measured by DLS, AFM and TEM ($p = 0.027$). The mean measurement from TEM was significantly lower than the ones from AFM but not DLS. There was no significant difference between the mean of BM2 measured by DLS, AFM and TEM sizing methods ($p = 0.126$).

Nanoparticle yield was higher when citrate method of synhesis was used, compared to the maltose method, as previously described and shown in Figure 3. However, the citrate method produced multiple nanoparticle populations, as demonstrated by the large size ranges measured by DLS, with six replicates (Table 4). Both Ag and BM2 nanoparticles had broad size ranges, i.e., 15.1–33.3 nm and 12.9–57.5 nm, respectively. Consequently, the citrate method proved unsuitable for synthesizing monodisperse nanoparticles, which are needed for studies of size-specific activities.

Table 4. **DLS size determination of nanoparticles synthesized using the tannic acid/sodium citrate method**. However, because of large variation it was difficult to consistently reproduce the particle size.

	Silver Nanoparticles			**BM2 Nanoparticles**		
Rep.	Size (nm)	ST.DEV.	%	Size (nm)	ST.DEV.	%
1	18.5	0.8	63.23	57.5	9.3	94.65
2	24.6	5.1	88.11	15.3	1.4	74.96
3	19.5	3.1	96.43	12.9	1.6	95.33
4	14.4	1.7	96.34	28.2	4.3	92.68
5	33.3	4.1	93.90	21.3	3.8	92.25
6	15.1	2.0	86.96	16.3	1.8	86.21
	Mean = 20.9 nm ± 7.1 nm			Mean = 18.8 ± 6.1 nm		

Glutathione-capped nanoparticle stability was assessed in both water, and in 1.5% BSA in PBS, by measuring the Zeta potential. Zeta potential values in Table 5 suggested that Ag nanoparticles were moderately stable, but that BM1 and BM2 may aggregate. Nevertheless, Table 4 and Figure 6 revealed that nanoparticles remained stable and did not aggregate in water or 1.5% BSA over 24 hours.

Table 5. **Zeta potential of synthesized ≈20 nm silver (Ag), BM1 and BM2 alloy nanoparticles**. Nanoparticle solution was appropriately diluted in 1.4% potassium chloride solution and Zeta potential measured on Nicomp ZLS3000. The mean represents three separate solutions analyzed. Zeta potential of ≈20 nm nanoparticles suggests that the silver nanoparticles have moderate stability, while the bimetallic silver-gold alloy nanoparticles may have weaker stability.

	Mean	ST.DEV.	Min	Max
		mV		
Ag	-26.66	-0.67	-25.83	-27.51
BM1	-8.74	-1.55	-7.15	-10.80
BM2	-11.13	-1.42	-10.19	-13.58

Colloids with Zeta potential ±20–29 mv are moderately stable and those with ±30 mV are highly stable (Battacharjee, 2016). BM1 and BM2 have Zeta potentials outside of the expected range for colloidal stability however, colloidal stability may also be supported by steric hindrance of nanoparticles (Fang et al., 2009). Steric hindrance can be inferred from time-course observations of UV-vis spectra (Figure 6). If the synthesized nanoparticles were to be delivered intravenously, they would interact with blood. Blood is a complex fluid that can be separated by volume into plasma (≈55%), and cellular components (≈45%) comprising various blood cells and cell fragments. The normal concentration of serum total protein is 60–80g/L which is ≈6–8% of plasma. The protein in plasma has two main fractions, albumin (≈60%) and globulins (≈36%) with a small amount of fibrinogen (≈4%) (Mathew and Varacallo, 2019). The normal range for albumin in adult human blood corresponds to 3.5–5 g/dL. In this study, we chose 1.5% BSA in PBS as representative of the lower limit, but within the normal range, of serum albumin.

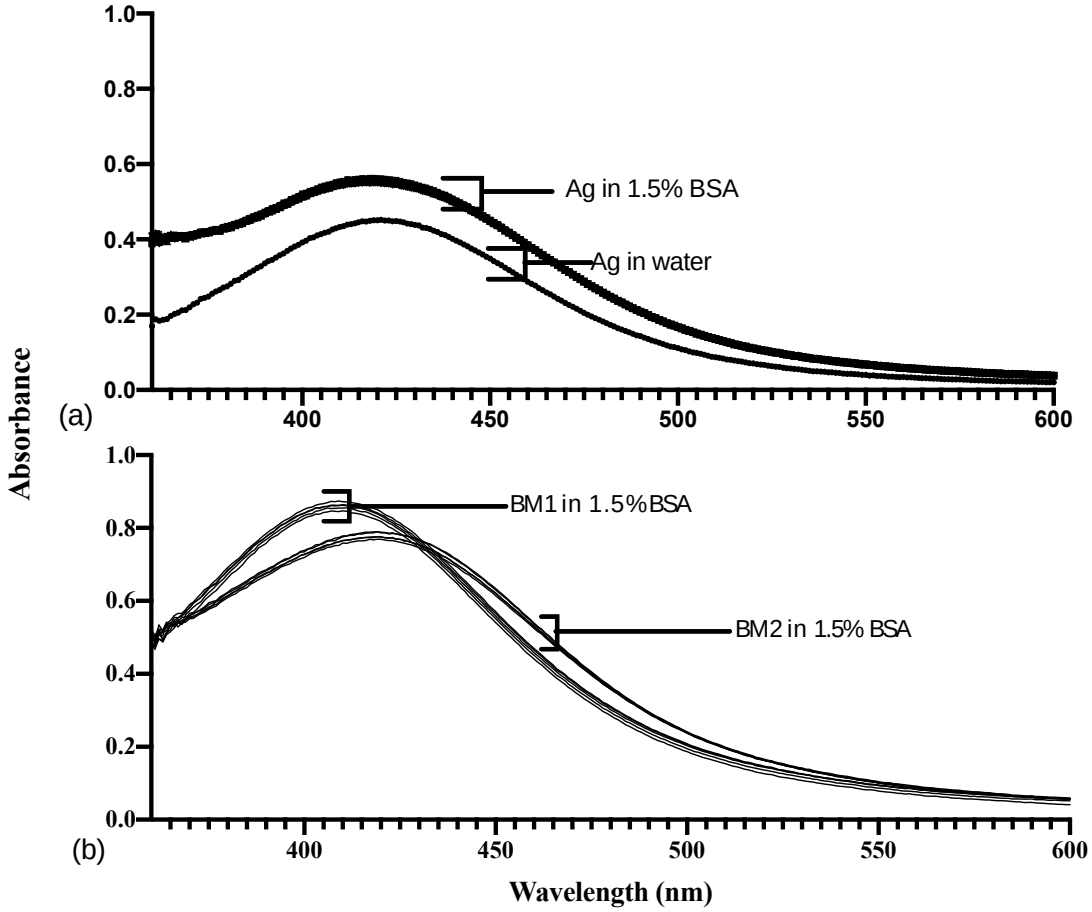


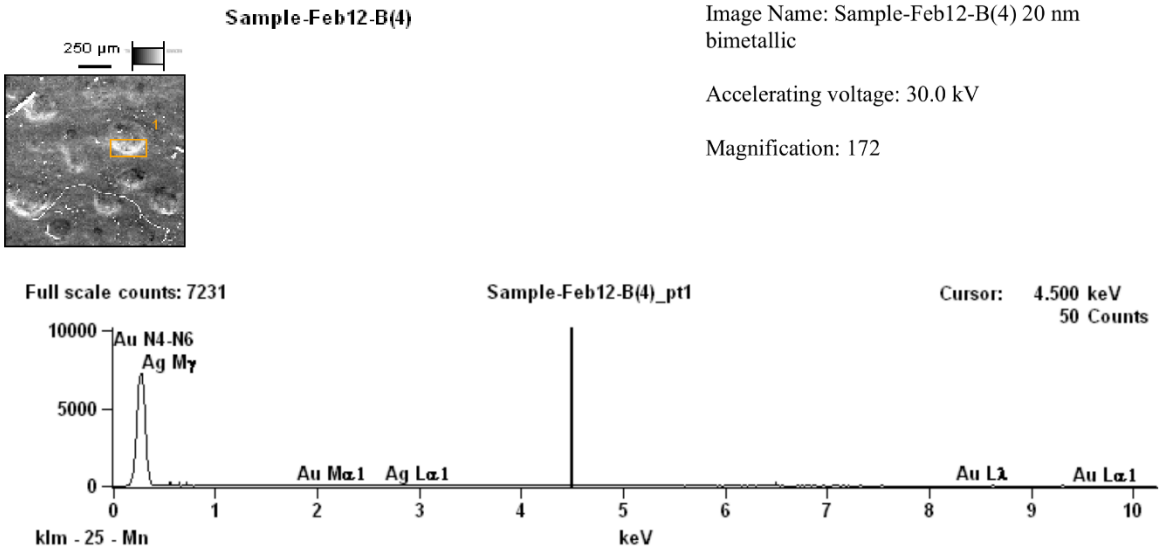
Figure 6. Stability of ≈20 nm glutathione-capped nanoparticles over 24 hours. (a) Silver nanoparticles in water and in 1.5% BSA. (b) Bimetallic nanoparticles in 1.5% BSA. Stock solutions of bimetallic nanoparticles, BM1 and BM2, were diluted to 10% in 1.5% BSA solution. UV-vis analysis was carried out with readings taken at t_o and every 3 hours for 24 hours. No significant change in absorbance or λ_{max} of either nanoparticle population was observed. Each nanoparticle species was tested twice.

Table 6 shows the absorbance at λ_{max} measured by UV-vis over 24 hours for ≈20 nm glutathione-capped silver and silver-gold bimetallic nanoparticles. Data show that neither Ag, BM1 nor BM2 absorbance spectra changed substantially over 24 hours, in either amplitude or λ_{max} position. This implies that progressive aggregation of nanoparticles is not observed.

Table 6. **UV-vis analysis of nanoparticle stability**. Synthesized ≈20 nm glutathione-capped silver and bimetallic silver-gold nanoparticle stability was tested in 1.5% BSA over 24 hours. Each nanoparticle species was evaluated once.

	Time	0 hr	3 hr	6 hr	18 hr	24 hr	Mean	St.Dev.
Ag	λmax	398	398	397	397	397	397.4	0.55
	Abs	0.643	0.615	0.626	0.648	0.645	0.635	0.014
BM1	λmax	409	410	411	410	409	409.8	0.84
	Abs	0.862	0.847	0.856	0.864	0.874	0.861	0.010
BM2	λmax	417	419	418	419	419	418.4	0.89
	Abs	0.769	0.777	0.776	0.790	0.790	0.780	0.009

Average fraction of gold was determined to range from ≈3–20% and ≈2–55% for the glutathione-capped and citrate-capped bimetallic nanoparticles, respectively (Figure 7). Representative data set obtained using SEM-EDS is shown in Figure 7. For each nanoparticle sample (Ag, BM1, and BM2), at least three different samples were analyzed.



Sample ID Feb12-B(4) 20 nm nanoparticle	*Ag-L*	*Au-L*	Sample ID Feb12-B(4) 20 nm nanoparticle	Ag	Au
Net Counts	1293	43	Compound	90.3	9.7
Weight %	90.3	9.7	Norm. Compound %	90.3	9.7
Atom %	94.5	5.5			
Formula	Ag	Au			

Figure 7. Representative SEM-EDS output of glutathione-capped bimetallic nanoparticles synthesized by the maltose method. A sample of synthesized nanoparticles was washed by centrifugation in MiliQ® water before placing on carbon tape on the SEM grid and analyzed by EDS. At least three regions of the sample were analyzed, and five separate Ag, BM1, or BM2 samples were used for each value reported.

Results of EDS analysis of the synthesized GSH-capped and citrate-capped nanoparticles are shown in Table 7. It appears that citrate-capped nanoparticles required more gold salt to achieve similar proportion of gold in the alloy, compared to the smaller 20 nm glutathione-capped nanoparticles. The citrate-capped nanoparticles tended to have bimodal size distributions with wide ranging particle sizes. Thus, these nanoparticles were not used for composition and size-dependent studies.

Table 7. **The ratio of silver to gold in bimetallic nanoparticles based on [$HAuCl_4$] added**. $HAuCl_4$ (0.25–25 $x10^{-4}$M) was added to a solution of synthesized silver nanoparticle seeds. The resulting bimetallic nanoparticle formed was analyzed for the ratio of silver and gold using SEM-EDS.

	Mean Percentage of Gold	
Final [$HAuCl_4$] ($x10^{-4}$ M)	**GSH-capped Nanoparticle**	**Citrate-capped Nanoparticle**
0.25	2.80	-
0.50	7.13	-
0.75	10.10	-
1.00	10.60	-
1.50	15.90	-
2.50	-	0.55
5.00	-	2.60
7.50	-	11.60
10.00	-	19.30
25.00	-	53.90

At 2.5 x 10^{-4} M $HAuCl_4$ GSH-capped silver nanoparticles were completely etched away. Alternatively, for citrate-capped nanoparticles, when less than 2 x 10^{-4} M $HAuCl_4$ was added, no bimetallic nanoparticles were formed. More gold is required to achieve a similar ratio of gold for the citrate-capped than for GSH-capped nanoparticles.

2.5 Discussion

Reproducibility is can be difficult for wet synthesis of nanoparticles. Numerous factors may affect size, shape, and concentration. Furthermore, the complexity of nanoparticle characterization can also lead to difficulties in reproducibility, as discussed

by Ozin (2013). Despite this, GSH-capped nanoparticles synthesized using the maltose method tended to be monodisperse and below 20 nm.

UV-vis analysis was helpful for comparison of the relative sizes of nanoparticles synthesized by the citrate or maltose methods. For nanoparticles of similar material, such as the Ag, larger particles tend to be red-shifted compared to smaller nanoparticles. This was observed in Figure 3, where the ≈ 20 nm GSH-capped nanoparticle had λ_{max} for Ag, BM1, and BM2 of 398, 403, and 405 nm, respectively. The citrate-capped nanoparticles had λ_{max} of 403, 409, and 419 nm for Ag, BM1 and BM2, respectively. This would suggest that initially, there is a dominant sub-population of < 20 nm citrate-coated Ag nanoparticles. The respective λ_{max} of 398 vs. 403, 403 vs. 409, and 405 vs. 419 nm for glutathione-capped vs. citrate-capped Ag, BM1 and BM2 nanoparticles, respectively, are consistent indicators of size differences. The change in λ_{max} from Ag to BM1 and then to BM2 was also consistent with the addition of gold to nanoparticle alloy. The λ_{max} of gold nanoparticles is red shifted relative to that of silver for similar sizes. The smoothness of the spectra in Figure 3 is consistent with the formation of silver-gold alloy, rather than separate nucleation and formation of gold nanoparticles. Simultaneous presence of silver and gold nanoparticles would be demonstrated by observation of a secondary peak to the right of the peak characteristic for silver.

Formation of the bimetallic nanoparticles BM1 from Ag, and BM2 from BM1, leads to a reduction in size because of the galvanic replacement of silver by gold. This was consistently demonstrated by all size determination methods employed. The mean size of nanoparticles generally follow a downward progression: Ag > BM1 > BM2 (Figure 5 and Tables 2, 3). Comparison of these sizes for glutathione-capped

nanoparticles revealed significant differences via each measuring method: $p = 0.003$, 0.035 and 0.027 for DLS, AFM and TEM, respectively. Nevertheless, significant differences in mean size measurements for the same nanoparticles, Ag and BM1, were obtained by different techniques, with $p \leq 0.001$ and $p = 0.027$, respectively. The differences in the mean size measurements of BM2, obtained by the three techniques, was not significant, $p = 0.127$.

Nanoparticle sizes were dependent on the synthesis method and not due to growth from aggregation during storage. The samples were sonicated before dilution for the purpose of Zeta potential measurements. Sonication was shown to possibly affect Zeta potential by increasing the metal release and oxide formation on particle surfaces (Skoglund et al., 2017). The Zeta potential readings, observed in present study, do not suggest that this happened to a significant degree. The inferred electrostatic stability suggests that only Ag nanoparticles are expected to be moderately stable, with a Zeta potential of -26.7 ± 0.7 mV. In contrast, Zeta potential measurements for both BM1 and BM2 suggest that these particles are likely to aggregate over time. However, electrostatic stability is not the only factor that affects colloidal stability. Steric effects may also play a role. In this context, UV-vis spectrometry can be used to assess steric stability. The GSH-capped nanoparticles, coated with Pluronic surfactants, were stable in both water and 1.5% BSA solution over 24 hours (Figure 6, Table 6). The combined effects of the electrostatic forces and steric hindrance from the Pluronic polymer tails prevented aggregation and the nanoparticles remained stable in storage solution.

Well-described monodisperse nanoparticles are of value because size is a significant contributing factor to function. Because of this, successful synthesis of

monodisperse nanoparticles is an important step in the study of specific effects of nanoparticles, and the role of size and composition in determining nanoparticle activity. Bimetallic nanoparticles were synthesized utilizing silver seeds, in order to study the effects of the addition of varying amounts of gold, for the preparation of bimetallic silver-gold alloy nanoparticles. Gold is known to passivate silver toxicity in silver-gold alloys (Grade et al., 2014; Padmos et al., 2015, Ristig, et al., 2015) through the control of silver ion release (Alissawi et al., 2013; Sotiriou et al., 2014). With conflicting results, this effect does not appear to be directly proportional to gold concentration in the bimetallic alloy, for the full range of 0–100% gold, or even for 0–50% gold. Because of this, both nanoparticle size and mean gold content were measured. Since, significant differences in measured sizes of the Ag, BM1 and BM2 nanoparticles were observed by DLS (p = 0.003), AFM (p = 0.035) and TEM (p = 0.027) (Table 2), it is not obvious that the functional effects are strictly due to gold content rather than due to changes in nanoparticle sizes.

Size determinations are affected by the sizing technique employed, as shown in Table 3. This may explain the functional differences reported for nanoparticles of presumably the same apparent size, as determined by different techniques, such as DLS or TEM. Varying the measurement technique can be expected to yield significantly different mean sizes for the same nanoparticles. Moreover, both intra- and inter-laboratory differences in size determination were reported, even when using the same method. Teulon et al. (2018) found that, for a unimodal population of polystyrene or SiO_2 Ludox® beads, the sizing techniques DLS, AFM and TEM were relatively close and within 25% of each other. However, when multimodal nanoparticles were evaluated

then the observed differences between techniques tended to be as much as 300%. These observations emphasize the importance of reporting size distributions and also the need for using multiple sizing techniques. Other possible causes of functional differences may include differences in the: a) nanoparticle concentrations, b) nanoparticle shapes, or c) nanoparticle-medium interactions.

Nanoparticle size is an important determinant of nanoparticle activity, but it is not clear to what degree is the size the principal factor. One of the most important applications of silver nanoparticles is based on their antibiotic properties, but this is limited by the toxicity of silver. Silver-gold bimetallic nanoparticles offer a possible solution to the problem of toxicity while maintaining the antibacterial activity of silver. Conflicting reports still need to be resolved on the role of gold in the bimetallic nanoparticles and on the amount of gold required to achieve an effect. To understand the roles of size and composition (gold content) on nanoparticle antibiotic activity, the potential of nanoparticles to inhibit bacterial growth will be analyzed. Biocompatibility will also be evaluated by assessing platelet activation in whole human blood in subsequent chapters.

2.6 Summary and Conclusions

Synthesis of monodispersed silver nanoparticles with uniform shape can be challenging. This is especially true when using commonly sourced materials and equipment in a resource-poor setting. The problem is compounded when bimetallic nanoparticles are subsequently made from silver seeds.

GSH-capped silver nanoparticles were synthesized and used as seeds for synthesis of silver-gold bimetallic alloy nanoparticles containing either 3% or 16% gold. The mean

sizes (mean±SEM) of nanoparticles were: 22.46±0.88, 25.57±2.55, 16.78±0.27 nm for Ag, 19.4±0.88, 20.11±1.31, 16.12±0.33 nm for BM1, and 16.88±0.88, 18.03±1.15, 15.79±0.27 for BM2, using DLS, AFM and TEM methods, respectively. The nanoparticles were stable in water, 2% Pluronic F127, and 1.5% BSA solutions. Significant differences in the measured sizes of the Ag, BM1 and BM2 nanoparticles, were observed by DLS ($p = 0.003$), AFM ($p = 0.035$) and TEM ($p = 0.027$). Measured sizes of the nanoparticles were significantly different, for each species of nanoparticles, using different measuring methods, such as DLS, AFM, or TEM. This suggests that size determinations of the same stock of nanoparticles using different methods can lead to different infered sizes. When these nanoparticles are subsequently used to investigate activity, similar activity may be reported for apparently differently sized nanoparticles, and vice versa. This tends to confuse studies of the relationship between nanoparticle size and its physiological effects in biological systems.

Nanoparticles need to be adequately characterized, with respect to their physicochemical properties, in order to correctly attribute to them the appropriate activities. Differences in activity between silver and silver-gold bimetallic alloy nanoparticles may result from differences in size as well as in the gold content.

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CHAPTER THREE

NOVEL ANTIBIOTIC SILVER-GOLD BIMETALLIC NANOPARTICLES

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3.1 Abstract

Antimicrobial activities of silver and silver-gold bimetallic nanoparticles (NPs) against *Porphyromonas gingivalis* (*W83 strain*) were compared under oxidative stress conditions. Glutathione-capped 15–20 nm silver and silver-gold bimetallic NPs (3 or 16% gold), stabilized in 2% w/v Pluronic F127, were incubated with planktonic *W83* in the presence of H_2O_2 (sub-inhibitory 0.1 mM, or inhibitory 0.25 mM). Bacterial inhibition was determined by measuring optical densities (ODs) of bacterial cultures at 18 and 24 hours. Cell viability was evaluated by a combination of colony-forming unit (CFU) determinations and by flow cytometry. Silver and bimetallic NPs (14 ng/mL) were bacteriostatic in the presence of 0.1 mM H_2O_2. Bimetallic silver-gold NPs, with16% gold content, were most effective. Flow cytometry analysis using standard dyes were inconclusive for determination of *W83* viability following nanoparticle treatment.

3.2 Introduction

Oral diseases are the most common non-communicable diseases worldwide, of which, periodontal disease afflicts 3.58 billion people (WHO, 2018). Over 60% of Americans (Rozier et. Al., 2017), and up to 15% of adults worldwide, suffer from advanced periodontal disease (Petersen & Ogawa, 2005), which comprises a range of chronic inflammatory diseases affecting tissue that supports the teeth. If untreated, periodontitis can lead to irreversible periodontal attachment loss, alveolar bone

destruction, increased tooth mobility, and subsequent tooth loss (Pihlstrom et al., 2005; Gerits, Verstraeten, & Michiels, 2017; Hajishengallis, 2014). Increased interest in periodontal diseases is motivated by evidence linking them to various vascular disorders (Hajishengallis et al., 2012; Olsen & Yilmaz, 2016; Velsko et al., 2014; Bartold & Narayanan, 2006).

Because much degradation of the tooth, gum, and mandibular bone health can be attributed to bacterial infections (Wade, 2013), oral treatments aim at preventing or controlling such infections. However, oral microflora comprise more than 700 bacterial species (Aas et al., 2005). Three of these organisms, *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia*, are implicated in adult periodontal disease. These organisms, named the red complex, have significant control over the bacterial communities and biofilm formation (Suzuki et al., 2013; Parahitiyawa et al., 2010; Darveau, 2010).

The oral bacterium, *P. gingivalis*, is a major cause of periodontal disease (Imamura, 2003; Imamura et al., 2001; Watanabe & Frommel, 1993). As a keystone pathogen, *P. gingivalis* enhances the establishment and persistence of microbial colonies while also modulating the inflammatory response even when in low abundance (Hajishengallis et al., 2012). Established colonies can form biofilm, are difficult to control, and may lead to periodontitis if untreated (Donlan, 2002). Although microbial colonies develop on periodontal surfaces, an access to the vascular system and entry into distant tissues can occur. This spread of infection may occur during routine dental checkups that incorporate probing of the gums, fillings, cleanings, or oral surgery. Routine flossing and tooth brushing can also lead to tissue and vessel microdamage,

permitting entry of *P. gingivalis* into circulatory system, which then can transport the bacteria to distant tissues and organs where cells can be infected (Radwan-Oczko et al., 2014). Several reports describe intercellular spread among gingival epithelial cells, vascular endothelial cells, and smooth muscle cells (Atanasova & Yilmaz, 2014; Bostanci & Belibasakis, 2012; Li et al., 2008; Velsko et al., 2014). *P. gingivalis* bacteria and their DNA was found in sclerotic plaques (Figuero et al., 2011; McNicol & Israels, 2010) and linked periodontitis to rheumatoid arthritis (Koziel et al., 2014). It was also suggested that *P. gingivalis* plays a role in development and progression of orodigestive cancers (Atanasova & Yilmaz, 2014) in general and in oral squamous cell carcinoma (Katz et al., 2011; Nagy et al., 1998) in particular. These studies suggest that control of *P. gingivalis* infection may improve oral health and is likely to prevent a variety of other regional and systemic diseases.

Current treatment approaches for periodontal disease include surgical and non-surgical scaling and root planning, antibiotic use and mechanical debridement, which are accomplished via regular maintenance dental care (Axelsson & Lindhe, 1981; Slots & Ting, 2002; Sweeting et al., 2008). Scaling and root planing can significantly disrupt the microbiota in the periodontal pocket and, combined with regular maintenance and good oral hygiene, may significantly reduce periodontal pocket formation (Mousques et al, 1980; Axelsson & Lindhe, 1981). While such care can reduce the presence of *P. gingivalis* and improve gum health (Sbordone, et al., 1990; Deas, & Mealey, 2010) it does not necessarily eradicate the microorganism, because *P. gingivalis* may "hide" in deep pockets, invade adjacent periodontal tissue and establish itself on root cementum (Deas & Mealey, 2010). Antibiotics can inhibit or kill some microorganisms that

combined treatment of scaling, root planing, and mechanical debridement cannot, especially because the chemicals can reach places where dental instruments do not. Indiscriminate use of antibiotics, however, may be more harmful than beneficial, even for newer antibiotics (Franci et al., 2015).

Microbial antibiotic resistance is becoming an increasing health problem. Bacterial isolates, even from children who did not receive regular oral/dental health check-ups and preventive care, had some antibiotic-resistant genes (Sanai et al., 2002). Apparently, all periodontal patients do not benefit from antibiotic therapy, and some adult patients with stable periodontitis and/or gingivitis get little to no benefit from it (Kapoor et al., 2012). However, when antibiotics are effective, they can reach regions that physical treatments cannot, including oral mucosa and vasculature (Slots & Ting, 2002). Local antibiotic application has also been proposed for better targeting and higher dosage delivery (Aljateeli et al., 2013; Nair & Anoop, 2012). Systemically administered antibiotics penetrate cells and distant tissues (Gerits et al., 2017).

Antibiotic resistance is a growing concern for *P. gingivalis* infections. On average, 20% of *P. gingivalis* isolates from patients with periodontitis are resistant to amoxicillin, clindamycin, metronidazole, penicillin and tetracycline (Ardila et al., 2010; van Winkelhoff et al., 2000). Antibacterial resistance, along with a variety of bacterial adaptive mechanisms, make biofilm removal difficult. As a result, new treatment options are needed to address oral infections and prevent biofilm formation (Stewart, 2002; Sweeny et al., 2004).

A variety of non-conventional treatments for *P. gingivalis* oral infections have been proposed including: inhibitors of bacterial quorum sensing, antimicrobial peptides,

plant-derived antibacterial agents, sugar alcohols and antibacterial coatings (Gerits, et al., 2017). Local drug delivery systems include examples such as: tetracycline-HCl with an ethylene/vinyl acetate copolymer periodontal fiber, doxycycline hyclate with a gel delivery system, and minocycline-HCl microspheres (Walker & Karpinia, 2002). Application of nanomaterials as therapeutic delivery agents may provide additional alternatives for prevention and control of periodontal disease. These materials include polymeric nanoparticles, nanofibers, quantum dots, liposomes, and nanocomposites/nanogels (Garg et al., 2018; Narang & Narang, 2015).

Compared to traditional antibiotics, nanomaterials offer numerous advantages such as: reduced toxicity, broad-spectrum application, low cost, and reduced likelihood of bacterial resistance (Cheng et al., 2015; Gurunathan et al., 2014; Lara et al., 2011; Pal, Tak, & Song, 2015; Vadlapudi & Kaladhar, 2014). Current efforts are focused on drug delivery to the site of persistent infection in periodontal pocket. Drug delivery improvements include: prolonged drug release using bioadhesive polymers, increased intrapocket drug penetration, and multiple drug loading via delivery systems using nanoparticles or hydrogels (Aminu & Toh, 2017). Several formulations of hydrogels and nanoparticles are already used (Hamidi et al., 2008).

When used together, debridement, topical chemical therapies, and antibiotics have the potential to control periodontal pathogens, including *P. gingivalis*, which is resistant to some antimicrobials (Slots & Ting, 2002). However, the best approach to control *P. gingivalis* is a combination of respective periodontal surgery, systemic antibiotic therapy, and good oral hygiene (Slots & Ting, 2002). Additionally, new and improved

nanomaterials, such as metallic nanoparticles, may provide the infection control that overcomes oral bacterial infections.

Metallic nanoparticles are potentially useful as dental materials in fillings and restorations, or in toothpaste or hydrogels to control infection, and for endodontics, implant dentistry and periodontology (Bapat et al., 2018). Use of silver has a long history, being the most widely used and studied antibacterial nanometal. Silver metal has broad antibacterial properties (Dhanalakshmi et al., 2013; Maillard & Hartemann, 2013; Sotiriou & Pratsinis, 2010; Swathy et al., 2014), and silver nanoparticles are effective antibiotics (Franci et al., 2015; Rai et al., 2014; Rai et al., 2009). They are active against drug-resistant bacteria (Smekalova et al., 2016; Amirulhusni et al., 2012), non-drug-resistant microbes (Amirulhusni et al., 2012; Markowska et al., 2013; Radzig et al., 2013; Xinping et al., 2011), Gram-positive and Gram-negative bacteria (Amato et al., 2011; Bondarenko et al., 2013; Gurunathan et al., 2014; Mohanty et al., 2012; Panáček et al., 2006; Taglietti et al., 2012), and free-living or biofilm integrated bacteria (Gurunathan et al., 2014; Markowska et al., 2013; Ouay et al., 2015; Qin et al., 2014; Velázquez-Velázquez et al., 2015). Silver nanoparticles also have anti-inflammatory (Kemp et al., 2009; Murphy et al., 2015) and wound healing properties (Orlowski et al., 2018; Ahmadi & Adibhesami, 2017; Akila & Nanda, 2012; Gunasekaran et al., 2012; You et al., 2017).

Silver ion (Ag^+) release is suggested as the major reason for the broad antimicrobial activity of silver, especially for nanoparticles smaller than 10 nm (Bartłomiejczyk et al., 2013; Dakal et al., 2016; Durán et al., 2016; Franci et al., 2015; Swathy et al., 2014). For nanoparticles larger than 10 nm, Ag^+ release and physical contact play comparable roles (Bondarenko et al., 2013; Sotiriou & Pratsinis, 2010).

Although it is not clear how Ag^+ works to kill bacteria, suggested mechanisms of action include destruction of the bacterial cell wall, disruption of cellular processes, and oxidative stress (Bapat et al., 2018; Garcia-Contreras et al., 2011). Ag^+ release and availability depend on the pH, oxidative environment, presence of sequestering ligands, agitation, temperature, and on the size and composition of nanoparticles (Alqadi et al., 2014; Amirjani et al., 2015; Anigol et al., 2017; Fleitas-Salazar et al., 2017; Jiang et al., 2011; Liu et al., 2017). This suggests that the environment into which the nanoparticles are placed has a significant contributing role toward nanoparticle efficacy and needs to be taken into account.

Despite the interest in silver nanoparticles, their toxicity towards eukaryotic cells is still poorly characterized. This will need to be corrected if they are to have medical use as antibiotics in humans. There are ways to control the toxicity of silver nanoparticles, including post-synthesis surface modifications. This approach can enhance antibiotic efficacy, improve biocompatibility and minimize undesirable outcomes (Kora & Rastogi, 2013; Liu et al., 2014; Yang et al., 2012; Chung et al., 2008; Shawcross et al., 2017). In this study, the nanoparticle surface was modified by alloying the silver seeds with gold. We chose this method because previous studies demonstrated that the antibacterial and anti-inflammatory characteristics of nanoparticles can be tuned by varying the ratio of silver nanoparticles to gold nanoparticles, or by varying the composition of silver-gold bimetallic nanoparticles (Bilous et al., 2018). Additionally, some silver-gold alloy nanoparticles exhibited better ion release control, providing improved antibacterial activity (Li et al, 2010; Grade et al., 2014; Padmos et al., 2015; Ristig et al., 2015).

Nanoparticles can be delivered in various forms, but colloidal suspensions have advantages over gels or polymers because suspended nanoparticles provide a larger surface area for microbes and Ag^+ to interact. Colloidal suspensions can also enter into crevices and irregular spaces where microbes may hide. Some silver-gold dental products have entered the market, for example the biocompatible Nanocare Gold®, a disinfectant for the periodontal cavity/tooth before restoration (Mackiewicz et al., 2015). As this nanotechnology approach is further developed, the number of products for medicine and dentistry will likely increase.

While bacterial infections on tooth surfaces generally form biofilms, effectiveness of antibiotics is tested using primarily planktonic cultures for minimal inhibitory and bactericidal studies (Larsen, 2002). Antibiotics are promising against *P. gingivalis*, but questions remain about continued effectiveness of these treatments (Japoni et al., 2011; Jaffin, 2011; Larsen, 2002; Herrera et al., 2010). Novel treatment methods are needed, that can clear oral biofilm and systemic infections, but these methods need adequate characterization so they can be correctly applied. The requirements include control/eradication of infection without (i) increasing the burden of antibiotic resistance, (ii) having serious but common side effects that outweigh the benefits, or (iii) toxicity to host cells. Nanomaterial based antimicrobials used alone, or in combination with conventional antimicrobials, are promising for hard to treat microbial infections. However, their use is contingent on fuller characterization of the mechanisms of action and the bacteriostatic and bactericidal levels.

In this study, the working hypothesis is that 20 nm silver-gold bimetallic nanoparticles have improved antibacterial activity over silver nanoparticles. It was

previously demonstrated that silver-gold bimetallic nanoparticles had controlled release of Ag^+ that sequestered on the nanoparticle surface because of the difference in the redox potentials between Ag (~0.8 V) and Au^{3+} (~1.5V) (Ramos et al., 2011; Xia et al., 2013; Zhang et al., 2007). Furthermore, adjusting the Ag/Au bimetallic nanoparticle alloy composition ratio provided further control of the rate of Ag^+ release for antimicrobial activity (Reidy et al., 2013). Adding a capping agent can also enhance biocompatibility and stability in physiological media (Mao et al., 2009; Yildirim et al., 2013).

Pluronic polymers are common capping agents (Pitto-Barry & Barry, 2014). We used Pluronic® F127 (typical composition PEO_{99}-PPO_{67}-PEO_{99}), a hydrophilic non-ionic surfactant polyol. Its thermo-responsive properties are derived from its chemical structure (Fakhari et al., 2017; Giuliano et al., 2018) and make it a suitable capping agent for nanomaterials in manufacture of medical equipment (Mao et al., 2009), and in drug synthesis and delivery (Basak & Bandyopadhyay, 2013; Bodratti & Alexandridis, 2018; Callan et al., 2017; Soni & Yadav, 2014). Moreover, Pluronic F127 is approved by the FDA for use in various forms (U.S. Food & Drug Administration, 2011) in clinical applications and to enhance biocompatibility. In spite of this, there is little information about Pluronic F127 toxicity studies (Cosmetic Ingredient Review Expert Panel, 2008).

3.2.1 **The Rationale for this Study**

The microenvironment of an active infection is one of oxidative stress ranging from sub-inhibitory to inhibitory against planktonic bacteria. In this study, an oxidative stress environment is examined, from sub-inhibitory (0.1 mM H_2O_2) to inhibitory (0.25 mM H_2O_2) conditions, to determine the effectiveness of the silver and bimetallic nanoparticles in this environment. Bimetallic nanoparticles with higher gold content are

expected to be more effective inhibitors of bacterial growth, presumably due to better control of Ag^+ release. Furthermore, the nanoparticles would be activated under oxidative conditions. Smaller bimetallic nanoparticle are expected to have higher H_2O_2 induced activation, due to their higher surface to volume ratio.

3.2.2 **Scope of this study**

In order to determine the antibacterial effects of single size silver and silver-gold bimetallic nanoparticles with varying gold content, 20 nm silver and silver-gold nanoparticles were prepared with gold composition ranging 0–20%. The antimicrobial activity of these nanoparticles was evaluated on planktonic *P. gingivalis* by measuring the reduction in the growth of treated bacterial culture, measuring the optical density of the culture at 600 nm (OD_{600}), following growth time course over 0–28 hours, under inhibitory (0.25 mM H_2O_2) or sub-inhibitory (0.10 mM H_2O_2) oxidative environments. Flow cytometrywas also used as an alternative method for *P. gingivalis* bacterial quantification, and to evaluate viabilities of nanoparticle-treated cultures.

3.2.3 **Objective(s) of this Study**

Objective: To determine the effectiveness of nanoparticles to enhance the antimicrobial effect of hydrogen peroxide against *P. gingivalis*. Aim one: To determine the reduction in bacterial growth, by measuring optical densities (600 nm) of bacterial cultures following incubation with specific nanoparticle concentrations under varying oxidizing conditions of 0.0–0.25 mM H_2O_2. Aim two: To assess flow cytometry as a tool in determining *P. gingivalis* viability following treatment with nanoparticles.

3.3 Materials and Methods

3.3.1 ***Reagents***

Ammonium hydroxide (28-30%), sodium hydroxide (≥98%), D-maltose (≥99%), silver nitrate (≥99%), gold (III) chloride hydrate ($HAuCl_4.3H_2O$; 99.999% trace metals basis), Pluronic F-127 ($EO_{100}PO_{65}EO_{100}$, MW ≈ 12500; batch number 038K0113) and reduced glutathione reagents were used as received (Sigma-Aldrich, USA). Milli-Q water (Millipore) was used in all experiments.

3.3.2 ***Bacterial Cultures***

The laboratory of Dr. Hansel Fletcher of Loma Linda University generously provided all *P. gingivalis W83* used in this study. *P. gingivalis* will be referred to as *W83* from this point in this chapter.

3.3.3 ***Maltose Method of Nanoparticle Synthesis***

Glutathione-capped nanoparticles were synthesized at room temperature by the reduction of $AgNO_3$ using maltose in an alkaline medium, as described previously (Holden et al., 2014). A suspension of silver nanoparticles was adjusted to an absorbance

of 10, at 400 nm, using 2% Pluronic F-127. Bimetallic nanoparticles were synthesized from silver nanoparticle seeds, by the galvanic replacement reaction between $HAuCl_4$ (0.1 M; 0 - 25μL) and silver nanoparticle seeds (10 mL), to make 3% gold (BM1) and 16% gold (BM2) bimetallic nanoparticles. The solution of 0.1 M $HAuCl_4$, which was added to the silver nanoparticle seeds, was brought up to a final concentration of 0.025 mM or 0.15 mM to make BM1 or BM2, respectively. Upon addition, the solution was mixed and incubated for 30 minutes at room temperature. Then, the samples were washed by centrifugation (10,000g, 10 minutes). Ag, BM1 and BM2 solutions were adjusted to an ultraviolet-visible (UV-vis) absorbance of 10, at 400 nm, by diluting with 2% Pluronic F127. Nanoparticles were characterized using UV-vis spectrometry, dynamic light scattering (DLS), atomic force microscopy (AFM), scanning electron microscopy with energy dispersive spectroscopy (EDS), transmission electron microscopy (TEM) and microwave plasma-atomic emission spectroscopy (MP-AES). All preparations were stored in black microcentrifuge tubes before use.

3.3.4 ***Culture Media and Agar Plates***

W83 growth media was prepared as follows. Brain heart infusion (BHI) media was supplemented with yeast extract (0.5%), vitamin K (0.5 μg/mL), hemin (5μg/mL), and cysteine (0.1%). BHI agar plates were made by adding agar (2% w/v) during the preparation of BHI media, autoclaved, cooled in a water bath to 55°C, and poured near a flame to prevent agar contamination. BHI broth and agar plates were stored at 37°C in an anaerobic chamber (10% H_2, 10% CO_2 and 80% N_2) until use.

3.3.5 *W83 Growth Inhibition Assay*

To determine inhibitory effects of nanoparticles on *W83* growth, pre-warmed BHI broth was inoculated with *W83* (final concentration 0.1% v/v), which then was allowed to grow to OD_{600} of approximately 0.2. Then, this *W83* culture was incubated with 14.8 ng/mL of nanoparticles (either Ag, BM1 or BM2) with or without sub-inhibitory hydrogen peroxide (Table 1), in an anaerobic chamber in 10% H_2, 10% CO_2 and 80% N_2 at 37°C for 18 hours. Then, the growth was assessed by measuring the scattering of the planktonic culture at OD_{600}.

Table 1. Treatment Plan for *W83* Cultures. BHI media was inoculated with *W83* and treated with either Ag, BM1, or BM2 in the absence or presence of 0.10 mM H_2O_2. Untreated *W83*, and *W83* treated with 0.20 mM H_2O_2, served as controls.

	Culture Sample								
	A	B	C	D	E	F	G	H	I
W83	+	+	+	+	+	+	+	+	+
Ag	-	+	-	-	-	-	+	-	-
BM1	-	-	+	-	-	-	-	+	-
BM2	-	-	-	+	-	-	-	-	+
0.10 mM H_2O_2	-	-	-	-	+	-	+	+	+
0.25 mM H_2O_2	-	-	-	-	-	+	-	-	-

All cultures in media and on agar plates were grown in an anaerobic chamber in 10% H_2, 10% CO_2 and 80% N_2 at 37°C. A culture of *W83* was grown overnight by inoculating 10 mL of fresh BHI broth with 200 µL of growing, exponential phase *W83*.

Fresh BHI (20 mL) was added to a 40 mL Falcon tube and inoculated with an overnight culture of *W83* to a final concentration of 1% (i.e., 200 µL of *W83*). The OD_{600} of the culture was determined, and the culture incubated at 37ºC. Subsequent OD measurements were made at 6 hours, and every 2 hours thereafter, until OD_{600} reached 0.2. Then culture was divided into eight 2 mL aliquots. One aliquot was untreated, one was treated with 0.25 mM H_2O_2, one each treated with either 0.148 ng/mL Ag, BM1 or BM2, and one each treated with 0.10 mM H_2O_2 and either Ag, BM1 or BM2 (Table 1). Total volume of treated *W83* was 2.5 mL. The samples were incubated overnight at 38°C for 16–18 hours, and the OD_{600} was measured to determine the difference in growth between the nanoparticle-treated and untreated cultures. A minimum of three replicates were made, for each treatment, and the whole experiment was repeated at least four times. The results are presented as mean absorbance and standard deviation for each treatment. The treatment efficacy was evaluated with 95% confidence intervals (CI).

3.3.6 *CFU Determination of W83 Viability*

CFU analysis was performed by separately pipetting 100 µL of treated or untreated *W83* culture onto an agar plate. Then, the culture was spread evenly on the plate and the plates incubated at 37-38°C in an anaerobic chamber at 10% H_2, 10% CO_2 and 80% N_2 for 7 days, or until CFU were visible. The CFU were counted and cell viability determined by calculating the Log_{10} reduction in bacteria. A one-way ANOVA was employed to test the significance of differences in mean absorbance for the cultures or CFUs for treated versus untreated *W83*. Where appropriate, Tukey's post-hoc multiple comparisons analysis was used to compare the group means of absorbance measurements

or CFU counts and to determine which specific treatments were significantly different. The Log reduction in bacterial growth was also calculated for CFU counts.

3.3.7 *W83 Viability by Atomic Force Microscopy*

Treated and untreated *W83* was imaged using AFM to validate CFU results and determine the physical/morphological effects of nanoparticles on bacteria. The work was carried out on a multimode 8 scanning probe microscope (Bruker, Santa Barbra CA) in the peak force tapping (k = 0.4 Nm-1, f – 70 kHz) mode. The samples were prepared as described earlier (Chapter 2). Briefly, the surface of an 18 mm mica disk (Ted Pella, Redding CA) was stripped with sellotape, then rinsed with 70% ethanol and allowed to air dry. The mica was silanized as follows: a) 40 µL volume of 0.5 mM (3-aminopropyl)triethoxysilane (APTES) (Sigma-Aldrich, USA), made up in ethanol, was pipetted onto a clean parafilm strip, b) the pre-cleaned mica was placed clean-surface-down on this APTES solution, and c) left to sit for 30-60 minutes. After silanization, the excess APTES was washed off with filtered deionized water. Treated or untreated *W83* was prepared for AFM imaging as follows: 0.5 mL of each sample was washed at 5000 rcf for 8 minutes, after which the supernatant was discarded, and the pellet re-suspended in 1 mL of filtered Milli-Q® water. A further ten-fold dilution of each sample was carried out in Milli-Q®. For imaging, 20 µL of diluted *W83* sample was pipetted onto a clean parafilm surface and the mica disk was placed silanized side down, on the sample for 45 – 60 min at room temperature. Then, the mica was rinsed with filtered Milli-Q® water and air-dried. The sample was imaged by AFM and the images processed using Gwyddion analysis tool (http://gwyddion.net).

3.3.8 *W83 Viability by Flow Cytometry*

Flow cytometry was used as a rapid and specific tool to evaluate the viability of nanoparticle-treated *W83*. Pre-warmed BHI broth was inoculated with overnight grown *W83* (final concentration: 0.1% v/v). Then the culture was allowed to grow to OD_{600} of approximately 0.2. This *W83* culture was then incubated with 14.8 ng/mL nanoparticles (either Ag, BM1 or BM2), with or without sub-inhibitory [H_2O_2] (Table 1), in an anaerobic chamber under 10% H_2, 10% CO_2 and 80% N_2 at 37°C for at least 18 hours.

To test the efficacy of the flow cytometry for distinguishing between live and killed bacteria, four volumes of 1 mL each of bacterial culture was washed at 5000 rcf for 8 minutes, and the supernatant was discarded. Then, the pellet was suspended either in 1 mL of 0.85% NaCl or in 70% isopropyl alcohol (live or dead culture, respectively) and incubated for 30 minutes. The samples were washed at 5000 rcf for 8 minutes to remove NaCl or isopropyl alcohol and the pellets re-suspended in 0.85% NaCl. Volumes (1 mL) of live, dead, and a 1:1 mixture of live and dead bacteria were prepared for flow cytometric analysis.

Two methods incorporating different fluorescent dyes were used to distinguish between live and dead *W83*. For the first method, propidium iodide (PI) and SYTO9 were used. The *W83* culture samples were diluted 1:99 in 1 x PBS and either unstained or stained alternatively with SYTO9, PI, or SYTO9 and PI. Forward scatter (FSC) and side scatter (SSC) properties were used to acquire 20,000 – 30,000 events and to gate for events of interest. *W83* was differentiated from other cells and debris and sellected using FSC vs SSC. Viable cells were distinguished from killed cells by gating for SYTO9 vs PI. For the second method, Violet Annexin V/Dead Cell Apoptosis Kit was used with

Pacific Blue Annexin V/SYTOX AADvanced Apoptosis Kit for Flow Cytometry, (Invitrogen Molecular Probes from ThermoFisher) to stain live, dead or a mixture of live and dead *W83* suspensions. All assays were done in triplicate and the experiment repeated at least three times. Flow cytometry results are presented as dot plots of mean fluorescent intensity (MFI).

Nanoparticles were synthesized and characterized by UV-vis spectrometry, DLS, AFM, and TEM. Studied nanoparticles were composed from silver (Ag), bimetallic silver and gold alloy (BM1) with 3% gold, and bimetallic silver and gold alloy (BM2) with 16% gold. Pluronic F127 from Sigma Aldrich was used to prepare 0–5% w/v solutions in filtered Milli-Q® water, and pH was adjusted to physiological range with 1M NaOH. Since Pluronic F127 contained 100 ppm Butylated hydroxytoluene (BHT) as a preservative, a control solution of BHT in filtered Milli-Q® water was also prepared in the same concentrations contained in the 0–5% w/v Pluronic F127.

3.4 Results

During active infection, host cells release reactive oxygen species, including H_2O_2, as part of their antimicrobial defense mechanism. This study looked at the effectiveness of silver and silver-gold bimetallic nanoparticles as enhancers of this antimicrobial defense against planktonic *W83*. Viability of *W83* was determined by conventional microbiological methods using OD measurements and CFU counts. Usefulness of flow cytometry in determining *W83* viability was also assessed following treatment with nanoparticles in an oxidatively stressed environment.

The synergistic effects of H_2O_2 and nanoparticles are shown in Figure 1.

Growth of *W83* was measured by spectrophotometry using overnight culture treated either with nanoparticles (panel (a)) or with nanoparticles in an oxidative stress environment due to added 0.10 mM H_2O_2 (panel (b)). The top panel (a) shows that 15 ng/mL NPs or 0.1 mM H_2O_2 do not inhibit *W83* growth. In contrast, panel (b) shows the conditions needed for growth inhibition of *W83*. The inhibitory effect is progressively increased in the presence of 0.1 mM H_2O_2 with Ag ≈ BM1 < BM2. BM2, in the presence of subinhibitory 0.1 mM H_2O_2, had equivalent inhibitory activity to 0.25 mM H_2O_2. Without nanoparticles, sub-inhibitory concentrations 0.1 mM H_2O_2 did not impair *W83* growth. However, one-way ANOVA analysis of the OD_{600}, following incubation of *W83* with nanoparticles with 0.1 mM H_2O_2, demonstrated significant inhibition of *W83* growth by all nanoparticles, $F(4, 14) = 74$, $p < 0.01$. Post-hoc Tukey's multiple comparisons were used to determine which specific treatment groups had significant differences (Table 2). BM2 antibacterial activity was (1) as potent as 0.25 mM H_2O_2, while (2) Ag and BM1 were similarly effective.

Table 2. Tukey's multiple comparisons test of nanoparticle treated *W83* in 0.1 mM H_2O_2. All nanoparticles significantly inhibit *W83* growth compared to untreated *W83* culture. There is no significant difference between activities of Ag and BM1, BM2 and 0.25 mM H_2O_2, suggesting similar inhibitory action against *W83*. BM2 were the most potent nanoparticles.

Compared Samples	Mean Diff.	95.0% CI of diff.	Significant?	Summary	Adjusted *P*-Value
W83 vs. Ag+W83	0.36	0.24 to 0.47	Yes	****	<0.0001
W83 vs. BM1+W83	0.48	0.36 to 0.61	Yes	****	<0.0001
W83 vs. BM2+W83	0.77	0.66 to 0.89	Yes	****	<0.0001
W83 vs. 0.25 mM H_2O_2+W83	0.78	0.66 to 0.89	Yes	****	<0.0001
Ag+W83 vs. BM1+W83	0.12	0.00 to 0.25	No	ns	0.0583
Ag+W83 vs. BM2+W83	0.41	0.30 to 0.53	Yes	****	<0.0001
Ag+W83 vs. 0.25 mM H_2O_2+W83	0.42	0.30 to 0.53	Yes	****	<0.0001
BM1+W83 vs. BM2+W83	0.29	0.17 to 0.42	Yes	****	<0.0001
BM1+W83 vs. 0.25 mM H_2O_2+W83	0.30	0.17 to 0.42	Yes	****	<0.0001
BM2+W83 vs. 0.25 mM H_2O_2+W83	0.00	-0.11 to 0.12	No	ns	0.9996

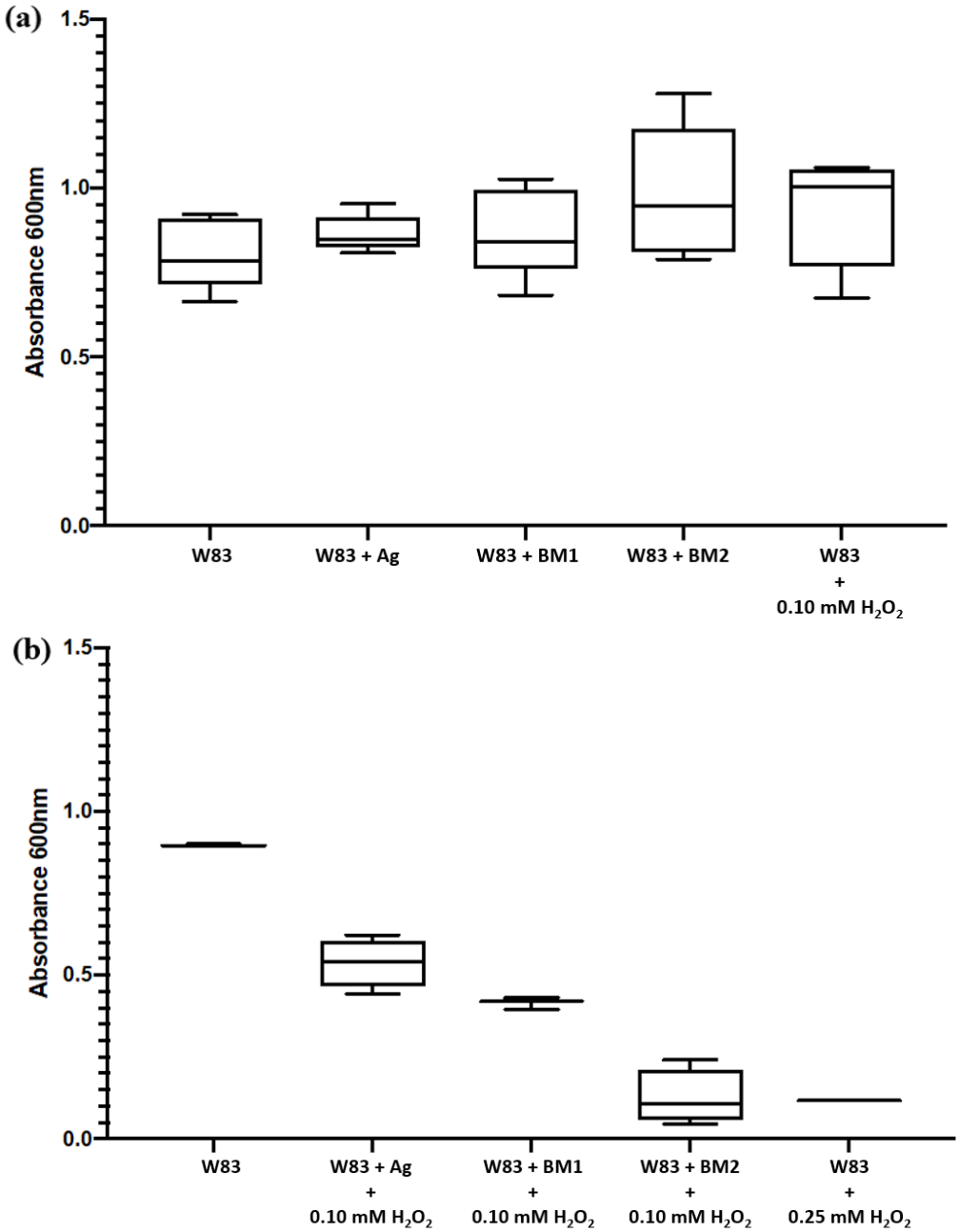

Figure 1. Synergistic effects of nanoparticles and H_2O_2 on inhibition of *W83* growth under anaerobic conditions. *W83* cultures, at OD_{600} of 0.2, were treated with (a) nothing, (b) nanoparticles with subinhibitory 0.10 mM H_2O_2, or (c) inhibitory 0.25 mM H_2O_2, while grown for 18 hrs at 38°C. Nanoparticle concentrations were 14.8 ng/mL of 20 nm silver (Ag), BM1, or BM2. OD_{600} was measured at 18hrs and corrected for scattering of nanoparticles. No inhibition occurred in the absence of 0.1 mM H_2O_2 (panel (a)). Significant growth inhibition occurred once 0.1 mM H_2O_2 was added to the culture exposed to Ag, BM1 or BM2 nanoparticles. BM2 inhibited growth comparable to that of 0.25 mM H_2O_2 suggesting it is a potent antimicrobial if in oxidative environment.

CFUs were measured to determine if *W83* inhibition, by NPs in the presence of 0.1 mM H_2O_2, was bactericidal or bacteriostatic. This was done by plating 100 μL of nanoparticle-treated culture on BHI agar plates, followed by incubation for 7 days at 38 ºC until CFU were observed. All treated samples had a reduction in CFUs of at least 50%, but BM2 achieved a reduction of 70% (Table 3). The comparable effects of Ag and BM1 may be attributed to the low amount of gold in BM1. Growth inhibition by nanoparticles was significant for Ag and BM1 ($p < 0.05$), and more highly significant for BM2 ($p < 0.001$). There was also significant inhibitory capacity of BM2 compared to BM1 ($p < 0.05$).

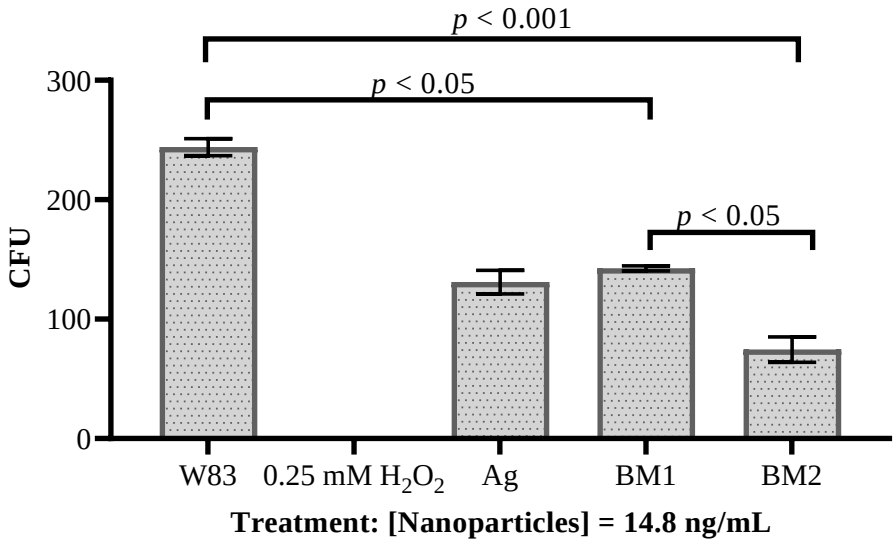

Figure 2. **Colony-forming units (CFU) from nanoparticle-treated or untreated *W83*.** Overnight culture of *W83* was grown to an OD_{600} of 0.2 and treated with 14.8 ng/mL of 20 nm silver (Ag), BM1, or BM2 nanoparticles and 0.1 mM H_2O_2, followed by incubation at 38 °C for 18 hours. Growth inhibition of treated *W83* compared to untreated *W83* was significant for Ag and BM1 ($p < 0.05$) and highly significant for BM2 ($p < 0.001$), based on a one-way analysis of variance.

There was no inhibition of nanoparticle treated *W83* in the absence of 0.10 mM H_2O_2 as the oxidizer. CFU analysis of these cultures showed no difference in viability when nanoparticle-treated cultures were compared to non-treated *W83*.

Table 3. **Colony-forming units (CFU) from treated and untreated *W83***. *W83* was untreated, or treated with 0.25 mM H_2O_2, or 14.8 ng/mL silver (Ag), BM1, or BM2 for 18 hours. Then, 100 μL of the cultures were plated and incubated at 37°C until CFUs were observed. BM2 caused at least a 10-fold reduction in CFU. Growth inhibition was 47, 42 and 70 % for Ag, BM1, and BM2, respectively.

Treatment	Mean CFU (SD)	Growth (+/- %)
W83	244 (7)	-
0.25 mM H_2O_2	0	-100
l4.8 ng/mL silver	131 (10)	-47
14.8 ng/mL BM1	143 (2)	-42
14.8 ng/mL BM2	75 (11)	-70

The CFU assay results were validated using AFM, which provided information of structural changes that may result from incubating *W83* with nanoparticles in the presence of 0.10 mM H_2O_2. Extensive *W83* membrane damage was observed, consistent with reduced viability. Furthermore, treated *W83* tended to clump together (Figure 3b and 3c), while untreated *W83* (Figure 3a) did not. The cause of this clumping is unclear, but it could be a protective response.

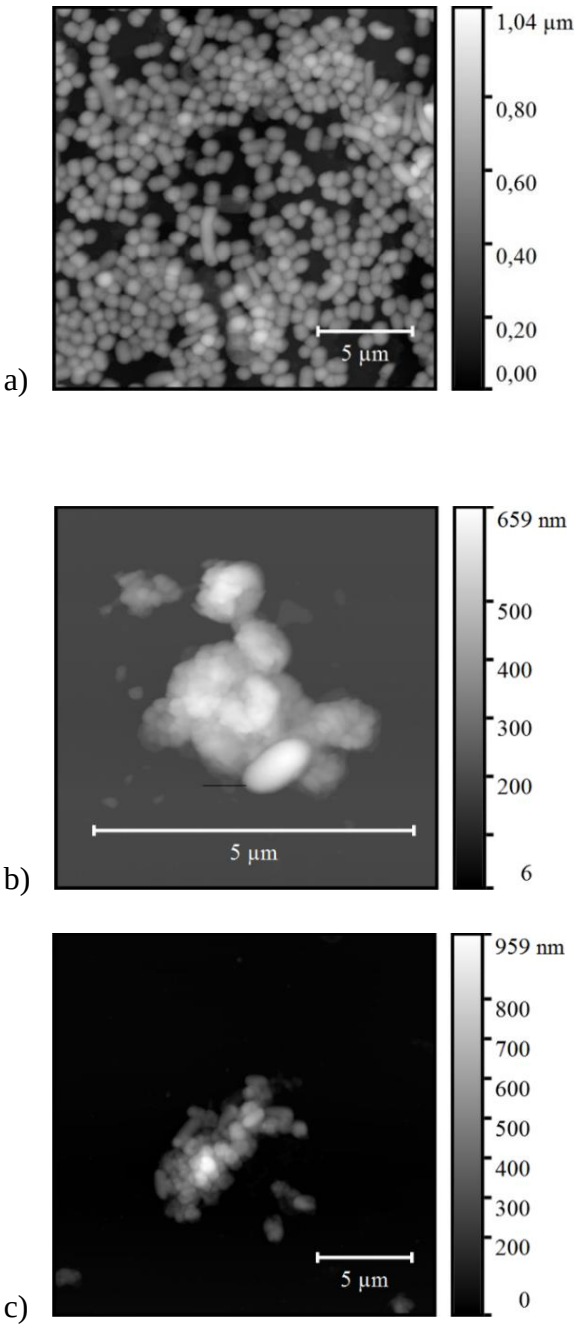

Figure 3. AFM analysis of nanoparticle-treated *W83*. The *W83* was (a) untreated or treated with 0.1 mM H_2O_2 and (b) Ag or (c) BM2 over 18 hrs. Samples were washed, diluted and imaged by AFM. Images show that untreated *W83* are uniform and intact whereas Ag and BM2 treated *W83* had ruptured cell membranes.

Validation of the CFU and AFM data was attempted by determining bacterial cell viability using flow cytometry analysis. Flow cytometry was first evaluated to see if it could distinguish between live and dead *W83* by comparing an overnight culture of the bacteria with isopropanol-killed culture. First, a comparison of observed events when PBS was run alone, versus when *W83* suspended in PBS was run, allowed distinguishing between noise in the test and the real population of *W83*. A gate was set to encompass all the recognized *W83*. Both live and killed bacteria samples were labeled with membrane-permeable SYTO9 as well ash membrane-impermeable propidium iodide (PI). The results are shown in Figure 4. Following incubation with 70% isopropyl alcohol the bacterial cellular membrane is damaged allowing propidium iodide to enter the cell. Propidium iodide is expected to displace SYTO9 from the nucleic acid due to its higher affinity. Using forward and side scatter, the dead cells could hardly be distinguished from the live cells (Figure 4(c)), when the cells were mixed in a 50:50 ratio (Figure 4(b)). Although there was an observable change in shape between Figure 4 (a) and (c), this was not sufficient to clearly correlate with either live or dead *W83*. However, using the differential nucleic acid dyes, the live cells (Figure 4(d)) were distinguishable from dead cells (Figure 4(f)) even when combined in a 50:50 mixture (Figure 4(e)). The two populations can readily be distinguished with the live population above the dead population (Figure 4(d)). In conclusion, viable and non-viable *W83* can be distinguished using flow cytometry when bacteria were killed using 70% isopropyl alcohol.

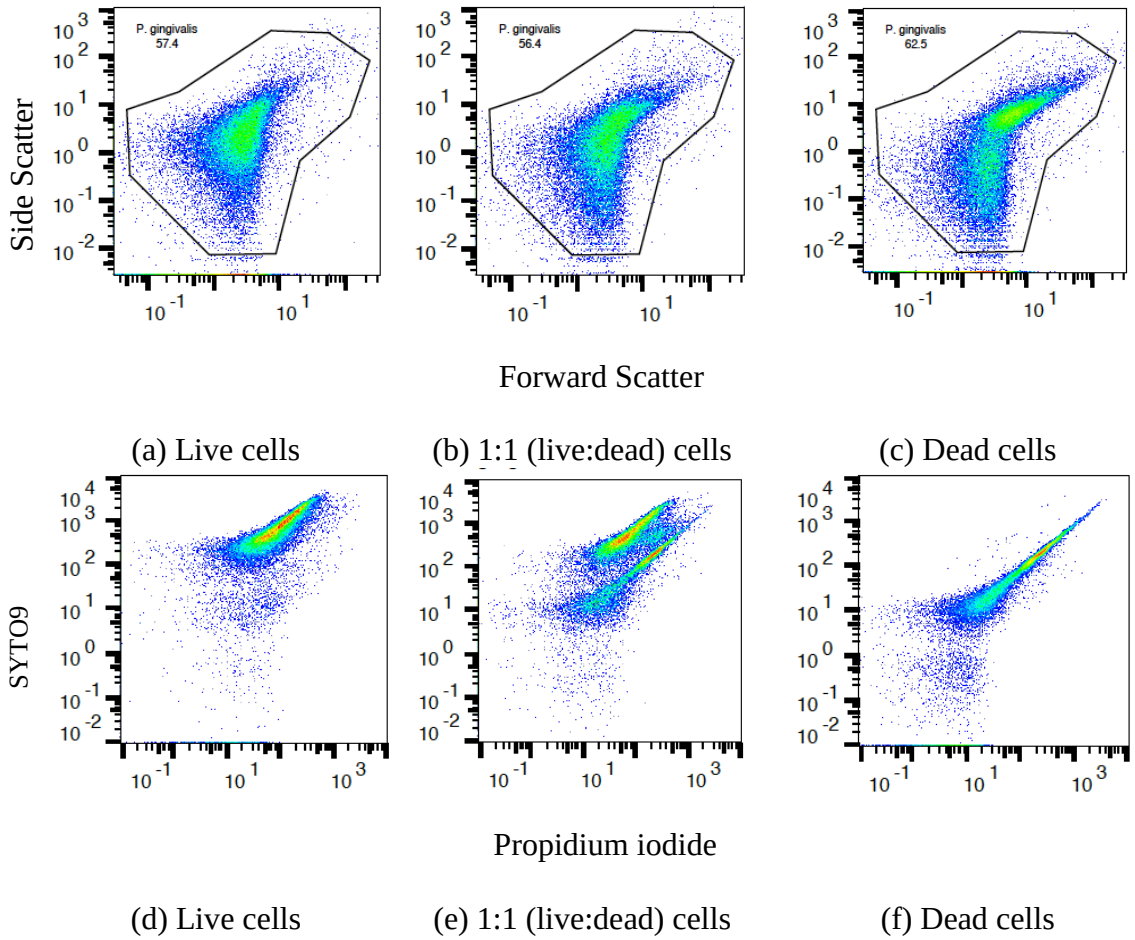

Figure 4. Live/Dead assay of alcohol-treated *W83*. The *W83* culture, in exponential growth phase, was washed to remove BHI and suspended either in 0.85% NaCl (live cells) or 70% isopropyl alcohol (dead cells) and incubated for 30 min at room temperature. *W83* was then centrifuged at 5000 rcf for 8 min and re-suspended in 0.85% NaCl. A 1:1 mixture of live and dead cells was prepared. Live, live/dead, or dead cells were incubated with SYTO9 and Propidium Iodide for 15 minutes in the dark at 25 °C. Samples were diluted and assessed by flow cytometry analysis. Forward and side scatter were uninformative (a) to (c). SYTO9-propidium iodide gating distinguished between (d) live and (f) killed cells. The 1:1 (live:dead) mixture had two clearly distinguishable populations (e).

After determining that the flow cytometry method using Syto9 and PI could distinguish between populations of live cells and killed (dead) cells, the method was employed to distinguish between nanoparticle-treated and untreated *W83*. The result, presented in Figure 5, suggests that the method was not suitable to distinguish between

the live and dead bacteria when *W83* culture was treated by nanoparticles or 0.25 mM H_2O_2 The untreated *W83* bacterial population had similar fluorescence and scatter distribution patterns with those of nanoparticle treated or H_2O_2 killed cultures. This analysis was attempted three different times with different cultures each time, yielding similar comparable results.

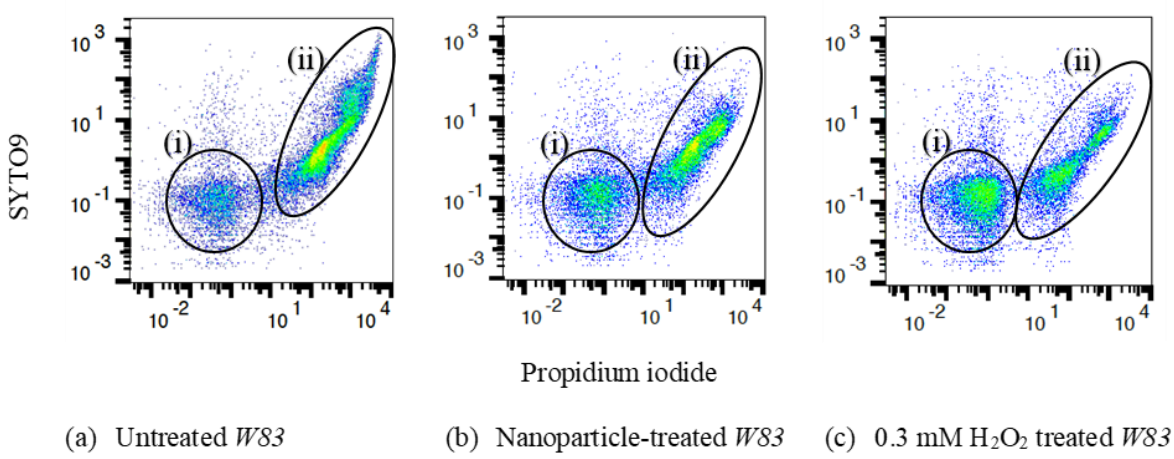

Figure 5. Live/dead assay of nanoparticle-treated *W83*. (a) Untreated *W83*, (b) Nanoparticle-treated *W83*, and (c) 0.25 mM H_2O_2-treated *W83* cultures were grown for 18 hours then centrifuged at 5000 rcf for 8 min and suspended in 0.85% NaCl. Samples were stained with SYTO9 and propidium iodide (PI) and incubated for 15 minutes in the dark at 25 °C. No clear distinction was observed between different treatments. Two populations (i) and (ii) were observed and gated off but there was no clear distinction between the three samples to help differentiate dead cells from cells that were alive.

3.5 Discussion

Antibacterial activity of silver nanoparticles was reported before (Swathy et al., 2014). However, their use for antibiotic purposes has been limited because of risks of cytotoxicity. Silver-gold bimetallic nanoparticles retain antibacterial activity, but can be tuned to reduce cytotoxicity and enhance biocompatibility (Padmos et al., 2015).

Synthesized nanoparticles, whether Ag, BM1 (3% gold) or BM2 (16% gold), had no observable antibacterial function of their own toward *W83*. In the presence of sub-inhibitory 0.10 mM H_2O_2, however, all nanoparticles had significant antibacterial activity. This increased activity was mediated by Ag^+ release, which was caused by the oxidizing agent H_2O_2. In turn, the released Ag^+ caused bacterial growth inhibition by several mechanisms including: a) blocking DNA transcription, b) interruption of bacterial cell respiration, c) inactivation of proteins and d) disruption of adenosine triphosphate synthesis (Ju-nam, & Lead, 2008). The presence of gold in bimetallic nanoparticles modulates Ag^+ release from these nanoparticles (Alissawi et al., 2013; Sotiriou et al., 2014). Previous studies reported that addition of gold to silver nanoparticles can improve biocompatibility of the bimetallic alloy, but at the expense of reduced antibacterial activity (Padmos et al., 2015). In contrast, our results demonstrated enhanced antibacterial activity with increasing gold content from 3% in BM1 to 16% in BM2. BM2 had significantly higher antibacterial activity than BM1, and was as effective for inhibition of bacterial growth as 0.25 mM H_2O_2.

An active bacterial infection is associated with an oxidizing environment due to a number of immune responses. During an inflammatory response, in the periodontal pocket, host cells produce and release hydrogen peroxide and other reactive oxygen species in an attempt to control the infection (Henry, McKenzie et al., 2012). If nanoparticles are administered as a local antibiotic, they would be activated under these circumstances. This suggests that nanoparticles work in a unique self-modulating manner with maximum efficacy where most needed. Additionally, the level of available H_2O_2 on its own may not be sufficient to kill the bacteria. The nanoparticles, however, can

enhance bacterial killing, in the presence of subinhibitory levels of H_2O_2, by mechanisms not yet fully described.

BM2 nanoparticles were somewhat smaller than BM1 or Ag (Chapter 2, Figure 5). Smaller nanoparticles tend to have higher antibacterial activity for several reasons including higher likelihood of entering bacterial cells (Barar, 2015) and disrupting cell surface events as well as internal biochemical pathways (Duran et al., 2016).

Due to size differences, the concentration of solutions in terms of nanoparticles/mL is BM2 > BM1 > Ag. While Ag^+ release is an important factor, nanoparticles themselves are in contact with and can adsorb to the bacterial cell. If the mechanism of action is contact based, a higher concentration of nanoparticles would tend to deliver more antibacterial activity. Since the observed antibacterial activity in the presence of hydrogen peroxide is BM2 > BM1 > Ag (Figure 1), this is consistent with particles/volume functional dependence, supporting a likely contact-based mechanism of action. Together, the smaller size, combined with higher nanoparticle concentration of BM2, may provide further explanation for the higher antibacterial activity. Imaging methods used for assessment of the nanoparticle activity focused on the bacteria and not on the localization of nanoparticles or their interactions with bacteria. Such information could provide a better understanding of the mechanism of nanoparticle based bacterial growth inhibition, and should be added in future studies.

The OD_{600} based estimates of bacterial cell viability were confirmed using CFU counts, which determined the numbers of viable cells in cell culture. CFU counts, however, may not distinguish between cell cytotoxicity and cell growth inhibition. Cells may still be viable even if they do not grow on agar plates. This inability to distinguish

between stasis and cell death is a shortcoming of the CFU method. Furthermore, the results in Figure 2, Table 3 suggest that nanoparticles may be bacteriostatic and not necessarily bactericidal. The AFM imaging, however, demonstrated significant morphological disruptions (Figure 3b and 3c). Such observations are more consistent with bacteria undergoing oxidative stress-related destruction, a suggested mechanism of nanotoxicity (Khanna et al., 2015; Wang et al., 2017). Therefore, AFM imaging provides direct evidence of bacterial destruction, while CFU analysis represents an indirect measure of viability.

Flow cytometry is a well established technique in immunology, but more recently has been applied also in microbiology. Flow cytometry can provide fairly rapid results for antibiotic studies with cell cultures. In contrast, the CFU method is labor-intensive, routinely takes a long time to complete, and is prone to contamination. Early reports of flow cytometry use in microbiology are from the 1970s (Hutter & Eipel, 1979). Development of a monoclonal antibody (mAb) OMR-Bg1E against *P. gingivalis* lipopolysaccharide was helpful to identify *W83* in co-culture with other bacteria (Kamiya et al., 1994). Later studies reported the use of flow cytometry to determine the adherence of FITC-labeled *P. gingivalis* to oral epithelial cells (Pathirana et al., 2007). Despite these attempts at using flow cytometry with *W83*, however, there are still no reports using flow cytometry to determine the viability of *W83* in response to various treatments. The manufacturer of a kit, for discriminating between live and dead bacteria by flow cytometry, reported the use of this LIVE/DEAD BacLight kit (L34856) on Gram-positive *Staphylococcus aureus* and Gram-negative *Escherichia coli* (Thermo Fisher Scientific, 2019). Similarly, Berney et al. (2007) reported that they were able to discriminate

between live and dead *E. coli*, *S. enterica* serovar *Typhimurim*, and *S. flexneri* using this kit. However, our attempts to use flow cytometry to confirm the results from OD measurement, AFM imaging and CFU for *P. gingivalis W83* were unsuccessful. We found that the Live/Dead™ BacLight™ Bacterial Viability and Counting Kit could not distinguish between live and dead treated *W83* (Figure 5). This was in spite of earlier successful *W83* viability tests, using the kit and protocol per manufacturer's recommendations, by killing *W83* with 70% isopropyl alcohol (Figure 4).

One explanation for this observed difficulty in distinguishing between live and dead *W83* could be that NPs interfere with the fluorescence-based test. Zucker, et al. (2013) reported interference by silver nanoparticles in their study of PVP or citrate-coated silver nanoparticle uptake into cells of a human-derived retinal pigment epithelial cell line. Of the three sizes they used (10, 50, and 70 nm), both the 50 and 70 nm nanoparticles caused increased far-red fluorescence in flow cytometric analysis in a dose-dependent manner, but 10 nm nanoparticles did not. While the reported far-red fluorescence was in the range > 670 nm, the maximum emission fluorescence used here for SYTO9 and propidium iodide were 503 and 617 nm, respectively. Therefore, the expected spillover or overlap in the emission spectra of nanoparticles and fluorescent dyes is likely minimal, necessitating a different explanation for the inability to distinguish between live and dead *W83*.

Fluorescent dyes, SYTOX advanced Dead Cell Stain and Pacific Blue Annexin V kit were considered. These tools have been used to assess cellular apoptosis and necrosis. Although, apoptosis is normally not used to describe bacterial cell death, and there are arguments against its use for bacteria (Hacker, 2013). Tanouchi et al. (2013) reviewed

numerous studies indicating that programmed cell death does occur in bacteria, and that such deaths may even be benefitial to a multispecies biofilm (Allocati, et.al., 2015). In view of such reports, it becomes reasonable to wonder if these tests for apoptosis may be useful in testing bacterial cell death mechanisms. Pacific Blue is a fluorophore conjugated to annexin V, a human vascular anticoagulant with a high affinity for phosphatidylserine, which difuses from the inner to the outer leaflet of the plasma membrane in apoptotic cells. The red fluorescent dye SYTOX advanced Dead Cell Stain is a high-affinity nucleic acid binding stain that easily penetrates cells with compromised plasma membranes but not healthy ones. Repeated attempts to evaluate apoptosis (via Annexin-V) and/or necrosis (via SYTOX) for *W83* proved unsuccessful. No cell viability discrimination was observed between *W83* populations stained with Annexin-V and SYTOX9. Following two attempted techniques to use flow cytometry to distinguish between viable and dead cells without success, it was concluded that, unfortunately, the current assays were unsuitable for *W83*. In this context, it is likely that further developments will be needed before flow cytometry can be used to rapidly distinguish between viable and dead *P. gingivalis*.

3.6 Summary, Conclusions and Future Studies

No prior reports were found about potentiation of antibiotic effects by nanoparticles in an oxidative stress environment. Present study demonstrated that silver and silver-gold bimetallic alloy nanoparticles can serve as potentiated antibiotics in an oxidative stress environment, and that higher gold content increased the antibacterial efficacy. Furthermore, these results differ from studies reporting that antibacterial activity is negatively correlated with increasing gold content in a silver-gold bimetallic

alloys due to incresed resistance to oxidation (Alissawi et al., 2013; Ristig et al., 2015). Current results suggest a potential application of nanoparticles against active inflammation with a developing oxidative stress environment. While the study demonstrates antibacterial efficacy of silver and silver-gold bimetallic nanoparticles, it does not explain the mechanisms of nanoparticle action. Nanoparticles may have a self-regulating mechanism, which is triggered by oxidative stress in the infection environment. Future studies should focus on a) the kinetics of Ag^{+} release in the presence and absence of hydrogen peroxide, and b) the characterization of the interactions between nanoparticles and bacteria and how these impact bacterial viability. Additional studies may further extend gold composition to find the optimal gold content for antimicrobial effect against *P. gingivalis*.

Flow cytometry analysis proved unworkable at this time for validation of bacterial viability assays using optical density spectrometry and colony-forming unit counts. However, flow cytometry remains an important tool in distinguishing viable from non-viable cells for many other eukaryotic and prokaryotic cells. The availability of *P. gingivalis*-specific antibodies suggests the possibility of using antibody-dependent techniques in microbial viability assessment. Challenges may include overlapping fluorescence from metallic nanoparticles and labeled bacteria. Such an overlap may prevent the distinction between populations of live versus dead bacteria. It will be important to develop techniques that can differentiate between nanoparticle fluorescence from fluorescently labeled antibodies targeting physiological or biochemical changes in cells in consequence to their interactions with nanoparticles.

Future studies may include more detailed time-course studies for nanoparticle treated bacterial populations over 24–48 hours, with bacterial samples assessed at 2 to 4-hour intervals. Furthermore, such studies could also involve avariety of imaging techniques over 24 to 48 hours, with the samples assessed at 2 to 4-hour intervals, to observe the specific interactions between nanoparticles and bacteria, with resulting changes in bacterial morphology and physiology. The elucidation of the specific role of the oxidative environment on nanoparticle activation and surface modification, with particular emphasis on the Ag^{+} ions in solution, would be helpful as part of a time course study. Imaging of nanoparticles and analysis of their physicochemical characteristics, as well as the changes that take place as a result of interactions with bacteria, are also of interest. Such information will help to better understand the antibacterial mechanisms of silver-gold bimetallic alloy nanoparticles and therefore help to design better antibiotic nanoparticles.

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CHAPTER FOUR

NOVEL HEMOCOMPATIBLE SILVER-GOLD BIMETALLIC NANOPARTICLES

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4.1 Abstract

Hemocompatibility of anaerobically synthesized silver and silver-gold alloy (3 or 16% gold) nanoparticles is assessed by determining their effect on platelet activation in fresh citrated human whole blood using flow cytometry. Using nanoparticle concentrations ranging from 0.00167 – 16.7 ng/mL suspended in 2% (w/v) Pluronic F127 we determined that platelet activation has a negative relationship to the concentration of nanoparticles. Antibody labeling was done with anti-human peridim-chlorophyll protein (PerCP)-conjugated anti-CD61 specific for platelet-specific glycoproteins to isolate platelets and phycoerythrin (PE)-conjugated anti-CD62P to identify activated platelets. Nanoparticles with up to 16% gold (16.7ng/mL) did not activate platelets. In contrast, Pluronic F127 activated platelets in a concentration-dependent manner between 0-5% w/v above baseline at values comparable to 3.0 μM ADP, a known activator of platelets. Pluronic F127 without nanoparticles has approximately 2.5-fold the platelet activating function compared to Pluronic F127 with nanoparticles. Future investigations are needed to fully characterize the effects of F127 on the hemostatic response.

4.2 Introduction

Periodontal disease affects over 3.6 billion people making this the most common communicable disease worldwide (WHO, 2018). Up to 15% of adults worldwide and 60% of Americans have periodontal disease (Rozier et al., 2017; Petersen & Ogawa, 2005). Periodontal disease leads to irreversible periodontal attachment loss, alveolar bone destruction, increased tooth mobility, and subsequent tooth loss if left untreated (Pihlstrom et al., 2005; Gerits et al, 2017; Hajishengalis, 2014). Periodontal disease has also recently been linked to some vascular disorders (Hajishengalis et al., 2012; Olsen & Yilmaz, 2016; Velsko et al., 2014; Bartold & Narayanan, 2006).

Current treatment methods for periodontal disease include surgical and non-surgical scaling, root planning, mechanical debridement, and antibiotic use (Sweeting et al., 2008). While antibiotics remain an important part of treatment, indiscriminate use of antibiotics may exacerbate development of antibiotic resistance which is encountered with increasing frequency in western nations including the United States (Franci et al., 2015; Rams et al., 2014). However, when effective, antibiotics can reach regions inaccessible by physical treatments, including oral mucosa and the vascular system (Slots & Ting, 2002). Systemically administered antibiotics can access numerous physiological compartments including the penetration of cell membranes (Gerits et al., 2017). Locally applied antibiotics may improve targeting. However, effectiveness is dependent on the application of correct dosage by clinicians (Aljateeli et al., 2013; Nair & Anoop, 2012). Together, debridement, topical chemical therapies, antibiotics, and the practice of good oral hygiene can potentially control even the resistant periodontal pathogens (Slots &

Ting, 2000). Developing new antibiotics remains a vital part of the overall objective of improving oral and systemic health.

As a keystone oral pathogen, *Porphyromonas gingivalis* (*P. gingivalis*) can significantly influence microbial colonization and inflammation, which are important components of periodontal disease development (Hajishengallis, 2012). Newer non-conventional approaches to oral infections include quorum sensing inhibitors, antimicrobial peptides, plant-derived antibacterial agents, sugar alcohols and antibacterial coatings (Gerits, et al., 2017). A recent addition to this field is the development of nanotech antimicrobials.

Nanomaterials offer numerous advantages over traditional antibiotics including: reduced toxicity, broad-spectrum application, low cost, and reduced bacterial resistance (Gurunathan et al, 2014; Lara et al., 2011; Pal et al., 2015; Vadlapudi & Kaladhar, 2014). Current efforts toward infection control is focused on delivery of drugs to the periodontal pocket, where infection often persists. Improvements in drug delivery may be achieved by a variety of strategies including: prolonged drug release using bioadhesive polymers, increased intrapocket drug penetration, and loading of multiple drugs into delivery systems made of nanoparticles and hydrogels (Aminu et al., 2017). Hydrogel nanoparticle formulations are already in use and were reviewed by Hamidi et al., (2008). Drugs targeting bacteria at primary infection site, the periodontal pocket, are the goal for effective control of dental infections.

Metallic nanoparticles offer some potential as dental materials for fillings and restorations, toothpaste and hydrogels, endodontics, implant dentistry, and periodontal care (Bapatet al., 2018). Silver has a long history as an antibiotic. It was the most

important antimicrobial agent before development of antibiotics (Alexander, 2009; Ebrahiminiezhad et al., 2016). Silver metal characteristically has broad antibacterial properties (Dhanalakshmi et al., 2013; Maillard & Hartemann, 2013). In this context, silver nanoparticles can be effective antibiotics (Franci et al., 2015; Rai et al., 2014; Rai et al., 2009) with activities against drug-resistant bacteria (Smekalova et al., 2016; Amirulhusni et al., 2012), non-resistant microbes (Amirulhusni et al., 2012; Markowska et al., 2013; Radzig et al., 2013; Xinping et al., 2011), Gram-positive and Gram-negative bacteria (Amato et al., 2011; Bondarenko et al., 2013; Gurunathan et al., 2014; Mohanty et al., 2012; Panáček et al., 2006; Taglietti et al., 2012), and biofilm integrated or free-living bacteria (Gurunathan et al., 2014; Markowska et al., 2013; Ouay et al., 2015; Velázquez-Velázquez et al., 2015). Silver nanoparticles also have some anti-inflammatory (Kemp et al., 2009; Murphy et al., 2015) and wound healing properties (Orlowski et al., 2018; Ahmadi & Adibhesami, 2017; Akila & Nanda, 2012; Gunasekaran et al, 2012; Orlowski et al., 2018; You et al., 2017).

The major impact of silver's broad antimicrobial activity, is due to release of Ag^+ ions. This is especially true for nanoparticles smaller than 10 nm (Bartłomiejczyk et al., 2013; Dakal et al., 2016; Durán et al., 2016; Franci et al., 2015; Swathy et al., 2014). For nanoparticles larger than 10 nm, Ag^+ release and physical contact are equally important (Bondarenko et al., 2013; Sotiriou & Pratsinis, 2010). Suggested mechanisms of Ag^+ action include: disruption of cellular processes, destruction of the bacterial cell wall, and oxidative stress (Bapat et al., 2018; Garcia-Contreras et al., 2011; Bapat et al., 2018). The release and availability of Ag^+ depends on pH, oxidative environment, presence of sequestering ligands, agitation, temperature, and the composition and size of

nanoparticles (Alqadi et al., 2014; Amirjani et al., 2015; Anigol et al., 2017; Fleitas-Salazar et al., 2017; Jiang et al., 2011; Liu et al., 2017).

Environmental factors can affect the Ag^+ release, leading to varying amounts of active Ag^+ ions, and to differences in scope of activity and results, produced by similar silver nanoparticles in particular tests. For example, it was reported that silver nanoparticles activated platelets, shortening coagulation time leading to the development of hemostatic blood clots (Fröhlich, 2016; Guidetti et al., 2012; Jun et al., 2011; Laloy et al., 2014), yet other reports claimed that silver nanoparticles cause increased coagulation time (Bandyopadhyay et al., 2012; Fröhlich, 2016; Major et al., 2016). Such conflicting reports reveal the need for precise characterization of nanoparticles and for adequate description of the environment they are used in.

Silver nanoparticles are toxic to eukaryotic cells and this poses a problem for use in humans (Prabhu & Poulose, 2012; Reidy et al., 2013). However, toxicity can be reduced by post-synthetic surface modifications to silver nanoparticles, which can enhance antibiotic efficacy, improve biocompatibility and minimize undesirable outcomes (Kora & Rastogi, 2013; Liu et al., 2014; Yang et al., 2012; Chung et al., 2008; Shawcross et al., 2017). While reducing toxicity toward animal or human host cells, the modifications should not significantly reduce the antibiotic effectiveness. Two examples of such modifications are: a) alloying with other metals, such as gold, and b) coating/capping of nanoparticle surfaces with surfactant.

Alloying silver nanoparticles with gold can enhance their antibacterial and anti-inflammatory characteristics. At the same time, silver-gold bimetallic nanoparticle toxicity can be tuned by varying the silver to gold ratio (Bilous et al., 2018; Li et al.,

2010). It was reported that an exposure to 10 - 12 nm 50:50 silver-gold nanoparticles, at 5-20 μg/mL for 24 hours, or at 15-60 μg/mL for 7 days, had no significant impact on the viability of human mesenchymal stem cells (Mahl et al., 2012). Padmos et al. (2015) synthesized ≈7 nm silver-gold alloy nanoparticles with silver:gold ratios ranging from 100:0 to 30:70. Bimetallic alloy nanoparticles had lower antibacterial activity, and much lower cytotoxicity. The antibacterial activity was dependent on the location of silver in the alloy, and the cytotoxicity was dependent on the overall gold concentration. Grade et al. (2014) and Ristig et al. (2015) confirm that bimetallic silver-gold alloys can maintain antibacterial activity, though it may be reduced, while exhibiting higher host biocompatibility.

Coating or capping nanoparticle surfaces can also enhance their biocompatibility. Pluronic polymers are common capping agents used for this reason (Pitto-Barry & Barry, 2014). Capping with Pluronic F127 did not restrict or reduce the antibacterial activity of silver-gold alloy nanoparticles (Holden et al., 2016).

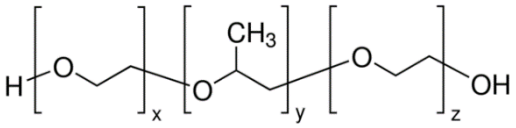

Figure 1. The general structure of Pluronic block copolymers. The Pluronic F127 (Sigma Aldrich) are typically x, z ≈100 and y ≈70, respectively.

Pluronic® F127 (PEO_x-PPO_y-PEO_z) where x, z ≈100 and y ≈70, is a triblock copolymer comprised of a central block of hydrophobic polypropylene oxide (PPO) flanked by two hydrophilic polyethylene oxide (PEO) chains. F127 is a non-ionic surfactant polyol with a molecular weight of ≈12.6 kDa. The surfactant and thermo-

responsive properties of Pluronic F127 are derived from its chemical structure (Fakhari et al., 2017; Giuliano et al., 2018) and make it a suitable capping agent in the synthesis of nanomaterials and manufacture of medical equipment (Mao et al., 2009), and in drug synthesis and delivery (Basak & Bandyopadhyay, 2013; Bodratti & Alexandridis, 2018; Callan et al., 2017; Soni & Yadav, 2014).

Pluronic F127 is approved by the Food and Drug Administration (FDA) for use in various formulations (U.S. Food & Drug Administration, 2019). The Cosmetic Ingredient Review Expert Panel (2008) reported a total of 31 uses for Pluronic F127 at concentrations ranging from 0–30% w/v in a range of products. The biocompatibility of Pluronic F127 makes it useful for dispersion of drugs (Yildirim et al., 2013), for reduction of the inflammatory potential of microspheres (Jackson et al., 2000), for reduced platelet adhesion to biomaterial device surfaces (Mao et al., 2009), and in medical treatments for embolism (Ohta et al., 2006). Pluronic F127 is approved for intratympanic, ophthalmic, oral, periodontal, and topical applications (Giuliano et al, 2018). It has been used as a temporary vascular occlusion tool following accidental vessel damage or surgery (Gucu et al., 2013; Raymond et al., 2004). A gel formulation was used to control temporary intra-arterial occlusion during surgery (Decrouy-Duruz et al., 2013; San Norberto et al., 2012). Polypropylene film coated with Pluronic 127 was found to prevent platelet adhesion (Hakani et al., 2018). Similarly, pluronic F127 reduced platelet adhesion when coating biomaterial device surfaces (Mao et al., 2009). Moreover, treating or conjugating Pluronic F127 with other substances, enhanced control of their physical properties and biocompatibility (Shachaf et al., 2010). In conclusion, it is well established that pluronic F127 is a versatile and widely used reagent.

In spite of its numerous biomedical uses, physiological effects of Pluronic F127 on its own is not reported. This lack of information about the concentration dependent effects of Pluronic F127 on platelet activation, and hence blood coagulation is of significant concern and warrants study. The current study reports the effects of addition of Pluronic F127, over the range of 0–5% w/v, to human citrated whole blood. Thus, these results will help to determine whether or not Pluronic F127 is hemocompatible at these low concentrations.

4.2.1 **Rationale for this Study**

The blood coagulation system is sensitive and responsive to foreign surfaces. In this context, nanoparticle applications for medical purposes may lead to their access to circulation. Because of this, adequate characterization of potential hemostatic impact for such nanoparticles is needed.

4.2.2 **Scope of Study**

In this study, ≈20 nm nanoparticles stabilized in 2% w/v Pluronic F127 were incubated with citrated human whole blood obtained from consenting donors. Hemocompatibility was determined by assessing platelet activation by flow cytometry. The biocompatibility of Pluronic F127 is also explored in the range of 0–5% w/v.

4.2.3 **Study Objectives**

A. To assess the effects of Pluronic F127-stabilized nanoparticles on platelet activation in citrated human whole blood. Platelet activation was evaluated with flow cytometry using platelet specific fluorescently labelled monoclonal antibodies. The

nanoparticles used were 20 nm in size, composed either of silver or silver-gold alloy with varying ratio of silver:gold and varying nanoparticle concentrations.

B. To assess the effects of the stabilizer, Pluronic F127, on platelet activation in citrated human whole blood. Platelet activation was evaluated with flow cytometry while varying the concentration of added Pluronic F127 from 0 to 5% w/v.

4.3 Materials and Methods

4.3.1 *Reagents*

Ammonium hydroxide (28-30%), sodium hydroxide (≥98%), D-maltose (≥99%), silver nitrate (≥99%), gold (III) chloride hydrate ($HAuCl_4.3H_2O$; 99.999% trace metals basis), Pluronic F-127 ($EO_{100}PO_{65}EO_{100}$, MW ≈ 12500; batch number 038K0113) and reduced glutathione, paraformaldehyde, and Tyrode HEPES buffer reagents were used as received (Sigma-Aldrich, USA). Milli-Q water (Millipore) was used in all experiments. Monoclonal antibodies, phycoerythrin (PE)-labeled anti-human CD62P and peridinin-chlorophyll protein (PerCP)-labeled anti-human CD61, were obtained from BioLegend, USA. A stock of 100 µM ADP (Sigma-Aldrich, USA) was made up with Milli-Q water. Diluted 10 µM ADP was made from the 100 µM ADP using Tyrode HEPES buffer.

4.3.2 *Nanoparticle Synthesis*

Glutathione-capped nanoparticles were synthesized and characterized as described previously in Chapter 2. Briefly, nanoparticle solutions of Ag, 3% gold (BM1) or 16% gold (BM2) were adjusted to an absorbance at 400 nm of 10 by addition of 2% w/v Pluronic F-127 solution. The concentrations of these stock solutions were determined by MP-AES (Chapter 2). Dilutions of nanoparticles for use in platelet activation tests were made as needed in 2% Pluronic F127. All reactions were carried out

at room temperature. Solutions were stored in black microcentrifuge tubes and used within 10 days of preparation.

4.3.3 *Blood Sampling*

Ethical approval was obtained from the Loma Linda University Health Institutional Review Board (IRB# 5150095). A total of 13 participants, that included 4 females and 9 males, were recruited to donate blood. Informed consent was sought and obtained from each participant before recruitment. The donor selection criteria were: non-smokers, no medications affecting coagulation or platelet functions, good hydration, age of 20–60 years, absence of illness and generally good health. Briefly, blood was drawn by venepuncture into VACUETTE® Blood Collection Blue Cap Tube containing 3.2% sodium citrate, mixed by gently inverting the tube a few times, then placed in a heating rack set to 37°C, and left for at least 30 minutes for platelet activity to normalize. Drawn blood samples were not pooled but tested separately.

4.3.4 *Flow Cytometry of Nanoparticle-Treated Citrated Human Whole Blood*

Percent platelet activation (PPA) was determined based on a protocol modified from the method of Frelinger et al. (2015). Nanoparticle species of choice (either silver, 3% gold or 16% gold bimetallic), or Pluronic F127 of varying concentration (0–5% w/v), was added to whole blood, followed by the addition of ADP or Tyrode HEPES buffer. A cocktail of fluorescently labeled monoclonal anti-human antibodies were used: PerCP-conjugated anti-CD61 targets platelet surface-specific glycoproteins and PE-conjugated anti-CD62P targets activated platelets (BioLegend, San Diego, CA). The reaction was allowed to run for 15 minutes in the dark, folloed by addition of 4% paraformaldehyde

(PFA) (one-part PFA to two-parts blood solution) fixative and incubation for 20 minutes in the dark. Then, the samples were diluted 75-fold in 0.1M PBS, pH 7.4, to a volume of ≈3 mL. PPA was obtained on a Miltenyi MacsQuant flow cytometer equipped with a 488 nm laser. For platelets, side-angle and forward angle scatter as well as fluorescence of PE (578 nm) and PerCP (675 nm) dyes were measured. CD62P PE fluorescence was used to determine relative PPA. Data were analyzed using FlowJo v9 (BD) software. Flow cytometry results are presented as mean fluorescent intensity (MFI), which is calculated by dividing the fluorescence of stained samples by the fluorescence of the negative control. Representative data are presented as dot plots.

4.3.5 ***Statistical Analysis***

One-way ANOVA was carried out to determine the significance of PPA measurements. Regression analysis was performed to demonstrate the association of Pluronic F127 concentration with PPA. Where appropriate, Welch's *t*-test was used to determine differences between mean PPA of treatment groups.

4.4 Results

Nanoparticles in Pluronic F127 aqueous solution did not significantly activate platelets in citrated human whole blood. Figure 2 shows the mean PPA (± SD) for nanoparticle-treated citrated fresh human whole blood. Platelet activation over the concentration range of nanoparticles tested (0.00167–16.7 ng/mL) was not significantly different from baseline (Figure 2, Table 1). In contrast, 0.33% w/v Pluronic F127 induced significant platelet activation ($p < 0.01$). In this context, there was a weak, though not significant, inverse relationship between percent platelet activation and

nanoparticle concentration. As nanoparticle concentration increased, percent platelet activation decreased, and vice versa.

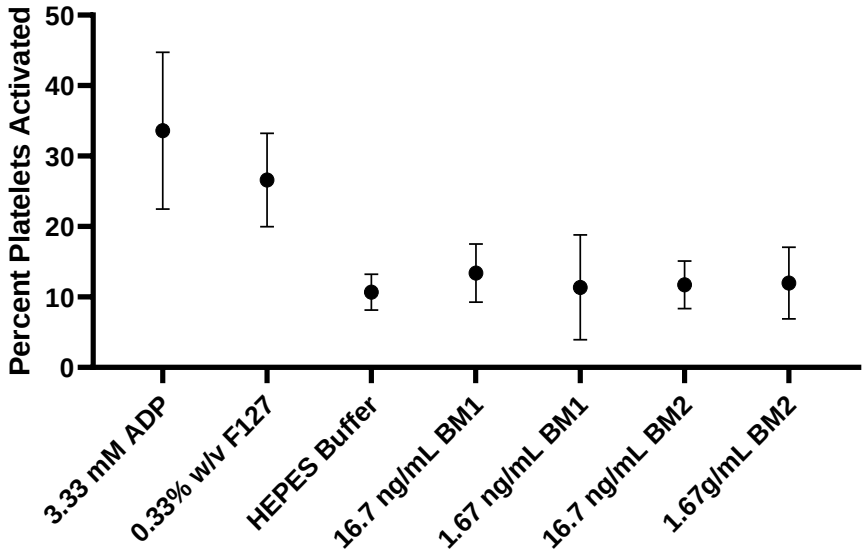

Figure 2. PPA by Pluronic F127-stabilized bimetallic nanoparticles. Citrated blood was incubated with 3.33 mM ADP, HEPES buffer, 0.33% w/v Pluronic F127, BM1 or BM2. PPA activation was determined by measuring the expression of CD62P, a platelet activation marker. PPA is presented as mean ± SD for all treatments. A one-way ANOVA showed that both ADP and Pluronic F127 had a significant effect on PPA compared to baseline activation under HEPES, $F(6,17) = 6.94$, $p < 0.01$. There was no difference between baseline activation and nanoparticle-induced activation ($N \geq 3$ for all treatments).

Welch's *t*-test was used to determine the difference between ADP and Pluronic F127 induced platelet activation. There was no significant difference in platelet activation by these two agonists, $t(4.9) = 1$, $p = 0.35$ indicating that Pluronic F127 is a potent activator of platelets at the concentration tested. We did not observe a gold ratio-dependent change in platelet activation levels for the different ratios of gold to silver in BM1 versus BM2. There was also no difference in mean platelet activation between silver and bimetallic nanoparticles.

Table 1. Bimetallic silver-gold nanoparticle-induced platelet activation in citrated human whole blood. BM1 and BM2 were incubated with citrated human whole blood, in the presence of fluorescently labeled antibodies targeting platelet, and activated platelet surfaces, for subsequent flow cytometry analysis. The results are represented as mean fluorescent intensities. Data were analyzed by one-way ANOVA. Percent platelet activation was lower for nanoparticles than for ADP agonist, but significantly higher than the HEPES buffer ($p < 0.05$). There is also a significant concentration-dependent effect of nanoparticles on platelet activation for both BM1 and BM2, $F(5, 10) = 29.45$, $p < 0.05$. Platelet activation was generally inversely related to nanoparticle concentrations for both BM1 and BM2.

	Blood Treatment	Platelet Activation (Mean ± SD)	N
Controls	HEPES Buffer	10.7 ± 0.2	2
	0.33% w/v Pluronic F127	26.6 ± 6.6	3
	3.33 mM ADP	28.4 ± 3.0	3
Nanoparticle Solutions	0.167 ng/mL Ag	13.4 ± 401	4
	0.00167 ng/mL BM1	19.9 ± 0.9	3
	0.167 ng/mL BM1	11.4 ± 7.4	3
	16.7 ng/mL BM1	10.9 ± 0.7	2
	0.00167 ng/mL BM2	19.1 ± 1.1	3
	0.167 ng/mL BM2	11.7 ± 3.4	4
	16.7 ng/mL BM2	12.0 ± 0.2	2

This further supports the hypothesis that the gold in the bimetallic silver-gold nanoparticle increases biocompatibility, which in this case is manifest as reduced platelet activation.

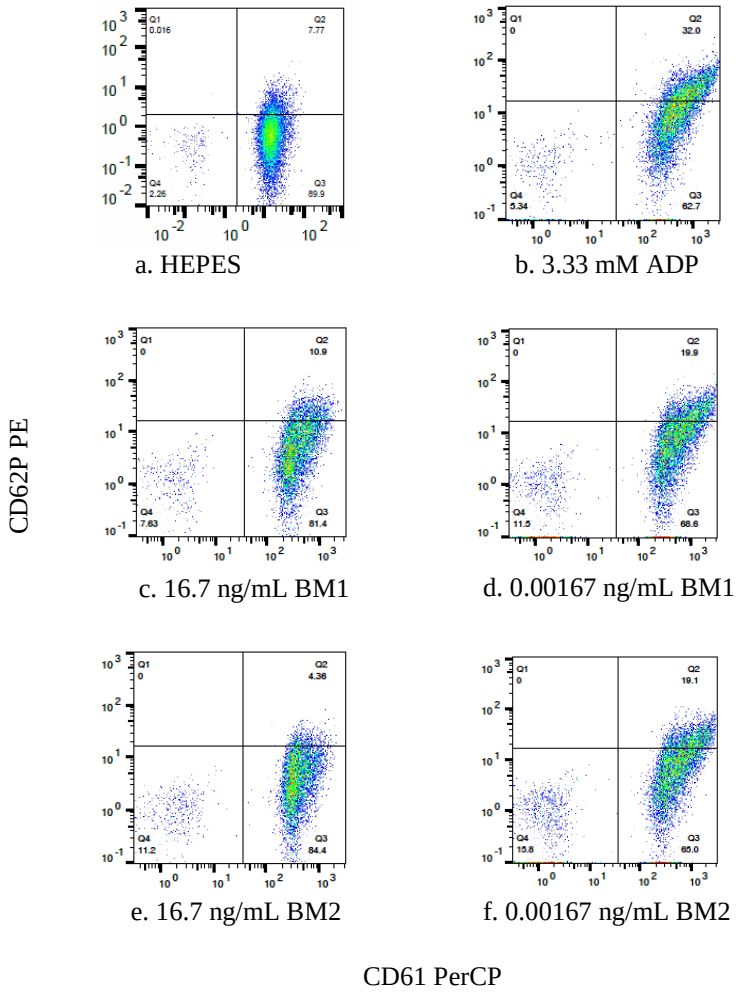

Figure 3. **Representative flow cytometry analysis of nanoparticle-induced platelet activation in fresh citrated human whole blood**. The blood was incubated with a) HEPES buffer, b) 3.33 mM ADP, c) 16.7 ng/mL BM1, d) 0.00167 ng/mL BM1, e) 16.7 ng/mL BM2, and f) 0.00167 ng/mL BM2. Following incubation, platelet activation levels were determined by flow cytometry. The result is the mean fluorescent intensity. Q1 represents PE-CD62P^{+}/PerCP-CD61^{-}; Q2, PE-CD62P^{+}/PerCP-CD61^{+}; Q3, PE-CD62P^{-}/PerCP-CD61^{+}; Q4, PE-CD62P^{-}/PerCP-CD61^{-} cells. Nanoparticle-induced platelet activation was lower than activation by 3.33 mM ADP for both BM1 and BM2 at all concentrations tested.

Results summarized in Figure 3 and Table 1 suggest that nanoparticles may have an attenuating effect on platelet activation and that Pluronic F127 on its own may activate platelets. To further probe the effect of Pluronic F127, it was incubated at varying

concentrations (0–5% w/v) in citrated human whole blood. Pluronic F127-treated blood was analyzed by flow cytometry and the data are given in Table 2 and Figure 4. The regression analysis of the effect of 0–5% w/v Pluronic F127 on platelets demonstrated that PPA was concentration-dependent up to 5% w/v. Above 5% w/v, further increase in Pluronic concentration did not produce a proportional increase in PPA (Table 2). Between Log([F127]) -2 and -8, PPA is directly proportional to Pluronic F127 concentration.

Table 2. **The effect of Pluronic F127 over 0–5% w/v (final concentration) on the activation of platelets in citrated human whole blood**. Pluronic F127 over a concentration range of 0–5% induces platelet activation in a concentration-dependent manner.

Log([F127])	% Act	P_{calc}	resid	$resid^2$	$(y-y_{av})^2$
-1.00	21.30	21.50	-0.20	0.04	22.56
-2.48	21.50	20.90	0.60	0.36	24.50
-4.78	16.70	17.18	-0.48	0.23	0.02
-6.78	14.80	14.19	0.61	0.37	3.06
-8.78	12.30	12.83	-0.53	0.28	18.06
-10.78	12.90	12.27	0.63	0.40	13.32
-12.78	11.60	12.02	-0.42	0.18	24.50
% Act(avg)	**= 16.55**		**Sum($resid^2$)**	**1.90**	**128.60**
			R^2	**0.9853**	

The effect of Pluronic F127 on platelet activation is dependent on concentration. Pluronic F127 at the highest concentration tested produced similar levels of platelet activation as the potent platelet activator ADP (Table 1). In Table 2 the percent platelet activation peaked at 21.3% for Log([F127]). This level is similar to ADP-induced platelet activation and significantly higher than that for BM1 and BM2 at 16.7 ng/mL (Table 1).

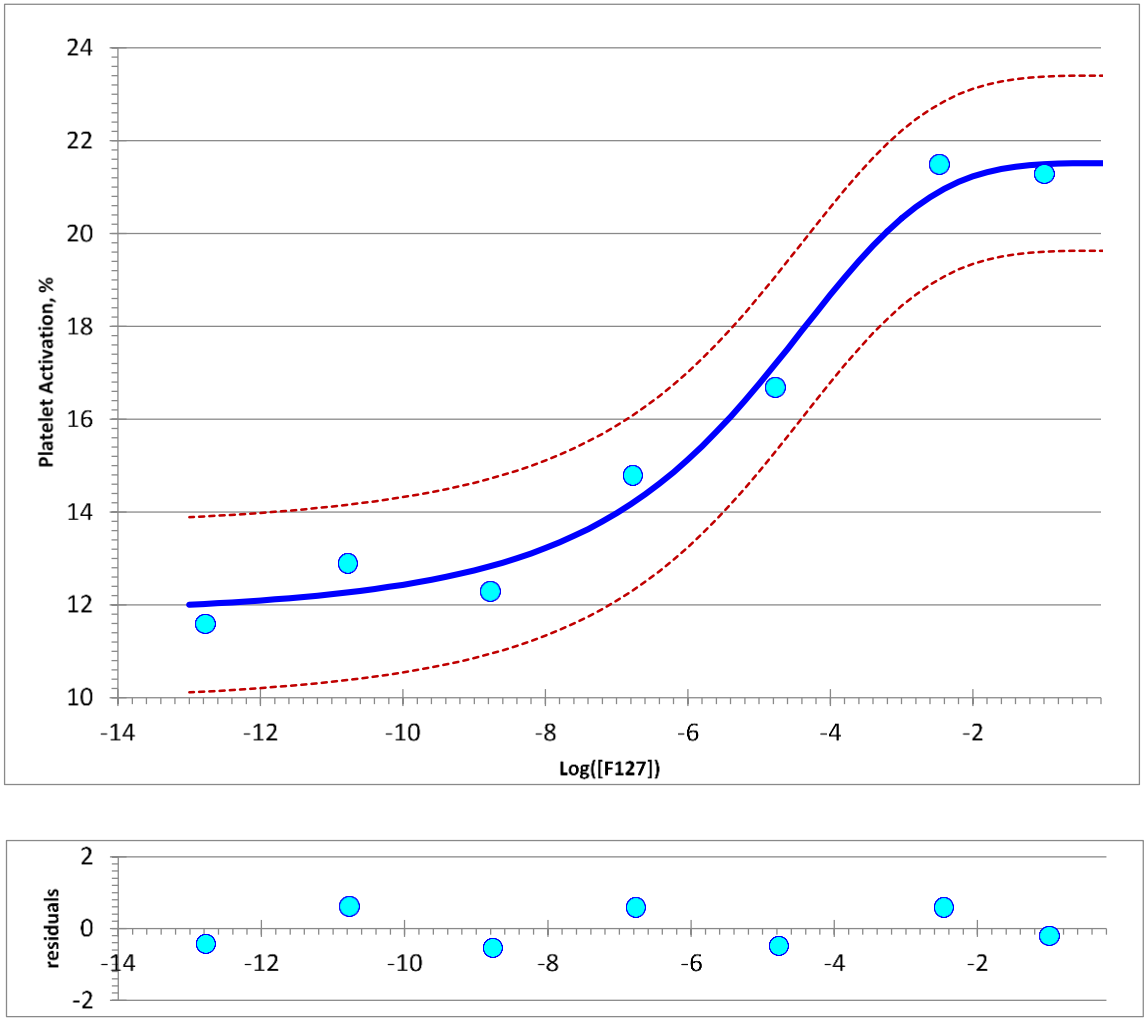

Figure 4. **Concentration dependent platelet activation by Pluronic F127**. A graph predicting PPA using logistic regression as determined by flow cytometry of citrated human whole blood incubated with 0–3% w/v Pluronic F127 at 25°C. Pluronic F127 causes concentration-dependent platelet activation over the tested range. EC_{50}(F127) = 8.4 x10^{-6} w/v; df = 4; SEM = 0.6886; $t_{critical}$ = 2.7764; CI = 1.9119. Percent platelet activation linear predictor = $\frac{initial+(final-initial)}{(1+\left(\frac{Log([F127])}{inflection}\right)^{p})}$, where initial = 11.724, final = 21.518, inflection = -5.076, and p = 3.761.

Flow cytometry analysis was also performed for the potential effects of butylated hydroxytoluene (BHT), used as a Pluronic F127 preservative at 100 ppm, corresponding to the concentrations of BHT contained in 0-5% Pluronic F127. BHT does not induce platelet activation above baseline (Table 3).

Table 3. Percent platelet activation by BHT. BHT-induced platelet activation was determined by assessing concentrations of BHT by itself in the amounts found in the Pluronic F127 sample from Sigma. BHT was 100 ppm in the Pluronic F127. The graph shows the mean (±SD) for N=5.

Log [BHT]g/µL	Mean	SD
-2.48	0.52	1.02
-2.78	0.04	0.07
-4.78	-0.26	0.53
-6.78	1.77	3.52
-8.78	-0.59	1.19
-10.78	-0.33	0.82

This implies that the platelet activation effects are due to Pluronic F127 rather than due to its preservative BHT. Therefore, as a component of the surfactant, BHT is not responsible for the platelet activity seen when citrated whole blood was incubated with 0-5% Pluronic F127, as shown in Figure 5.

4.5 Discussion

The use of nanoparticles is limited by our understanding of the physiological effects nanoparticles have inside the body, including their potential impact on the hemostatic system (Fröhlich, 2016**)**. Silver nanoparticles, in particular, have broad

applications as antibiotics. However, they have known eukaryotic cellular toxicities (Prabhu & Poulose, 2012**)**. Developing biocompatible silver nanoparticles is important for full integration of nanoparticles into clinical treatments of systemic infections. It was reported that silver nanoparticles alloyed with gold have reduced cytotoxicities while maintaining antibacterial activity (Mahl et al., 2012; Padmos et al., 2015). Silver-gold nanoparticles with ≈3% (BM1) and ≈16% (BM2) gold, in this study, were tested over a concentration range of 0.00167–16.7 ng/mL, representing the concentration range shown to have antibacterial activity (Chapter 3). Ag, BM1 and BM2 produced similar platelet activation to control in citrated human whole blood, without inducing significant platelet activation above baseline ($p<0.01$) (Figure 2, Table 1). The percentage of activated platelets was negatively related to the concentration of nanoparticles applied and the amount of gold in the bimetallic. This suggested that there is a platelet activating effect due to the solution containing the triblock copolymer F127. Pluronic F127 was tested over the range of 0–5% with citrated human whole blood, demonstrating concentration-dependent platelet activation (Figure 4).

Previous studies reported that Pluronic F127 is a suitable biocompatible surfactant and it was approved for use by the FDA (2019). It has been used in numerous applications, including studies with platelets to reduce platelet adhesion (Hakani et al., 2018; Mao et al., 2009). Our results contrast with some reported effects of Pluronic F127 on platelets. This may result from several differences in the previous studies. First, the studies report using Pluronic F127 as a coating which we can assume is a thin corona for nanoparticles or thin layer for other surfaces (Mao et al., 2009). This differs from our study where Pluronic F127 is in solution in which nanoparticles are suspended.

Secondly, the concentration range reported in those studies is much higher than used in this study. For example, in Hakani et al. (2018) at 3% w/v Pluronic F127 showed platelet activation but at higher concentrations activation decreased until it was almost non-existent at 20% w/v. Ahmed et al. (1999) suggest that for Pluronics, the ability to inhibit platelet aggregation depends on their tendency to self-assemble into micelles and on their relative binding rate to platelet surfaces.

Based on previously reported studies about the effects of Pluronic on platelet aggregation, and from current results of platelet activation, several possible mechanisms are suggested. First, nanoparticles may compete with Pluronic F127 for platelet surface binding sites and once bound, they prevent Pluronic F127 from binding. Second, nanoparticles bind to platelet surface and directly attenuate platelet activation. Both of these scennarios are consistent with the observation that solutions with low nanoparticle concentrations tended to induce higher platelet activation. Third, nanoparticles may be endocytosed by platelets and then may prevent platelet activation by disrupting internal cellular pathways. According to this mechanism, platelets are not activated even when Pluronic F127 binds to platelet surfaces.

Testing these hypotheses could be achieved using high-resolution microscopy such as TEM to identify nanoparticles at surface receptors and phase-contrast microscopy to determine receptor abundance (Hajtuch et al., 2019). Alternatively, blocking platelets' surface receptors before incubation with nanoparticles or Pluronic F127 could be used to determine if surface receptors are involved with activation. Using specific receptor antagonists, we could determine which specific receptors were involved in the nanoparticle- or Pluronic F127-mediated platelet interactions. A number surface receptor

ligands are currently available for specific surface receptors, such as the P2Y12 receptor for ADP, and others are being developed (Laine et al., 2016; Wijeyeratne & Heptinstall, 2011). Microscopy could also be used to determine whether nanoparticles in this study are endocytosed and how this process takes place. Confocal microscopy has been used to study the internalization of nanoparticles (Chung et al., 2008).

4.5.1 *Implications of this study*

Nanoparticles, when carefully synthesized, can be made to be biocompatible. Furthermore, nanoparticles can be tuned to achieve desired functionality. These functions include biocompatibility and possible platelet antagonists. In our studies, we observed that Ag, BM1 and BM2 nanoparticles reduced platelet activation to baseline levels compared to a solution of Pluronic F127 without nanoparticles. The amount of gold in the bimetallic did not impact the hemocompatibility of nanoparticles with respect to platelet activation. Alternatively, our results indicate that Pluronic F127, at concentrations 0–5%, activated platelets in a concentration-dependent manner (threshold $>10^{-5}$% w/v), thus posing a potential physiological threat of thrombosis associated with its use. Pluronic F127 was not reported before to cause platelet activation in the 0–5% w/v concentration range. Therefore, further studies are needed to determine the mechanisms of this effect. Presented results tend to suggest that nanoparticles are antagonizing platelet activation. This also needs further mechanistic investigation.

4.5.2 *Limitations of this study*

The nanoparticles studied were of one size, and the composition was limited to three options of relative gold:silver ratios. In this sense, this work represents a pilot

study. A more comprehensive study should include a more thorough examination of varying the nanoparticle size and composition, and its impact on platelets in citrated human whole blood. Further concern is the potential concentration effect of nanoparticles. While the concentration of the nanoparticles incubated with the blood was calculated, it also would be helpful to monitor the number of nanoparticles interacting with platelets. Furthermore, the working assumption is that platelets are relatively uniform and that, therefore, nanoparticle interactions with them will be comparable. Current understanding is that platelets comprise different functional subgroups, potentially impacting the observed nanoparticle effects. Moreover, the physical interactions between platelets and nanoparticles still remain to be characterized.

Finally, nanoparticles can be altered following incubation with the blood. Further, nanoparticles in the blood are known to develop a corona, which potentially affects their activity. As a result, upon addition to blood, the effective nanoparticle concentration and surface properties may be changing over time.

4.6 Summary, Conclusions and Future Studies

There may be some gold-dependent reduction in platelet activation, ordinarily observed with silver nanoparticles, when they are alloyed with gold. As a result, improved biocompatibility is anticipated, particularly with respect to hemostasis. Then, because of improved biocompatibility with gold alloy formulations, and because of the antimicrobial properties of silver, the silver-gold bimetallic nanoparticles can be used more broadly in medical applications. In contrast, Pluronic 127 may not be suitable for use at low concentrations for the delivery of nanoparticles due to its potential to impact hemostasis through activation of platelets.

The mechanism of nanoparticle-induced platelet activation is not well described, and this would be helped through studies of (1) the action of uncoated nanoparticles on blood cells in general and platelets in particular, (2) the effect of varying silver:gold ratio of bimetallic nanoparticles on platelet activation, and (3) the nanoparticle size-dependence for platelet effects, (4) detailed time course morphologic/structural investigations of Pluronic F127 impact on platelets, (5) biochemical changes in platelets as a result of interaction with Pluronic F127, and (6) the relationship between micellization of Pluronic F127 and its effects on blood cells including platelets. Additionally, *in vitro* detailed concentration tests are needed between 0–5% w/v Pluronic F127 to more fully characterize the surfactant effects on platelet function, clot formation, and stability, in the context of whole blood.

4.7 References

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CHAPTER FIVE

THESIS SUMMARY, CONCLUSIONS AND FUTURE DIRECTIONS

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Nanotechnology applications in biomedicine include treatments, which may help to address specific problems such as drug resistant microbes or specific targeting of cancer cells. Silver nanoparticles offer potential for treatment of some bacterial infections, but toxicity issues are concerning. Various approaches during and post-synthesis have been attempted to improve their biocompatibility. Current work presents efforts to improve biocompatibility via synthesis of 20 nm glutathione-capped silver-gold alloy bimetallic nanoparticles. These nanoparticles were tested for their antibacterial efficacy against the oral pathogen, *Porphyromonas gingivalis W83,* and for hemocompatibility in citrated human whole blood. Observations are presented, demonstrating antibiotic efficacy as well as enhanced biocompatibility.

Chapter two describes nanoparticle measurements, demonstrating that measured mean diameter is dependent on the method used. Characterizing the same nanoparticles, using three methods (DLS, AFM, and TEM), it was observed that the measured sizes were significantly different. It was also found that silver nanoparticles tended to be larger than the bimetallics BM1 and BM2, so that Ag > BM1 > BM2. This suggests that conflicting results from various labs may in part be due to nanoparticle size differences as well as their composition. Higher activities of smaller nanoparticles could in part be due to their higher surface/volume ratios. Some groups report different rates of activity for similar sized nanoparticles with different silver-gold composition. However, if the

physical dimensions are not fully described, then it is not certain that functional differences are necessarily attributable to the metal composition rather than size differences, as shown in chapter three.

A suitable stabilizing and storing agent is needed to preserve physicochemical characteristics of nanoparticles. Pluronic F127, at 2% w/v, stabilized nanoparticles and did not significantly alter their physicochemical properties.

Chapter three reports that oxidation from subinhibitory 0.10 mM hydrogen peroxide mediated antibiotic effectiveness of sub-20 nm silver-gold bimetallic nanoparticles. This antibacterial function was positively correlated with gold content. Some of the increased activity may also be due to nanoparticle size differences. The surface/volume ratio decreases with increased gold content, so that Ag < BM1 < BM2. The antibacterial activity, stimulated by subinhibitory hydrogen peroxide, followed similar trend. Since the concentration of nanoparticles was based on OD measurements, it follows that smaller nanoparticles will require larger numbers to achieve similar OD. This implies that smaller nanoparticles, having higher surface/volume ratio and larger numbers, will release more Ag^+ ions. Antibacterial activity was limited in the absence of an oxidizing agent. During infection, the host cellular reactions include production and release of reactive oxygen species, including hydrogen peroxide, as part of their immune response. The hydrogen peroxide induced oxidation of silver, resulting in a release of Ag^+, which have antibacterial activity. The bimetallic nanoparticles in this size range may be physiologically self-regulating, so that the most potent antibacterial activity occurs during active infection that produces reactive oxygen species.

Chapter four examines the hemocompatibility of the silver containing nanoparticles. The glutathione-capped 20 nm silver-gold bimetallic nanoparticles were hemocompatible to citrated human whole blood without significant platelet activation. The stabilizing agent, Pluronic F127, however, activated platelets in a concentration-dependent manner at concentrations below 5% w/v. This may challenge the suitability of its use in clinical applications. Further characterization of this effect is needed to determine its mechanism of action.

Finally, the bimetallic nanoparticles are demonstrably countering the platelet activating effects of the triblock copolymer Pluronic F127. Further in vitro experiments are needed to fully characterize this unexpected observation.