The effects of silver nanoparticles on the expression of protein biomarkers of cell stress, apoptosis and inflammation by the human liver cancer cell line, HepG2

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Declaration

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I, Tina Volkmann (student number: 3252594) declare that the mini-thesis on **"The effects of silver nanoparticles on biomarkers of inflammation and cell stress in HepG2 human hepatoma cells with and without inhibition of clathrin-mediated endocytosis"** is my work, that it is free of plagiarism and that all the sources used have been indicated and acknowledged by complete references.

Signed: 

Tina Volkmann

Abstract

Nanoscience is the study of phenomena and objects at the nanoscale (around 1-100 nm), so-called nanomaterials. These nanomaterials exhibit novel properties that are often very different to those of the bulk materials used for their synthesis. Hence, nanoparticles are widely commercialised, especially silver nanoparticles (AgNPs) due to their antimicrobial properties and some other useful phenomena. This commercialisation leads to inevitable exposure to the environment and humans, which leads to inhalation, ingestion or dermal uptake of AgNPs by the human body culminating in distribution to several major organs, including the liver. Both chronic and acute exposure to AgNPs have been linked to detrimental effects in both *in vitro* and *in vivo* studies. These include oxidative stress, induction of inflammation, DNA damage, cell death and many others. The results vary greatly since several properties and aspects of the AgNP as well as the nanoparticle synthesis and toxicity assay methods used, such as capping agent, cell type, exposure route, exposure environment, cellular uptake pathway – to name a few have a significant effect on the outcome. The purpose of this study was to determine the effect of AgNP exposure, as well as the modulation of these effects by clathrin-mediated endocytosis (CME) inhibition, on HepG2 human hepatoma cells *in vitro*. Biomarkers of inflammation and cell stress were investigated.

Treatment with the AgNPs for 48 hrs showed a concentration dependent reduction in viability of HepG2 cells, which was partially mitigated by pre-incubation with chloropromazine (CPZ), a pharmaceutical CME inhibitor. The culture supernatants were assayed for inflammatory markers: nitric oxide, interleukin-6, interleukin-8, macrophage inflammatory protein 1 beta, and macrophage migration inhibitory factor (MIF) - of which only MIF was detected upon AgNP exposure. The cell lysates were tested for cell stress biomarkers: superoxide dismutase 2 (SOD2) and heat shock protein 27 (HSP27/HSPB1), of which only HSP27 was detected and upregulated upon AgNP exposure. The inhibition of CME significantly ($p<0.001$) lessened the viability reducing effects, as well as MIF and HSP27 upregulation compared to the CPZ free AgNP (50 µg/mL) exposure samples. Although this study was not able to completely determine the mechanism of cytotoxicity induced by AgNPs, it showed that CME is involved in the upregulation of cytotoxicity, MIF and HSP27, - biomarkers indicative of cytotoxicity, inflammation and cell stress caused by AgNPs in HepG2 liver cells.

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Abbreviations

°C	degrees Celsius	ISO	International Standardization Organisation
µg	micrograms	kv	kilovolt
µg/mL	micrograms per millilitre	LDH	lipid dehydrogenase
µl/well	microliter per well	LSPR	localized surface plasmon resonance
µm	micrometre	mA	milliampere
A/A	aggregates/ agglomerates	MAPK	mitogen-activated protein kinase
ADP	adenosine diphosphate	MIF	macrophage migration inhibitory factor
Ag	silver	mL	millilitre
Ag+	silver ions	MoA	mechanism of action
AgNPs	silver nanoparticles	mRNA	messenger RNA
ANOVA	analysis of variance	MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
APP	acute-phase proteins	mV	microvolts
APR	acute phase response	NaCl	sodium chloride
ATCC®	America Type Culture Collection	NF-κB	nuclear factor κB
BSA	bovine serum albumin	nm	nanometre
CCL4/MIP-1β	macrophage inflammatory protein 1 beta	NMs	nanomaterials
cDMEM	complete DMEM	NO	nitric oxide
CM	cytoplasmic membrane	NPs	nanoparticles
CME	clathrin-mediated endocytosis	OD	optic density
CO2	carbon dioxide	PBS	phosphate-buffered saline
CPZ	chlorpromazine	PDI	polydispersity index
CvME	caveolae-mediated endocytosis	PEG	polyethylene glycol
CXCL8/IL-8	interleukin-8	pg/mL	picograms per millilitre
d_H	hydrodynamic size	PI3-K	phosphoinositide 3-kinase
diH2O	milli-Q ultrapure water	PVP	polyvinylpyrrolidone
DLS	dynamic light scattering	PVP-AgNP	PVP capped AgNPs
DMEM	Dulbecco's modified eagle medium	rcf	relative centrifugal force
DNA	deoxyribonucleic acid	ROS	reactive oxygen species
EC	European Commission	SA	surface area
EDX	energy-dispersive X-ray	SAED	selected area diffraction
ELISA	enzyme-linked immunosorbent assay	SD	standard deviation
EM	electron micrograph	SEM	scanning electron microscopy
ER	endoplasmic reticulum	SOD	superoxide dismutase
FBS	foetal bovine serum	TEM	transmission electron microscopy
Hrs	hours	UFPs	ultrafine particulates
HSA	human serum albumin	UV-vis	ultraviolet-visible spectroscopy
HSP27/HSPB1	heat shock protein 27	XRD	x-ray diffraction
IC_{50}	inhibitory concentration 50	XTT	2, 3-bis (2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium- 5carboxanilide
IL-6	interleukin-6	ζ	zeta-potential
iNOS	inducible nitric oxide synthase	λ_{max}	lambda max

Chapter 1 Literature review

1.1 Nanoscience

Nanoscience is the study of phenomena and objects at the nanoscale (around 1-100 nm), whereas nanotechnology is the use and manipulation of matter that is in the nanoscale (International Organization for Standardization, 2015). The European Commission (EC) defines a nanomaterial (NM) as:

> "*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*" (EU, 2011).

This definition considers individual particles or aggregates that become weakly bound together as agglomerates as long as their external surface area remains similar to the sum of the surface areas of the individual parts. While aggregates are strongly bound or even fused particles. While several definitions exist, all agree that any one of the particle's dimensions has to be within the nanoscale to qualify as a NM. In contrast to this, a nanoparticle (NP) further has all three dimensions in the nanoscale (International Organization for Standardization, 2015).

At such a small scale, size- and structure-dependent properties and phenomena are different from the same material, as single atoms or the material at the macro scale (International Organization for Standardization, 2015; Rasmussen *et al.*, 2018). These novel properties can be physical, chemical or biological in nature. Such novel biological activities and physicochemical properties include a higher surface area to volume ratio which leads to increases in surface reactivity due to a larger portion of energetically unstable atoms on their surface. Other modifications of properties include structural colour or electronic properties such as conducting and insulating behaviour, and many others (Nel *et al.*, 2006; Smita *et al.*, 2012; Syafiuddin *et al.*, 2017).

In addition to the novel properties, scientists and engineers can use different synthesis methods or modify certain aspects of the synthesis to achieve changes in properties despite using the same core material (Smita *et al.*, 2012). For example, varying the temperature or chemicals used during certain steps during production can change the size and shape of silver nanoparticles (AgNP) produced which in-turn changes the colour of the differently sized colloidal suspensions

(Wu *et al.*, 2016; Mohammadzadeh-Asl *et al.*, 2018). During the synthesis process, NPs are often 'capped' with a material to confer increased stability in liquid or another property such as targeting a specific location in the human body. In general, properties depend highly on the surface chemistry of the NMs. This surface chemistry, in turn, can be altered by the environment it is found in (Smita *et al.*, 2012; Moore *et al.*, 2015). This tunability of the properties is what NM manufacturers exploit when designing NMs for a specific purpose or harnessing such dynamic properties in nano-enabled (or composite) materials (Smita *et al.*, 2012). The logical consequence being applications in almost any sector, from the industrial to household sector (Sanyasi *et al.*, 2016). The Nanotechnology Products Database (https://product.statnano.com/) lists a total of 8856 consumer products. In 2018, the global nanotechnology market was worth $2.0 billion and is expected to increase to $2.1 billion by 2023 (BCC Research, 2019). Therefore, leading to increased intentional or unintentional exposure to consumers and the environment.

1.2 Nanotoxicology

Weighing up both the benefit and the potential hazards of emerging technologies always remains important; especially given nanotechnology's speedy and vast development, distribution and resulting exposure to humans and the environment. There are various potential routes for NP exposure, especially in consumer products. Packaging is often impregnated with AgNPs to extend shelf-life as it confers antimicrobial properties to the packaging but at the same time AgNPs can cause contamination of packaged food due to leeching and therefore ingestion by people (Llorens *et al.*, 2012). Nanotechnology's enhanced or even unexpected properties can be considerably beneficial not only in commercial but also in medical applications. Often NMs for medical applications are designed to have a complex surface to evade phagocytosis, translocate to specific tissues or organs, or cross the blood-brain barrier, to mention a few. Yet, this benefit may be countered by unintended consequences either during the intended application or at the end of the life cycle (Donaldson et al., 2004; Oberdörster, Oberdörster and Oberdörster, 2005a). Additionally, the small size of NPs allows for interaction and likely entry into the body and specifically entry at the cellular level leading to several interactions and possible interferences with the biological system (Oberdörster, Oberdörster and Oberdörster, 2005). For example, 20-30 nm sized carbon NPs translocated, upon inhalation, to the olfactory bulb; while particles >1

µm were not able to enter since the olfactory nerve axon is 100-200 nm in size. Similarly, C60 molecules of 0.7 nm showed cell penetration, likely via ion channels or pores found in the cell membrane (Buzea, Pacheco and Robbie, 2007). Given, the ubiquity, size and ability to enter cells and interact with sub-cellular structures it is necessary to consider the potentially detrimental effects of NMs. Studies designed to evaluate safety fall under nanotoxicology; which is defined as:

> *"[the] science of engineered nanodevices and nanostructures that deals with their effects in living organisms"* (Oberdörster, Oberdörster and Oberdörster, 2005).

Standardized terminology and methodology are needed to enable regulation of effective and valid nanotoxicology investigations of nano-scaled materials (Gatti and Montanari, 2015; Gao and Lowry, 2018). The International Standardization Organisation (ISO) has put forward several standards for different terms, assessments, and NM types, while the European Commission has given support to several nanotoxicity projects (Riediker and Katalagarianakis, 2013; ISO, 2020). Furthermore, the European Union has put some regulations in place and provided several recommendations, which are revised every few years. Yet, challenges with the guidelines exist, *e.g.* cost, equipment availability and required expertise (European Commission, 2012; Gao and Lowry, 2018; Rasmussen *et al.*, 2018, 2019; www.oecd-ilibrary.org).

As one of the first nanotoxicological studies, pulmonary toxicity by ultrafine particulates (UFPs) of heterogeneous mixtures (<100 nm diameter) was examined. It was at first found and later confirmed, that asthma risk, in both adults and children, correlates to environmental exposure to UFPs in urban air (Kaiser *et al.*, 2004; Buzea, Pacheco and Robbie, 2007; Kotecha *et al.*, 2019). Regarding the pulmonary toxicity, in support of physiological responses recorded in animal studies, *in vitro* studies showed data indicative of oxidative stress, production of inflammatory cytokines and apoptosis in response to such ultrafine particles (Buzea, Pacheco and Robbie, 2007). This shows how subliminal exposure to NPs from various sources, can have detrimental effects which are not necessarily evident as overt consequences.

Another such example of physiological modulation was performed in mice. Mice were exposed to AgNPs with different surface modifications. The NPs showed antimicrobial activity to select

gut microbiome bacteria and behavioural changes. No causal relationship was established but points to the need for further investigation (Javurek *et al.*, 2017).

1.3 Silver nanoparticles (AgNPs)

Since ancient times, silver has mostly been used for medical purposes, while some applications are still in use today *e.g.* since the 1880's a silver nitrate eye drop solution has been used in neonates for the prevention of gonococcal ophthalmia neonatorum (Alexander, 2009; Konop *et al.*, 2016). These uses are predominantly due to the antimicrobial properties of silver. The colloidal suspensions of silver, which often contain AgNPs, have been in use well before the advent of the germ theory (Nowack, Krug and Height, 2011; Konop *et al.*, 2016). AgNPs, in comparison to macro silver, retain and show enhanced antimicrobial activity among many other properties of interest. Therefore, silver is among the most common NMs found in consumer products and has widespread uses (Royce *et al* 2014, http://product.statnano.com/).

AgNP's interesting and useful properties include: thermal properties, electrical properties, catalytic abilities, photocatalytic abilities (Syafiuddin *et al.*, 2017; Gellé and Moores, 2019) and optical and plasmonic properties. The latter is likely of the best known AgNP properties. At the nano-scale, due to quantum entrapment of the surface electrons, AgNPs exhibit localized surface plasmon resonance (LSPR). Such AgNP surface electrons absorb electromagnetic radiation in the ultraviolet-visible (UV-vis) region around 380-450 nm (Syafiuddin *et al.*, 2017). Given a narrow size distribution of a AgNP sample, a characteristic maximum absorption will occur at a specific wavelength (lambda max; λ_{max}), forming a peak on the spectrograph. The height, intensity and breadth of the UV-vis spectrograph correlate with shape, size and the medium of suspension (Moore *et al.*, 2015). A red-shift (longer wavelengths) in $\lambda_{max,}$ as well as the broadening of the peak, is associated with a size increase, due to surface adsorption by organic species or aggregation of the AgNPs (Mohammadzadeh-Asl *et al.*, 2018). Furthermore, irregularly shaped AgNPs can exhibit two or more plasmon resonance peaks depending on particle symmetry. Due to such interesting optical properties, AgNPs are widely used for ultrasensitive bio-detection (Syafiuddin *et al.*, 2017), together with immune or aptamer detection methods (Liang *et al.*, 2012), for biological labelling and solar energy harvesting (Escoubas *et al.*, 2019).

Yet, the oldest and most studied property of AgNPs is their antimicrobial property. At the nanoscale, this antimicrobial activity is not only retained but enhanced. This, in short, is due to the relatively increased surface area, silver ion (Ag^+) dissolution *i.e.* Ag^+ release from the AgNP surface, and other less investigated properties such as crystalline structure (George *et al.*, 2012; Konop *et al.*, 2016). AgNPs are effective as antiviral, antifungal, antiparasitic, and antibacterial agents (Ravindran, Chandran and Khan, 2013).

The antiviral property is exemplified in its interaction with the HIV-1 virus, to which it binds preferentially at the gp120 surface glycoprotein acting as an initial replication stage viricidal agent or by inhibiting entry into the host cell (Elechiguerra *et al.*, 2005). For a more detailed discussion refer to Lara *et al.*, 2010; Rai *et al.*, 2016. Furthermore, the antifungal activity of AgNPs was demonstrated on pathogenic *Candida spp*. The minimum fungicidal concentration was considerably lower than the levels at which the AgNPs show cytotoxicity to human fibroblasts *in vitro*. This indicates that the AgNPs are more efficient than the ionic form of silver which was an effective fungicide only close to the cytotoxic concentrations used (Panáček *et al.*, 2009). Similarly, larvicidal effects have been established towards different mosquito species (Firdhouse and Lalitha, 2015) and the malaria pathogen, *Plasmodium falciparum* (Dutta *et al.*, 2017). Most exploited, though, is likely the antibacterial property of AgNPs.

AgNPs have been shown to be effective against several pathogenic bacteria such as *Escherichia coli* (Gram-negative) and *Staphylococcus aureus* (Gram-positive) (Qasim *et al.*, 2018). With and without antibiotics, AgNPs were found to be effective against multidrug-resistant bacteria (Katva *et al.*, 2017; Baptista *et al.*, 2018; Ipe *et al.*, 2020). AgNPs are efficient antibacterials due to the increased surface area resulting in greater contact area, increasing reaction capacity and being small in size enables interaction with the bacteria. The exact mechanism of action (MoA) is under debate but is likely combinatory in nature and depends on more factors than just the core material - silver. AgNP's antibacterial MoA can be discussed as caused by a) Ag^+ dissolution and b) directly by the AgNPs activity (Yin *et al.*, 2020). Several papers and reviews are available for further reading (Durán *et al.*, 2016; Kędziora *et al.*, 2018; Siddiqi, Husen and Rao, 2018; Tang and Zheng, 2018; Gu *et al.*, 2019; Yin *et al.*, 2020). Briefly, Ag^+ released from the AgNPs can adhere to the cell membrane and cell wall of bacteria, which can alter permeability and interfere with the

bacterial envelope. After entering the cell, Ag^+ may generate reactive oxygen species (ROS), hinder adenosine diphosphate (ADP) production, cause problems with dioxiribonucleic acid (DNA) synthesis, and denature cytoplasmic ribosomes resulting in the inhibition of protein synthesis. Furthermore, AgNPs have been shown to denature the cytoplasmic membrane (CM) by accumulating in pits on the cell wall and causing the denaturation of the CM. This allows the penetration or changes in the CM's structure which in turn can result in cell or organelle lysis. Also, the accumulation on the cell wall is thought to interfere with transport into or out of the cell (Bondarenko *et al.*, 2018). Furthermore, AgNPs have been shown to disrupt the protein substrates involved in bacterial signal transduction leading to cell apoptosis and cell growth cessation (Yin *et al.*, 2020). The dosage, as well as the morphology, size, charge and surface modification, affect its antibacterial activity (Liu *et al.*, 2010).

Due to all of the above-mentioned properties, its medical applications include infection prevention (*e.g.* surface coatings in hospitals, catheters, contact lenses, wound dressings and implants) or disinfectants; innovative bone tissue engineering; as adjuvants in vaccines; cancer treatment; or dental applications (Santoro, Duchsherer and Grainger, 2007; Xu *et al.*, 2013; Aw-Yong *et al.*, 2018; Bapat *et al.*, 2018; Hasan *et al.*, 2018; Hussain *et al.*, 2019). AgNPs can also be found in everyday items such as cosmetics, antimicrobial surface coatings (*e.g.* baby bottles, packaging, textiles), and water purification (Llorens *et al.*, 2012; Wu *et al.*, 2016; Xu *et al.*, 2017; Deshmukh *et al.*, 2019; Singh *et al.*, 2019). Given the vast applications of AgNPs, being exposed to them is almost inevitable.

1.4 AgNP toxicity

1.4.1 Uptake, distribution and fate

The abundance of AgNPs is considered to be an environmental hazard and known to potentially lead to detrimental human health effects (Bakshi, 2020). Generally, humans are exposed to AgNPs by different routes and AgNPs can be taken up by each of them. These are mainly, ingestion and dermal uptake, to a lesser extent inhalation, as well as injections - mostly for therapeutic and imaging reasons (Dobrzyńska *et al.*, 2014; George *et al.*, 2014; Qiao *et al.*, 2015; Wang, Xia and Liu, 2015). Injected AgNPs bypass the dermal barrier. For diagnostic purposes and imaging of tumours AgNPs are injected due to preferential localisation to a tumour.

Unfortunately, only 1-10 % of the total dose is found to localise to the tumour (target) (Fischer *et al.*, 2010). *In vivo studies* show that AgNPs, upon gaining access to the organism, can circulate, migrating to organs like the spleen, lungs, kidneys, brain and liver (Tomankova *et al.*, 2015; Bergin *et al.*, 2016; Wilding *et al.*, 2016). AgNPs may affect the exposure site or enter the systemic circulation distributing to and accumulate in secondary organs persisting, according to some studies, up to four months or possibly longer, especially given continuous exposure (Lee *et al.*, 2013; Ferdous and Nemmar, 2020). Individuals with pre-existing non-communicable diseases are more vulnerable to AgNP toxicity (Jia *et al.*, 2017; Kermanizadeh *et al.*, 2017).

1.4.2 Pathophysiological effects of AgNPs

An *in vivo* and clinical research review found acute AgNP exposure caused systemic neurotoxicity and death while chronic exposure caused local inflammation, organ damages and argyria (Wang, Xia and Liu, 2015). Argyria is a greyish-blue discolouration of the skin or eyes, caused by the sub-dermal deposition of silver particles or accumulation in other tissues. Its incidence has reportedly increased in recent years, likely due to voluntary and often excessive intake of colloidal silver (Kwon *et al.*, 2009; Chung *et al.*, 2010; Lencastre, João and Lobo, 2013). Furthermore, burn wound treatment with silver-impregnated dressings has been found to lead to argyria and more health-threatening conditions. For instance, a patient showed raised liver enzymes upon treatment with silver nanocrystal impregnated burn wound dressings (Trop *et al.*, 2006). Similarly, Weldon *et al.*, (2016) found the liver to be the most vulnerable target organ upon incidental inhalation exposure of AgNPs in the occupational environment.

The endothelia of the liver are non-continuous, therefore, small NPs can pass through the vascular fenestrations of 50-100 nm leading to direct contact (Filipak Neto *et al.*, 2018). The liver plays an integral part in normal physiology (carbohydrate, lipid and protein metabolism; detoxification of both endogenous and exogenous metabolites; biosynthesis of important serum components) and immunological functions. It is an immunologically complex organ that plays a crucial role in the acute phase response (APR), upon pathogen invasion or tissue injury. The liver's main parenchymal cells, hepatocytes - secrete many innate immune phase proteins such as bactericidal proteins, opsonins, fibrinogen and acute phase proteins (APP) *e.g.* C-reactive protein, inflammatory proteins, protease inhibitors, cytokines and chemokines (Bode *et al.*, 2012;

Robinson, Harmon and O'Farrelly, 2016; Zhou, Xu and Gao, 2016). The cytokines, small glycoproteins, modulate the interaction between cells and regulate immunity and inflammation. Chemokines also are cytokines, which promote and regulate the migration of cells (Dembic, 2015). Under physiological conditions, inflammation is part of the innate immune response and serves a protective purpose upon injury. To maintain liver homeostasis, several liver-resident cells (hepatocytes, immune cells stellate cells) are activated to secrete inflammatory mediators which amplify inflammation and activate the adaptive immune response (Murphy and Weaver, 2017). This leads to either the resolution of inflammation and return to homeostasis or persistent inflammation and therefore progressive liver damage (Robinson, Harmon and O'Farrelly, 2016).

Therefore, AgNPs toxicity in the liver is potentially far-reaching and of concern. Given the liver's detoxification function, role in immunity and the high likelihood as an accumulation location, the liver is especially vulnerable to AgNP exposure-damage (Cha *et al.*, 2008; Ebabe Elle *et al.*, 2013; Tomankova *et al.*, 2015; Lai *et al.*, 2020).

While *in vivo* studies are very important in assessing biodistribution, tissue injuries and many other aspects of AgNP toxicity, *in vitro* studies are of interest. Due to: the global *three R* principle (replace, reduce, refine) initiative – replacing *in vivo* studies with appropriate *in vitro* models where possible (Mohr, 2013; Graham and Prescott, 2015); *in vitro* studies providing a less complex environment and being suitable for mechanistic elucidation at the molecular level and they may be less cost-intensive (Boverhof et al., 2014). A non-exhaustive list of *in-vitro* studies of the effects of AgNP exposure on mammalian cell lines is summarised in Table 1.1. It provides a brief summary and insight into the various types of studies having been performed. The sizes range significantly as do the capping agents, cell lines, exposure concentrations and duration as well as the endpoints tested to determine potential adverse effects.

Table 1.1 A non-exhaustive list of toxicological investigations and outcomes of AgNPs in vitro in mammalian cell lines.

AgNPs		**Cells**	**Exposure**		**Main findings**	**References**
Size (nm)	Capping agent	Type	Duration & concentration			
20 & 50	Citrate	HepG2 human hepatoma cells	4 & 24 hrs	0.5-25.0 µg/mL	Cytotoxic at > 5 µg/mL AgNP.	(Sahu *et al.*, 2015a)
				2.5 µg/mL (sub-lethal)	Transient upregulation of stress response genes *e.g.* heat shock proteins and metallothioneins. Cells exposed for 4 hrs to 20 nm AgNP - p53 signalling and NRF2-mediated oxidative stress pathway affected.	
17–50	Chitosan	HepG2 human hepatoma cells	48 hrs	20–100 µg/mL	IC_{50}: 48 µg/ml AgNPs Upregulated caspase 3 and 9 protein (apoptosis).	(Priya, Vijayakumar and Janani, 2020)
10, 50, & 100	Polyvinyl-pyrro-lidone (PVP)	HepG2 human hepatoma cells	24 hrs	≤10 µg/mL Sub-lethal	Highest uptake and accumulation of 10 nm Autophagy and enhanced lysosomal activity at noncytotoxic concentrations (1 µg/ml; all sizes), Vesicle-engulfed 10-nm AgNPs induce cytotoxicity by a mechanism involving perturbations in the autophagy-lysosomal system and inflammasome activation.	(Mishra *et al.*, 2016)
30	Citrate & poly(ethylene glycol) (PEG)	HaCaT human keratinocytes	24 & 48 hrs	0, 0.5, 5, 10, 25, 50, 75 & 100 µg/mL	IC_{50}: 40 µg/ml & 37.4 µg/ml cit-AgNPs (24 & 48 hrs) PEG-AgNPs (24 & 48h) below IC_{50} cit-AgNPs most cytotoxic and active MCP-1 decreased by both, no change in other cytokines (IL- 1β, IL-6, IL-10 and TNF-α) Similar ROS production by both cit-AgNPs induced apoptotic/necrotic death and arrested cells at G2 further and earlier than PEG-AgNPs.	(Bastos, Ferreira de Oliveira, *et al.*, 2016)
30	Citrate & PEG	HepG2 human hepatoma cells	24 & 48 hrs	0, 1, 5, 10, 15, 25 & 50 µg/mL	IC_{50}: ± 11 µg/ml cit-AgNPs & PEG-AgNP (24h) Similar uptake – only at 11 µg/ml for both Both decreased expression of apoptotic cascade genes Both AgNPs increased S and G2 phase concentrations and reduced cyclins gene expressions; cytostatic.	(Bastos, Ferreira-de-Oliveira, *et al.*, 2017)
30	Citrate	RAW 264.7 murine macrophages	24 & 48 hrs	0, 0.5, 5, 10, 25, 50, & 75 µg/mL	IC_{20}: 60 µg/ml AgNPs (high tolerance) Intracellular ROS formation Impaired cell cycle (sub-G1)-phase and increased DNA fragmentation.	(Bastos, Duarte, *et al.*, 2017)

25-35	Curcumin For comparison: not mentioned	Representative Gram (+) and (-) bacteria	16-18 hrs	0.6 – 1000 mg/L	Curcumin coated AgNPs (Cur-AgNPs) performed better in minimum inhibitory concentration (MIC), time kill kinetics, post agent effect, and were about 20% more effective in inhibiting biofilm formation than chemically synthesised AgNPs (Chem.syn-AgNPs).	(Jaiswal and Mishra, 2018)
		HaCaT human keratinocytes THP1 human macrophages	24 hrs	0.075 -10 mg/mL	Cur-AgNPs were cytotoxic at a concentration much higher than the bacterial MIC. Cur-AgNPs reduced IL-6 and TNF-α (pro-inflammatory cytokines) in macrophages relative to Chem.syn-AgNPs.	
20 and 35	Not stated *citrate stabilised	Human embryonic stem cell (hESC) differentiation system	18 days	0.001, 0.01, and 0.1 µg/mL AgNPs or $AgNO_3$	Both AgNPs and $AgNO_3$, endoderm and mesoderm specification affected, not ectoderm. Human embryoid body-derived primary germ layers. Differentiation interference (cardiocytes and hepatocytes).	(Hu *et al.*, 2020)
		hepatocyte-like cells and cardiomyocytes	day 2 & 12	0.001–0.5 µg/mL AgNPs or $AgNO_3$	Differentiation of hepatocyte-like cells was impaired by both AgNPs and $AgNO_3$ Cardiac differentiation affected by both AgNPs and $AgNO_3$ - in opposite ways.	
14	Sodium citrate *endotoxin-free (below 0.2 EU of endotoxin/mL)	primary monocytes & Whole blood	24 hrs	1.3, 4 and 12 µg/mL	AgNPs did not kill or activate monocytes – no production IL-6, IL-1α, IL-1β, and IL-36γ AgNP's transient AgNP induced inflammation-enhancing effect, yet did not interfere with the resolution of the inflammatory reaction.	(Li *et al.*, 2016)
6.5 - core 14 - d_H	surfactant-coated (polyethene glycol-25 glyceryl trioleate, polyethene lycol-20 sorbitan monolaurate)	Caco-2 cells differentiated - polarized monolayer of small intestinal epithelium-like cells & HepG2 human hepatoma cells	24 hrs	0.1, 1, 10 & 100 µg/mL 2.5 & 25 µg/mL AgNP 0. 5 and 5 µg/mL $AgNO_3$	Differentially expressed proteins - related to protein folding, protein synthesis/modification & cellular assembly and organisation. Differentially expressed proteins - proteins linked to chaperone function, several redox-related and metal ion binding proteins, and proteins related to structural functions and the cytoskeleton. Bioinformatic analyses suggests similar biological and/or toxicological processes were triggered in both cell types.	(Oberemm *et al.*, 2016; Braeuning *et al.*, 2018)

1.4.3 Proposed AgNP toxicity mechanism and examples of biomarkers

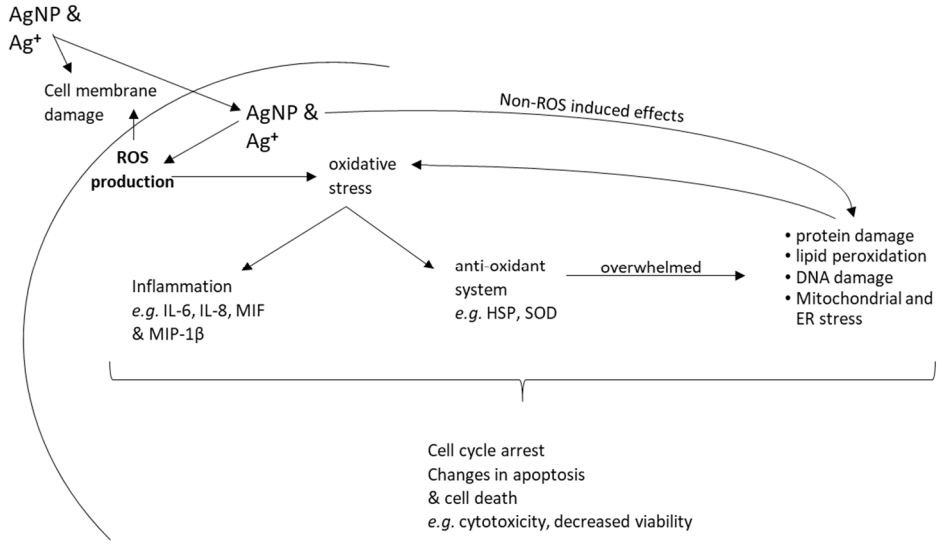

Figure 1.1 Proposed AgNP induced detrimental cellular effects and their mechanisms with examples of biomarkers

ER: endoplasmic reticulum; HSP: heat shock proteins; SOD: superoxide dismutase; IL-6: interleukin 6; IL-8: interleukin 8; MIP-1β: macrophage inflammatory protein; MIF: macrophage migration inhibitory factor

Figure 1.1 briefly shows proposed toxicity mechanisms of AgNPs and examples of *in vitro* biomarkers. AgNP exposure *in vitro* leads to ROS production, DNA damage, cell cycle arrest, apoptosis modulation, inflammation induction and cytotoxicity in various mammalian cell lines Table 1.1.

Oxidative stress due to ROS induced by AgNPs and Ag^+ released from the AgNP's surface (i.e. dissolution), is the most agreed on mechanism (Haase et al., 2012; Singh and Ramarao, 2012; Prasad et al., 2013; Dayem et al., 2017; Braeuning et al., 2018). Briefly, AgNPs have been found to interact with membrane proteins causing activation of signalling pathways which can lead to ROS generation. Upon uptake, further and possibly excess amounts of ROS generation can occur. The production of ROS is an integral part of normal cell physiology and is highly regulated, by the stimulation of several cellular protective responses upon oxidative stress, such as anti-oxidant enzymes, general stress pathway, apoptosis and inflammation (Hussain *et al.*, 2005; Avalos *et al.*, 2014). Excessive ROS can overwhelm the redox homeostasis of the cell and lead to non-selective oxidative biomolecule damage and organelle structure damage, such as mitochondrial or endoplasmic reticulum stress, resulting in protein damage, lipid peroxidation, DNA damage and membrane disruption leading to cell death

(Cameron, Hosseinian and Willmore, 2018; Bahl *et al.*, 2020; Priya, Vijayakumar and Janani, 2020; Yu *et al.*, 2020).

Here, the different protective responses and examples of biomarkers used to investigate them are briefly summarised and pointed out from research. For instance, Sahu *et al.* (2015) found stress response gene *e.g.* heat shock protein (HSP) upregulation, in HepG2 human liver cells, by AgNPs (20 & 50 nm) exposure for 4 and 24 hrs at the sub-lethal concentration of 2.5 µg/mL. Several HSPs groups exist, such as the small HSPs (sHSPs) group which includes HSP27/HSPB1, which not only acts as an antioxidant but also as aids in refolding of denatured proteins as a molecular chaperone (Vidyasagar, Wilson and Djamali, 2012; Srivastava, Sharma and Priya, 2017).

Furthermore, oxidative stress can activate inflammatory signalling cascades such as mitogen-activated protein kinase (MAPK), nuclear factor kappa B (NF-κB) and phosphoinositide 3-kinase (PI3-K) which culminate in cytokine and chemokine production (Kermanizadeh *et al.*, 2012; Mishra *et al.*, 2016; Bastos, Ferreira-de-Oliveira, *et al.*, 2017). Healthy tissue can suffer damage upon overexpression of inflammatory cytokines (Ilinskaya and Dobrovolskaia, 2014). Therefore, modulation of the inflammatory response is used as a measure of AgNP toxicity by determining inflammatory biomarker responses (Bastos, Brown, *et al.*, 2016; Bastos, Ferreira-de-Oliveira, *et al.*, 2017). IL-6 is a pleiotropic inflammatory cytokine. Hepatocyte synthesised IL-6 and mediates liver repair and regeneration and acts to further amplify the APR (Castell *et al.*, 1989; Jin *et al.*, 2006; Robinson, Harmon and O'Farrelly, 2016). Chemokines also used as biomarkers are CXCL8/IL-8 and CCL4/MIP-1β. CXCL8/IL-8 attracts and activates neutrophils while CCL4/MIP1-β attracts several kinds of immune cells and contributes to macrophage activation and acts as immune surveillance of the liver (Rowell *et al.*, 1997; Zhang *et al.*, 2003; Dembic, 2015). Macrophage migration inhibiting factor (MIF) is constitutively expressed in hepatocytes and stored in pre-formed intracellular pools. MIF is an integral part of the host's response to tissue invasion or injury, and its release stimulates the migration of innate immune cells and activates inflammatory pathways. In sepsis, it forms part of the hyper-inflammatory response. MIF is considered an inflammatory mediator cytokine with chemokine-like function and usually plays an integral role in the liver's response to stress - chronic or acute (Wheelhouse *et al.*, 2006; Grieb *et al.*, 2010; Prucha, Bellingan and Zazula, 2015; Marin *et al.*, 2017).

Anti-oxidant enzymes such as superoxide dismutase (SOD) are typically upregulated upon loss of redox balance induced by AgNPs (Oberemm *et al.*, 2016; Braeuning *et al.*, 2018). Similarly, nitric oxide (NO) production is upregulated by the production of inducible nitric oxide synthase (iNOS) enzyme, which can be stimulated by AgNP exposure and therefore serve as biomarkers for potential toxicity (Zuberek *et al.*, 2017).

Autophagy, another cellular defence mechanism was increased upon exposure of HepG2 liver cells to a sublethal concentration (1 µg/mL) of polyvinylpyrrolidone (PVP) capped AgNPs (PVP-AgNP). Yet, higher concentrations led to toxicity by interrupting the autophagy-lysosomal system and causing inflammasome activation (Mishra *et al.*, 2016). Furthermore, citrate-capped AgNPs induced apoptotic and necrotic cell death in addition to causing cell cycle arrest at the G2 stage (Bastos, Ferreira de Oliveira, *et al.*, 2016).

ROS independent mechanisms of action have also been noted *e.g.* acute calcium response activation (Haase *et al.*, 2012). Furthermore, developmental toxicity has also been found (Hu *et al.*, 2020). The mechanism and specific effect caused by AgNPs depend on several factors namely intrinsic AgNP characteristics, the cell type and uptake pathway, among others (Bahl *et al.*, 2020). Despite extensive research into the cytotoxicity mechanism of NPs the exact cause remains unclear (Liu and Tang, 2019).

1.5 Cellular uptake mechanisms and pathways

The entrance of NPs into the cell plays a role in determining their function and the potentially ensuing toxicity (Halamoda-Kenzaoui *et al.*, 2017). Usually, cells prevent the uptake of foreign particles and the traffic in and out of cells is highly regulated. Uptake of extracellular molecules and substances occurs through passive transport, special transport and the energy-dependent process of endocytosis – where extracellular molecules are internalized in CM derived transport vesicles with the help of specific receptors (Zhao *et al.*, 2011; Liu and Tang, 2019). Endocytosis is the predominant method of NP uptake, yet, direct penetration or insertion has been reported (Liu and Tang, 2019). In general, endocytosis occurs in different stages. Firstly, the cell protrudes a portion of its CM, including lipids and proteins, beginning to engulf the extracellular molecules together with the surrounding extracellular fluid. This engulfing continues and leads to a membrane bound vesicle that is pinched-off into the cell and is known as an endosome, which has different destinations in the cell, either releasing its cargo to various parts of the cell or fusing with a lysosome for degradation (Donaldson, 2013).

Endocytosis is the preferred uptake method of metal NPs. Which pathway specifically is used is dependent on several NP physicochemical characteristics as well as the cell type. Endocytosis is most commonly classified, into phagocytosis – large particle uptake; and pinocytosis – cellular drinking, *i.e.* solutes and fluid uptake. Phagocytosis is performed by phagocytes, cells specifically equipped to perform this function (*e.g.* neutrophils, macrophages, monocytes, and dendritic cells). While pinocytosis, which all cells perform, is further subdivided according to the partaking proteins into clathrin-dependent endocytosis (*i.e.* clathrin-mediated endocytosis (CME)), caveolae-dependent endocytosis (CvME), clathrin- and caveolae- independent endocytosis, and macropinocytosis (Sahay, Alakhova and Kabanov, 2010). Macropinocytosis and phagocytosis, both tightly regulated, take up larger size substances and therefore involve large-sized endosomes (*e.g.* macropinosomes 0.2-10 µm). While both take up large volumes relative to cell volume, phagocytosis internalizes large particles (*e.g.* bacteria). Whereas, macropinocytosis is concerned with fluid-phase engulfing *i.e.* containing solutes and possibly even bacteria. Furthermore, its CM shows distinct ruffling during the uptake process (Kumari, Mg and Mayor, 2010). CvME, which will internalise extracellular molecules through characteristic flask-shaped invaginations, referred to as caveolae which caveolin, a cholesterol-binding protein, forms part of (Harush-Frenkel *et al.*, 2008; Panzarini *et al.*, 2018). The best known and most studied pathway is CME (Ahmed *et al.*, 2017). CME allows for concentration of, and efficient internalisation of CM bound proteins. The cytosolic protein clathrin polymerizes upon receptor binding and subsequent cytosolic adaptor protein recruitment, forming a basket-like coat on the internal aspect of the CM which facilitates the vesicle formation and fission (Donaldson, 2013).

Pharmacological inhibitors, among other methods, can be employed to further investigate the different uptake pathways of NPs (Wang, Xia and Liu, 2015). For instance, chlorpromazine (CPZ) is a pharmacological inhibitor of CME and is commonly used in uptake pathway studies of NPs. Such chemical inhibitor methods are fast and inexpensive methods to study particle uptake compared to other endocytosis techniques (Iversen, Skotland and Sandvig, 2011).

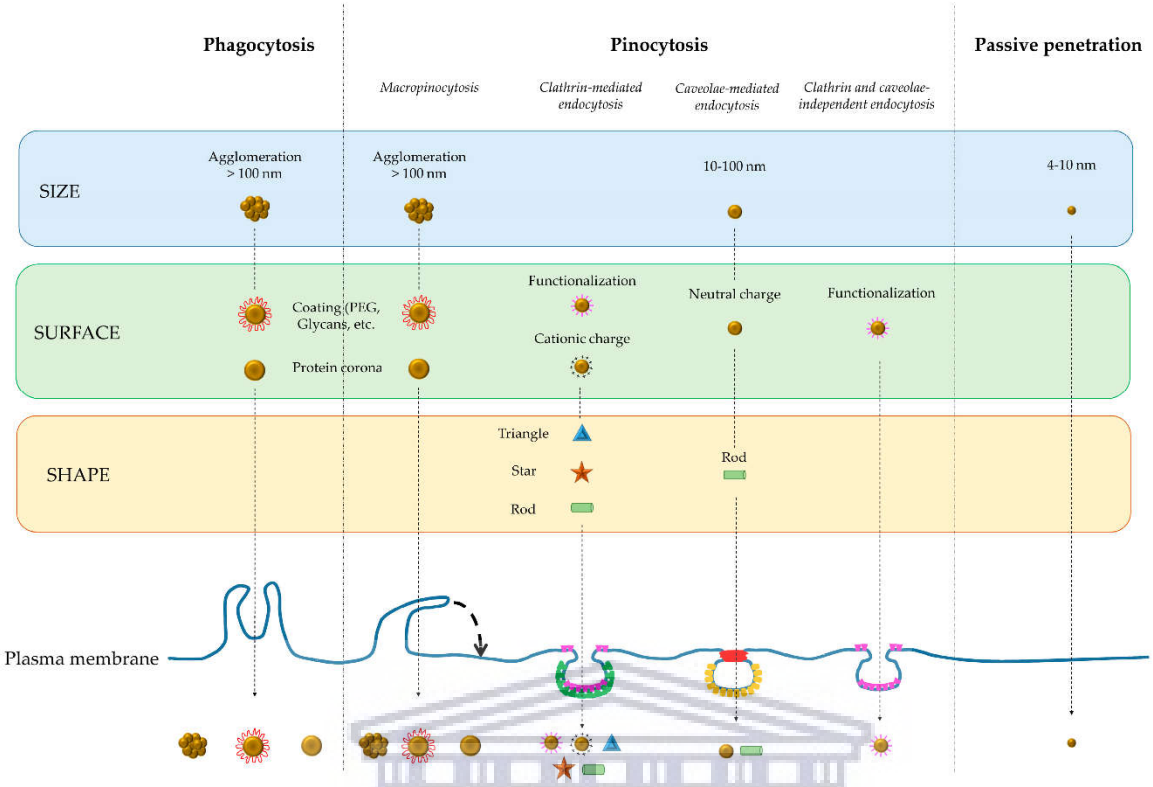

Figure 1.2 Internalisation pathways of different NP types. (Panzarini *et al.*, 2018)

Figure 1.2 shows the described classification of the cellular uptake pathways of different NP types, showcasing examples of how size, surface features (functionalisation, protein corona and charge) and shape of different NPs have been found to affect uptake pathways (Foroozandeh and Aziz, 2018; Panzarini *et al.*, 2018; Liu and Tang, 2019).

Different NPs are taken up by different mechanisms and therefore their destinations inside the cell vary. Generally, NPs will be found in endocytic vesicles at first and can escape and distribute in the cytosol and interact with intracellular components, *e.g.* the mitochondria, the nuclear region and the nucleus (Panzarini *et al.*, 2018; Liu and Tang, 2019). Kang *et al.* (2010) demonstrate that a difference in length can affect the localisation of shorter and longer single-walled nanotubes; the short single-walled nanotubes (diameter: 1-3 nm, length: 50 nm) entered the nucleus of HepG2 cells, in contrast to longer ones (length: 100-200 nm) only being found in the cytoplasm.

Herewith, examples of how some of the different NP characteristics have been found to affect NP uptake. Macropinocytosis and phagocytosis are employed for agglomerated NPs as well as those with specific coatings or protein coronas (Panzarini *et al.*, 2018). Inhibition of CME,

in B16 murine melanoma cells, affected a decrease in uptake of all four AgNP sizes (5 nm, 20 nm, 50 nm and 100 nm). While the smaller three AgNPs (5 nm, 20 nm and 50 nm) were taken up less upon CvME pathway inhibition and the larger two AgNPs (50 nm and 100 nm) uptake also decreased upon macropinocytosis inhibition (Wu *et al.*, 2019). Xie et al. (2017) investigated uptake pathways in RAW264.7 murine macrophages of differently shaped gold NPs. Care was taken to ensure the NPs had similar sizes and surface charges in an attempt to exclude confounding factors. The authors found, that all NPs regardless of shape, made use of CME. Furthermore, the gold nanorods could be taken up by both CvME and CME. While size has shown a generally accepted trend in terms of uptake type, amount and even effect, the surface charge in comparison has not been so clearly defined. Cationic NPs are taken up by CME, whereas, neutrally charged NPs are often taken up by CvME, in some studies (Harush-Frenkel *et al.*, 2008). Yet, Fröhlich (2012) performed a literature review and concluded no clear trend or rule could be determined. Nanotoxicity is complex due to the dynamic characteristics of NPs.

1.6 Relevant aspects for consideration in nanotoxicity and AgNP toxicity

Considerable amounts of data have been produced from nanotoxicological studies and gaps in knowledge have been identified along with inconsistencies and discrepancies about the potential risk of NMs (Smita *et al.*, 2012; Gatti and Montanari, 2015). The toxicity of NMs is determined by numerous aspects, such as the base material used and variations of physical and chemical properties. This produces a substantial number of variables and potential hazards that need to be considered (European Commission, 2012).

Yet, several shared physical and chemical characteristics of NMs allow grouping to some extent. Therefore, it may be possible to read-across or anticipate certain trends in toxicological outcomes based on trends of the NP's physicochemical characteristics (Oberdörster *et al.*, 2005; Giusti *et al.*, 2019; Bahl *et al.*, 2020). Below are generalisations and considerations for nanotoxicity that have been elucidated in and are based on the evidence and discussions found in the literature.

1.6.1 Importance of characterisation

NP characterization is considered an essential part of nanotoxicology to gain a greater understanding of the cause of the effect due to NM–biological system interaction (Powers,

Carpinone and Siebein, 2012). Therefore, characterisation should be performed prior to experimentation and not only relied on the manufacturer's data, preferably by a validated and standardised method (Ferdous and Nemmar, 2020).

Furthermore, the complex environment of biological systems complicates matters considerably and a straight-forward characterization protocol is not easily established. The complex behaviour and dynamic changes occurring in biological environments, for instance, cell culture media, lead to considerable changes to NM's surface properties and behaviour *e.g.* surface charge, aggregation, dissolution behaviour, or chemical reactivity. Therefore, modulate toxicity behaviour (Winkler, 2016; Quik *et al.*, 2018). Hence in nanotoxicity, the particle properties need to be determined before, during and after exposure to the biological environment, to accurately and fully define its toxicity profile.

Additionally, the dynamic evolution (transformation) of NMs during exposure to different environments can be influenced significantly by even the slightest variation in laboratory procedure, such as sonication type for resuspension of NP powders (Cupi, 2015; Haase and Lynch, 2018). Furthermore, the procedure each individual or laboratory follows may affect the measured outcome (Gatti and Montanari, 2015). In several round-robin studies performed by Haase and Lynch (2018), it was found that the variation in characterisation data alone can lead to contradicting conclusions on what characteristics a NP displays which in turn, may affect the usefulness of the results generated.

A NP's characteristics not only are important for gaining insight and understanding into the detrimental effects in general, such as cytotoxicity but also the mechanism of action *i.e.* how the NPs cause such effects; for instance considering internalization methods and sub-lethal modulation of protein synthesis (Powers *et al.*, 2006; Winkler, 2016; Quik *et al.*, 2018; Johnston *et al.*, 2020).

1.6.2 Shape, size and surface area

The shape of the NM affects toxicity (Stoehr *et al.*, 2011). Silver quasi-spherical particles (± 45 nm) & nanowires (~83 nm x 6.8 µm) versus nanocubes (~37 nm) were found to be more toxic in model plant species, while all morphologies demonstrated similar bactericidal efficacy (Gorka *et al.*, 2015). Furthermore, the shape also affects both the uptake pathway and uptake

quantity (Xie *et al.*, 2017). Similar to shape, the size of NPs can affect the uptake, and therefore affect the toxicity outcome caused by NPs.

At the nano-level (1-100 nm), the chemical and physical properties are significantly different relative to the macro-scale. Therefore, it stands to reason that the size of NPs has a considerable effect on their interaction with cells and other biological species, especially considering that the size of NPs is comparable with that of cellular molecules, e.g. cell membrane thickness (10 nm). Generally, it is understood that the smaller AgNPs, the more toxic they are and the more easily they enter into the cell (Avalos *et al.*, 2014). In two cell types, 4.7 nm AgNPs were much more toxic, and induced an increased production of ROS, compared to 42 nm AgNPs (Avalos *et al.*, 2014). Zhang, Gao and Bao (2015) demonstrated, by combining theoretical physics with research data, that <5 nm NPs can enter cells non-specifically, by passive penetration; while larger particles entered via various uptake pathways.

Related to size is the specific surface area (SA) of NPs. The same amount of base material, such as silver, in macro particle form, will have a smaller total SA compared to AgNPs. Therefore, the smaller the NPs the larger the SA, and hence more available SA to react and interact with, in a given volume or area (Donaldson *et al.*, 2004). This, in turn, can potentially heighten the activation of toxicity mechanisms, such as oxidative stress and ROS generation. Therefore, the SA available to interact with the NP's environment plays a role in NP toxicity (Oberdörster, Oberdörster and Oberdörster, 2005; Bahl *et al.*, 2020). Schmid and Stoeger (2016) found that SA, compared to volume or mass, was the most effective dose metric in a rat study assessing acute lung NP toxicity. When considering chemical regulations, it is common to use mass-based metrics and therefore thought to be easier. Mass-based doses may lead to smaller particles providing a greater number of particles per mass unit (Drasler *et al.*, 2017). Therefore, while it is not necessarily yet known what the best dose descriptor for NMs is, the dose metric that best correlates with the end-point of interest should be preferentially used *e.g.* SA/volume (Kuhlbusch, Wijnhoven and Haase, 2018). Moreover, when considering dosage real-world applicable levels need to be used, *i.e.* the biologically relevant levels - the concentration or amount of NPs one is likely to be exposed to and not an unlikely high exposure (Johnston *et al.*, 2020). Furthermore, SA can also be influenced by the NP aggregation and agglomeration state.

1.6.3 Aggregation and agglomeration

The high SA of NMs is often associated with increased surface reactivity; the tendency of the surface to undergo a chemical reaction with external molecules, atoms or other NPs (Carenco, 2018). Essentially, the surface reactivity determines how easily particles can, among other consequences, agglomerate, *i.e*. bind reversibly, or aggregate, *i.e.* fuse irreversibly; forming a secondary structure composed of the primary NPs (International Organization for Standardization, 2015; Carenco, 2018). Furthermore, aggregates/ agglomerates (A/A) of NPs can exhibit characteristics that are different from or similar to those of the primary NPs. Aggregation reduces the SA, effectively similar to an increase in primary particle size. It is generally believed that the smaller the NM is the greater its toxicity, therefore, one can argue that the larger the aggregates are the less toxic (Murugadoss *et al.*, 2020). In contrast, agglomerates may retain the high SA and therefore reactivity. Accordingly, Murugadoss *et al.*, (2020) found no difference in toxicity outcomes tested between large and small titanium dioxide agglomerates for all but one tested outcome.

Commonly in the literature, the terms A/A have been interchangeably used (Zook *et al.*, 2011; Albanese, Tang and Chan, 2012; Bélteky *et al.*, 2019). Generally, not sufficient energy is supplied in nanotoxicological experimental and physiological conditions it is likely that agglomeration is predominantly present. Agglomeration is readily influenced by both physicochemical NP characteristics and environmental conditions (Zook *et al.*, 2011). This, in combination with the dynamic behaviour of agglomerates, being able to de- and re-agglomerate can modulate the toxicity outcome considerably (Bruinink, Wang and Wick, 2015). Furthermore, the agglomerates are usually taken up by pathways specific to large molecules. Halamoda-Kenzaoui *et al.*, (2017) found that the agglomeration state of silica NPs influenced not only the uptake pathway, mainly increasing the extent to which macropinocytosis was performed, but also led to cellular uptake increase in general, compared to the non-agglomerated particles. Zook *et al.* (2011) found larger AgNP agglomerates to result in decreased haemolysis relative to smaller AgNPs. In contrast, Murugadoss *et al.* (2020) evaluated titanium dioxide NP agglomerates with different primary particle sizes and agglomerate sizes. The authors found large agglomerates to be less or equally toxic, compared with the small agglomerates and suggested that the differences seen may be due to primary particle size differences. Additional investigations are required to

definitively determine whether the A/A may or may not be relevant for toxicological NP studies and to, if possible, generalise the expected outcome from A/A behaviour (Zook *et al.*, 2011; Carenco, 2018; Murugadoss *et al.*, 2020).

Another result of A/A formation is a reduction in NP stability in solution. The larger the secondary structures are the more likely they are to come out of suspension. Furthermore, a higher NP concentration leads to a propensity towards agglomerate formation. This is especially significant in *in vitro* studies, where a high number of sedimented A/A may accumulate on the surface of the cells, possibly preventing nutrient uptake or causing increased interaction and uptake (Bahl *et al.*, 2020). This may show higher toxicity, especially at the highest exposure concentrations, and not be due to the increase in concentration itself but rather due to the sedimentation of the NP A/A onto cell cultures *in vitro*. A commonly employed strategy to decrease the extent and the number of agglomerates is the sonication of NP solutions. It is also frequently used to redisperse NP powders in a solution before exposure in toxicity experiments. Sonication itself though, might alter the surface properties and therefore toxicological behaviour of NPs and should be taken into consideration (Pradhan *et al.*, 2016). Another method used to prevent A/A, especially considering biomedical applications of NMs, are stabilising surface agents *i.e.* capping agents – which are often used in the production process. Since the surface dictates interaction, surface modifications can be used to influence stability and reduce A/A propensity.

1.6.4 Surface features, surface modifications and dissolution behaviour

Several surface features and surface modifications influence nanotoxicity and cellular uptake, leading to different toxicity outcomes (Nguyen *et al.*, 2013; Prasad *et al.*, 2013; Kwok *et al.*, 2016). These include intentional and incidental surface modifications and surface charge (Prasad *et al.*, 2013). Briefly discussed here are intentional surface modifications and surface charge; since dissolution behaviour is greatly determined by surface properties, it too is included below.

In addition to stabilizing agents, surface functionalisation can be used to target specific cells in the body, enhance the therapeutic ability of NPs or increase NP persistence in the circulatory system. The latter of which, can be achieved by capping NPs with e.g. polyethylene glycol (PEG) or PVP (Shukla *et al.*, 2012; Ahmed *et al.*, 2017; Khoobchandani *et al.*, 2020).

These are examples of intentional surface modifications. Incidental surface modifications are of interest in nanotoxicity as well and are briefly discussed in 1.6.5.

Ahmed *et al.* (2017) investigated the uptake mechanisms and cytotoxicity of AgNPs with different surface functionalisation in HepG2 cells. Among other surface functionalisation species investigated were: PVP, one of the commonly used stabilising agents; and bovine serum albumin (BSA), mimicking high protein solutions that NPs are often found in, both in *in vitro* and *in vivo* research studies. The cytotoxicity potential of the BSA-coated AgNPs was considerably pronounced over that of the PVP-coated AgNP (Ahmed *et al.*, 2017). The authors, furthermore, found that the primary uptake mechanisms were macropinocytotic and CME. The uptake of PVP and BSA coated AgNPs, upon inhibition of the CME pathway, was reduced by >80 % and > 50% respectively. Therefore, different capping agents can lead to modified uptake behaviour, which in turn, could trigger different signal transduction cascades downstream.

Metal NPs can release ions in solution. For instance, AgNPs can release Ag^+ which in turn can: cause nucleation and new AgNP synthesis, form precipitates or give rise to ROS upon reacting with molecular oxygen (Fu *et al.*, 2014; Smith *et al.*, 2018). This dissolution behaviour of metal NPs depends on the material, size, capping agent and environment of the NPs, to name a few (Liu *et al.*, 2012). When dissolution occurs inside cells, after cellular uptake, the phenomena is aptly named, the Trojan-horse effect since the potentially toxic Ag^+ can now cause damage inside the cell. The debate whether AgNP toxicity is, and if to what extent, caused by Ag^+ release, or both is still ongoing (Sahu *et al.*, 2014; Xu *et al.*, 2015; Smith *et al.*, 2018).

Surface charge is also thought to influence NP uptake and effect. Since the cellular surface is negatively charged, it can be expected and has been shown, that a negatively charged NP surface would limit cell-particle interaction due to electrostatic repulsion. The inverse is also true, positively charged NPs interact more easily with the negatively charged cell surface and has been shown to lead to increased uptake in comparison to uncoated and negatively charged NPs (Hühn *et al.*, 2013; Ahmed *et al.*, 2017). The addition of specific surface species often confers a specific surface charge (mV, zeta-potential (ζ)) to the NPs. While surface charge is one of the most influential NP characteristics, limiting or favouring interaction with cells, results have been inconsistent (Forest and Pourchez, 2017). The nature of the protein corona, which adsorbs onto the NP surface in the biological environment, is of significant

importance as well and will be considered in the next section since it can be modulated in the exposure environment.

1.6.5 Exposure environment

The physicochemical properties of NPs briefly discussed so far are largely determined by intrinsic NP factors due to base material or synthesis method. Beyond this, the NP encountered environments can modify such characteristics.

For instance, the reduced pH encountered by NPs in endo-lysosomes can increase the ion release rate. This so-called endosome-lysosome Trojan Horse Effect is likely to increase the toxicity of metal-containing NPs once taken up into the cell (Sabella *et al.*, 2014). Similarly, temperature, oxygen and light can potentially increase or decrease the ion dissolution rate of metal NMs (Fu *et al.*, 2014).

Furthermore, it is known that biomolecules and ions may adsorb onto the surface of NPs. Proteins, which are abundant in biological environments, can adsorb onto NPs. This is known as the protein corona, which subsequently can affect dissolution and determines the biological identities of NPs in physiological conditions, or modulates the available protein concentration in a biological environment (Riaz Ahmed *et al.*, 2017). Therefore, it is important to determine NP characteristics in an exposure environment, or as close as possible, to the environment toxicity studies are conducted in. (Xue *et al.*, 2016). Furthermore, these adsorbed proteins can include impurities, such as endotoxin, a component of Gram-negative bacterial cell walls (Schulze *et al.*, 2008). Endotoxin induces pro-inflammatory cellular responses and even aggravates the inflammatory effect the NPs alone would have caused. Therefore, the inflammatory markers produced in a given experiment may be false or inaccurately high, consequently interfering with the study results (Oberdörster *et al.*, 2005; Schulze *et al.*, 2008).

1.6.6 Cell type

Lastly, different cell types interact differently with NPs, leading to different uptake pathways and subsequent cellular effects (Kettler *et al.*, 2014). This likely is due to the differences cell types have in their surface receptors, membrane compositions and functions, among others. The cell type and its function dictate what uptake pathways, or a combination thereof, are predominantly employed by cells (Liu *et al.*, 2013). Phagocytic cells, for example, are

specialised immune cells that phagocytose large molecules by engulfment with subsequent uptake. Non-phagocytic cells, in comparison, make use of other uptake mechanisms. Furthermore, gold NP aggregates were found to be taken up more readily by the melanoma MDA-MB 435 cell line than in the HeLa cervical cancer cell line and A549 adenocarcinoma human alveolar epithelial cell line. The authors attributed this to the A549 cells making use of receptor-independent uptake mechanisms and both the other via receptor-mediated uptake mechanisms (Panzarini *et al.*, 2018).

Considering the brief information provided it is evident that several variables influence NP toxicity. Therefore, despite the vast amount of research that has been performed in recent years, further research is required.

Chapter 2 Problem statement, aim, objectives and hypothesis

2.1 Problem statement

At the nano-scale (1-100 nm) nanoparticles (NPs) gain novel physiochemical properties, while retaining some of the bulk-scale ones. For instance, silver NPs (AgNPs) gain novel properties while retaining an enhanced effectiveness of their anti-microbial property (Koduru *et al.*, 2018). The novel properties make nano-enabled products profitable and therefore large amounts of new products are available (Johnston *et al.*, 2020).

Simultaneously, several concerns have been raised about the safety of such products for the environment and human health, especially of AgNPs. Little to no regulation is in place, dependent on the country and the lack of adequate regulation is of concern (Musee, Brent and Ashton, 2010). The rapid increase in nanotechnology is not always accompanied by nanotoxicological investigations. This is complicated by a lack of sufficient detection and characterisation techniques and particularly insufficient reproducible and validated method for such toxicological studies (Krug, 2014; Gatti and Montanari, 2015; Johnston *et al.*, 2020).

The ubiquitous use of AgNPs can be found in many industrial and consumer products *e.g.* clothing, food packaging; and biomedical or therapeutic products *e.g.* wound dressings, implants, vaccines and bio-assays (Liu and Xu, 2014; Stark *et al.*, 2015; Burdușel *et al.*, 2018). Furthermore, AgNPs have been detected in the environment including water and soil, and are likely to bioaccumulate (Fabrega *et al.*, 2011; Mahdi *et al.*, 2018). Therefore, exposure to humans, either deliberate - such as for therapeutic reasons, or incidental – food packaging and environmental exposure is inevitable. Once exposed, whether via the skin, inhalation, ingestion or direct injection, AgNPs have been found to move to secondary organ sites via the circulatory and lymph system. The liver has been found to be one of the main target organs (Trop *et al.*, 2006; Ebabe Elle *et al.*, 2013; Chen *et al.*, 2016; Weldon *et al.*, 2016). Accumulation in the liver leads to interaction between the liver cells and AgNPs, possibly changing the normal function of the liver resulting in hepatotoxicity by modulating biochemical and physiological functions. The liver is the primary detoxification organ, stores glycogen, and secretes many proteins especially those forming part of innate immune system *e.g.* complement factors and acute phase proteins. The liver is predominantly composed of hepatocytes, the liver's primary functional unit. Concerns regarding the potential toxicity in

hepatocytes have been raised, but studies have produced varying results (Zhou, Xu and Gao, 2016; Yao *et al.*, 2019).

Reports include cytotoxicity and often reactive oxygen species (ROS) induced effects leading to pro-inflammatory cytokine release, upregulation of cellular anti-oxidant defence mechanisms, often leading to DNA damage and cell cycle arrest or cell death. Low level chronic inflammation in the liver has been linked to several inflammation-based liver diseases. Therefore, such AgNP induced changes could not only modulate the immune response but also make the liver more prone to liver disease and injury (Castell *et al.*, 1989; Klaper *et al.*, 2014; Robinson, Harmon and O'Farrelly, 2016). Other ROS-independent mechanisms have also been found but are less studied (see Chapter 1 *Literature review*). Different properties of AgNPs have been found to modulate the toxicity potential. Intrinsic properties - such as, core size, shape, chemical composition; and extrinsic properties - those that can be modulated by the environment of the nanoparticle (NP), modulate the potentially detrimental outcome and to some extent explain the varying AgNP toxicity data produced (Rose *et al.*, 2012; Casals *et al.*, 2017).

The endocytosis mechanism of AgNPs have also been reported to modulate the toxicity outcome in nanotoxicological studies. Clathrin mediated endocytosis (CME) is one of the most studied and relevant mechanism of endocytosis (Francia *et al.*, 2019). While several studies have investigated the relationship between NP properties and uptake pathways, the resulting data is often contradicting (Francia, Montizaan and Salvati, 2020). Yet, nanotoxicity is significantly influenced by the uptake mechanism and elucidation of the internalisation pathway and its effects in liver cells have been scarcely investigated.

Therefore, this is a further research contribution on AgNP uptake and resulting effects in liver cells. By coupling the investigation of biomarkers for inflammation, cell stress and cytotoxicity modulation in the HepG2 human liver cell line with the inhibition of CME prior to AgNP exposure. Providing greater insight into the effect of uptake pathway used for AgNPs and the modulation on inflammation, cell stress and cytotoxicity.

2.2 Aim, objectives and hypothesis

The aim of this thesis is to investigate the effect AgNP uptake mechanism, specifically CME, has on the potentially toxic effects. These were measured as viability, pro-inflammatory

biomarkers and cells stress biomarkers in HepG2 human hepatoma cell line, which was previously described as being representative of primary liver cells.

- ✓ To determine at what concentration AgNPs are cytotoxic to HepG2 human hepatoma cells.
- ✓ Investigate sub-lethal effect, making use of inflammatory response (Nitric oxide, IL-6, CXCL8/IL-8, CCL4/MIP-2β and MIF) and cell stress (SOD2, HSP27/HSPB1) biomarkers.
- ✓ To elucidate whether the measured effects are modulated by CME inhibition to gain further insight into the mechanism of effects.

H_0: AgNPs do not affect viability, inflammation and cell stress biomarkers and are not modulated by CME inhibition.

H_α: AgNPs affect viability, inflammation, and cell stress biomarkers and the effects are modulated by the CME of the AgNPs.

Chapter 3 The effect of silver nanoparticles on inflammation and cell stress biomarkers in HepG2 cells with and without chloropromazine pre-incubation

3.1 Abstract

The purpose of this study was to determine the effect of silver nanoparticle (AgNP) exposure as well as the modulation of these effects by clathrin-mediated endocytosis (CME) inhibition on HepG2 human hepatoma cells *in vitro*. Biomarkers of inflammation and cell stress were investigated. Since, AgNPs are ubiquitously used commercially and for personal use, exposure to humans is inevitable. The liver has been identified as one of the main organs AgNPs locate to, upon gaining entrance into the human system. Furthermore, health concerns have been raised concerning AgNPs. Here, 48 hr treatment with the AgNPs showed a concentration dependent reduction in viability of HepG2 cells, which was partially mitigated by pre-incubation with chloropromazine (CPZ), a pharmaceutical CME inhibitor. The culture supernatants were assayed for inflammatory markers: nitric oxide, interleukin-6, interleukin-8, macrophage inflammatory protein 1 beta, and macrophage migration inhibitory factor (MIF) - of which only MIF was detected upon AgNP exposure. The cell lysates were tested for cell stress biomarkers: superoxide dismutase 2 (SOD2) and heat shock protein 27 (HSP27/HSPB1), of which only HSP27 was detected and upregulated upon AgNP exposure. The inhibition of CME significantly ($p<0.001$) lessened the viability reducing effects, as well as MIF and HSP27 upregulation compared to the CPZ free AgNP (50 µg/mL) exposure samples. Although this study was not able to completely determine the mechanism of cytotoxicity induced by AgNPs, it showed that CME is involved in AgNP cytotoxicity and upregulation of MIF and HSP27 in HepG2 liver cells.

3.2 Introduction

Nanoparticles, due to their small size, exhibit unique physicochemical properties (Rasmussen *et al.*, 2018). These novel properties can be modified by varying synthesis methods and capping agents. This ability to harness such dynamic abilities has led to nanomaterial applications in almost all sectors of life (Smita *et al.*, 2012). Silver nanoparticles (AgNPs) are one of the most widely used nanomaterials, from the household, personal care products to medical applications. AgNPs retain their antimicrobial ability while exhibiting novel properties, such as optical and catalytic abilities (Syafiuddin *et al.*, 2017). Due to these and

other properties AgNP containing consumer products are numerous, and many lead to deliberate or accidental AgNP exposure to humans (Llorens *et al.*, 2012).

Concerns have been raised, regarding the safety of AgNPs (Javurek *et al.*, 2017; Hadrup, Sharma and Loeschner, 2018). Upon entry into the human body (*via* inhalation, oral, dermal or intravenously) AgNPs can distribute and accumulate in several organs, one of which is the liver (Wang, Xia and Liu, 2015). The liver has been found to be particularly vulnerable to AgNP exposure (Trop *et al.*, 2006; Weldon *et al.*, 2016). The liver is not only cardinal in normal physiological functions of the body and is responsible for most detoxification, it also is of immunological importance (Bode *et al.*, 2012; Zhou, Xu and Gao, 2016). The liver produces several components of the innate immune response and acute phase proteins. These are predominantly produced by hepatocytes, the functional units of the liver, which make up most of the cells in the liver (Bode *et al.*, 2012; Yao *et al.*, 2019). Damage by inflammation or cell stress to these cells can therefore have detrimental effects for the body.

The reported effects caused by AgNPs exposure are highly variable (Bastos, Brown, *et al.*, 2016; Bastos, Duarte, *et al.*, 2017; Bastos, Ferreira-de-Oliveira, *et al.*, 2017). This variability in data produced is often attributed to the different characteristics of the AgNPs tested and the modulation of these characteristics by different exposure environments, synthesis methods and many more factors (Prasad *et al.*, 2013). Generally speaking, AgNPs have been found to induce reactive oxygen species (ROS) leading to oxidative stress in cells. Usually this induces the upregulation of several cellular protective responses. These include anti-oxidant enzymes, general stress pathway, apoptosis and inflammation (Hussain *et al.*, 2005; Avalos *et al.*, 2014). ROS-independent mechanisms have also been found to cause AgNP induced toxicity, but ROS-mediated oxidative stress is the most widely accepted (Haase *et al.*, 2012; Dayem *et al.*, 2017; Braeuning *et al.*, 2018). Furthermore, AgNP toxicity is modulated by the mechanism of cellular uptake (Halamoda-Kenzaoui *et al.*, 2017).

Given the importance of the liver, its vulnerability to AgNP exposure and the great influence the endocytic pathway plays in determining the manifestation of toxicity requires further investigation. The aim of this study was to determine the effects of AgNP, at different concentrations with and without inhibition of clathrin-mediated endocytosis exposure, on HepG2 hepatocytes. Specifically by determing at what concentration AgNPs are cytotoxic to

HepG2 cells, investigating sub-lethal effect, by making use of inflammatory response (Nitric oxide, IL-6, CXCL8/IL-8, CCL4/MIP-2β and MIF) and cell stress (SOD2, HSP27/HSPB1) biomarkers, and determining whether the measured effects were modulated by CME inhibition to gain further insight into the mechanism of the measured effects.

3.3 Materials and methods

Unless otherwise stated laboratory equipment was provided by and analysis performed at the Departments of Medical Bioscience and Biotechnology at the University of the Western Cape (UWC), Cape Town, South Africa (SA). Transmission electron microscopy (TEM) and scanning electron microscopy (SEM) was performed at the Electron Microscope Unit also at the UWC. X-ray diffraction (XRD) analysis was done by i-Themba LABS, Materials Research Department, SA.

3.3.1 Silver nanoparticles (AgNPs)

3.3.1.1 AgNP storage, dispersion and specifications

Powdered silver nanoparticles (AgNP) were stored in a dark environment once obtained from Sigma Aldrich (Product no. 576832-5G; CAS no. 7440-22-4). All specifications provided by either the manufacturer, previously performed and published by our laboratory (Walters, Pool and Somerset, 2013; Walters *et al.*, 2017), as well as those performed for this writing are summarized in Table 3.1.

Table 3.1 Specifications of AgNPs
Unless otherwise indicated, as provided by the manufacturer. Experimental values (a), previously measured values already published (b (Walters, Pool and Somerset, 2013; Walters *et al.*, 2017)). Additional comments in discussion (c).

Physico-chemical properties	**Measurement technique**	**Specifications of AgNPs**
Size	SEM	<100nm[b]
Diameter (nm)	TEM	50 – 100 nm [b]
Morphology	TEM/SEM	Spherical [a]
Purity/chemical composition	EDX	99.5 % pure Ag; elemental silver [a,b]
Morphology (powder)	TEM/SEM	highly clustered powder [a,b]
Crystalline nature	PXRD	crystalline nature confirmed [a,b]
Surface area	BET	7.5329 m^2/g ± 0.0028 /g [b]
Maximum absorption (λ_{max})	UV-Vis	Inconclusive [c]
Zeta potential (charge) (mV)	PALS	-21.7 ± 0.3 [a] in dH_2O
Hydrodynamic size (d_H)	DLS	135.3 d.nm ± 15.4 [a] in dH_2O
Polydispersity index (Pdi)	DLS	0.497 ± 0.124 [a] in dH_2O

The grey powder was delivered with polyvinylpyrrolidone (PVP) as a dispersant, which upon consultation with Sigma Aldrich was confirmed to be the likely coating agent with ~0.2 % PVP and therefore adsorbent present.

3.3.1.2 Dispersion and use

Probe ultra-sonication (Misonix Microson XL2000, Qsonica; 3 mm probe diameter; immersion depth ~750 mm) was used for stock solution preparation of 1 mL. The AgNP powder was weighed out and suspended in autoclaved deionized water (diH_2O; Milli-Q®, Merck) with a stock concentration of 10 mg/mL. Sonication was done on ice at about 20 sec intervals (with 1 min interruptions allowing sample and probe cooling) for 5 min total. Stock solutions were prepared at room temperature on the day of exposure, a maximum of 20 min in advance, and working dispersions were prepared at the required concentrations in prewarmed supplemented tissue culture media.

3.3.2 AgNP characterization

3.3.2.1 Transmission electron microscopy (TEM) and energy dispersive x-ray (EDX) analysis

The analysis of the powdered AgNP was performed by the Analytical imaging facility of the Physics department at the UWC. Their procedure is to operate a FEI Tecnai G2 20 field-emission gun (FEG) TEM in bright field mode at an accelerating voltage of 200 kV. Energy dispersive x-ray (EDX) spectra were collected using an EDAX liquid nitrogen cooled lithium doped silicon detector. Samples were prepared by drop-coating one drop of specimen solution 5 mg/mL of NP powder in 1 mL EtOH (70 % alcohol) onto a carbon coated copper grid. Drying for 10 min under a Xenon lamp, where after the sample coated grids were analysed under the microscope. The post-dispersion samples (same batch of AgNP) were analysed by Walters *et al.* (2017).

3.3.2.2 Scanning electron microscopy (SEM) and X-ray diffraction (XRD)

The size and shape of the AgNPs was further analysed using a scanning electron microscope (SEM) with a Zeiss Gemini Auriga equipped with a CDU-lad detector at 25 kV. The sample preparation was performed by placing the AgNP powder onto the carbon surface of SEM stub. The XRD analysis was performed by i-themba LABS, Materials Research Department using a D8-Advance diffractometer (Bruker AXS, Germany) with Cu-k radiation at voltage 40 kv and current 40 mA to determine the structure of the NPs and EVA software (Bruker).

3.3.2.3 Hydrodynamic size (d_H), zeta-potential (ζ) and polydispersity index (PDI)

According to the manufacturer's instructions 1 mL of AgNPs solution was placed into machine-model-specific polystyrene cuvettes (Malvern) or capillary tubes (Malvern) for DLS and ζ measurements respectively. Using the phase analysis light scattering mode at 25 °C

analysis and the translational diffusion coefficient set according to its aqueous environment. The AgNP were dispersed, centrifuged, resuspended, and diluted as described above. Measurements were performed by the Zetasizer NanoZS® (Malvern Instruments Ltd.), the average of 3 measurements was used and expressed as mean ± standard deviation (SD).

3.3.3 Cell Culture

3.3.3.1 Cell Line

The human hepato blastoma cell line, HepG2 (*ATCC®* HB-8065™) cells were acquired from the American Type Culture Collection (ATCC; Virginia, USA). HepG2 cells are a well-studied and characterised cell line that is hepatitis negative. It has been widely used and considered a good *in vitro* model for several assessments, including nanotoxicity screening for hepatotoxicity studies (Kermanizadeh *et al.*, 2013; Xue *et al.*, 2016; Wang *et al.*, 2019).

3.3.3.2 Cell Culture Conditions

HepG2 cells were grown and cultured aseptically in 75cm^2 treated tissue culture flasks (SPL Lifesciences) under standard conditions [37 °C with 5 % carbon dioxide (CO_2) in saturating humidified air]. Sub-culturing and seeding were performed according to the ATCC's specifications at 70-80 % confluence once every three to four days. The cells were maintained in complete media (cDMEM): Dulbecco's Modified Eagle's Medium (DMEM; Lonza) with 10 % (v/v) heat inactivated foetal bovine serum (FBS) (HyClone), 1 % (v/v) antibiotic / antimycotic (Sigma Aldrich), 0.5 % (v/v) gentamycin (Sigma Aldrich) and glutamax (Sigma Aldrich).

3.3.3.3 Seeding and exposure to AgNPs

At about 70-80 % confluence the cells were trypsinised with trypsin-EDTA (Gylson) in sterile phosphate buffered saline (PBS; Lonza) with 0.025 % trypsin and 0.01 % EDTA) at 37 °C in humidified air according to standard procedure, neutralized and centrifuged at 618 rcf for 10 min (C-28A, BOECO, Germany) to bring cells out of suspension. The supernatant was discarded and cells resuspended in complete media and subsequently diluted to 1 x 10^5 cells/mL seeding density. Seeding and exposure to the AgNPs occurred in 96, 48 and 6 well plates (Nunclon, Sigma-Aldrich) at a cell density of 1 x 10^5 cells/mL by the addition of 100, 200 and 1600 µl/well respectively. Due to their doubling time the cells were allowed to attach and duplicate for 24 hrs. Subsequently, half the plates were pre-exposed to 5 µg/mL Chlorpromazine (CPZ) (in pre-warmed complete media) for one hour and then exposure to various concentrations of AgNP (0-400 µg/mL AgNP) for 48 hrs at standard tissue culture

conditions followed. The 48 hr exposure period was based on previous exploratory exposures that showed the most response at this exposure duration. Cells exposed to AgNPs with 5 µg/mL and without CPZ [(+) CPZ and (-) CPZ respectively] were used as negative controls.

3.3.3.4 Sample collection and preparation

Supernatants were collected, centrifuged at 12100 rcf for 1 min (Super mini centrifuge, miniStar Plus) in order to remove the remaining AgNPs. Some studies have found considerable interference from AgNPs (Riaz Ahmed *et al.*, 2017) due to SPR band overlap with the typical absorbance wavelengths used during the measurement.

The nitric oxide (NO) assay was performed immediately and the rest of samples were frozen at -80°C for subsequent enzyme linked immunosorbent assays (ELISAs). The supernatants and cells from the 96 and 48-well plates were used for sodium 3'-[1-[(phenylamino)-carbony]-3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene-sulfonic acid hydrate (XTT) cell viability determination. Whereas, the cells from the 6-well plates were used to isolate the intracellular protein containing fraction, where cell scraping was performed on ice. Cells were washed 3 times with Dulbecco's phosphate buffered saline (DPBS) prior to scraping, scraped in lysate buffer (LB) (0.1 % w/v protease and phosphatase inhibitor (Sigma Aldrich) with 0.01 % v/v Tween^{20} (Merck) in PBS). The cell scrapings were stored in Eppendorf tubes (Greiner, Bio-one) and kept on ice prior to sonication (QSonica, LLC. Misonix sonicators, XL-200 Series). The sonicated samples were then centrifuged (Super mini centrifuge, miniStar Plus) at 12100 rcf for 1 min for cell debris removal, the cell lysate supernatant was removed, and total protein concentration determined before being stored at -80 °C. The cell fracture supernatants were evaluated by ELISA for cell stress biomarkers (HSP27/HSPB1 & SOD2). Whereas, the cell supernatants were evaluated for inflammatory markers: NO, IL-6, CXCL8/IL-8, CCL4 /MIP-1□□and MIF. All samples were assayed in triplicate.

3.3.4 Cell lysate total protein determination

The total protein was determined for standardization of subsequent tests. To determine the protein content the commercial Bradford reagent (Biorad, Sigma) (1 in 5 v/v in diH_2O) was used. A standard curve was produced by performing doubling dilutions with an initial concentration of 1000 µg/mL human serum albumin (HSA) in PBS and the Bradford assay was performed as previously described in (Bradford, 1976) according to manufacturer's instruction. Subsequent to diluted Bradford reagent addition absorbance was read at 595 nm

(FLUOStar Omega). The determined standard curve was used to calculate the protein concentration as standardization of samples for ELISAs.

3.3.5 Cell Viability

A chromogenic water-soluble XTT (Roche) kit was used to determine the cell viability and proliferation test was used to asses HepG2 viability. It is a widely used viability assay and similar to assays using different dyes (*e.g.* MTT and WST-1). The tetrazolium salt is reduced to formazan dye by dehydrogenase enzymes of metabolically active cells. Therefore, the spectrophotometrically measured intensity is directly proportional to the viable cells and relative proliferation rates, expressed as a percent of the negative control (Paull *et al.*, 1988; Wang, Yu and Wickliffe, 2011).

The assay was performed according to the protocol provided by the manufacturer with minor modifications to ensure as little interference by the AgNPs as possible. Shortly, after the experimental supernatants were removed the cells were washed thrice with 100 µl/well of warmed PBS in order to remove any AgNPs which possibly could interfere with the results. Immediately, 150 µl/well of the XTT reagent mixture (0.3 mg/mL in complete media) was added and immediately again 50 µl from each well transferred into a 96-well flat bottom plate (Greiner) and read at 450 nm (FLUO star Omega, BMG Labtech) and served as a blank measurement. After incubation in the dark at about 37 °C for 60 min, 50 µl per well were removed and transferred to another 96-well flat bottom plate and read at 450 nm. Results were expressed as % viability relative to the 0 µg/mL AgNP (negative control) of both (+) 5 µg/mL CPZ and (-) CPZ as 100 % viability ± SD.

3.3.6 Inflammatory and cell stress markers

3.3.6.1 Nitric Oxide (NO)

NO is a small highly reactive free radical molecule that is not only present at physiological levels but can be induced as it plays a role in the inflammatory process. The Griess assay was performed on the supernatants from the 96-well plate samples (post centrifugation) to determine the secretion of NO by means of measuring nitrite levels. For the assay, 100 µL of the respective supernatant was added to each well of a 96-well flat bottom plate (Sigma Aldrich). A standard curve (doubling dilutions) with an initial concentration of 100 µM sodium nitrite (Sigma-Aldrich) was used to determine the concentration present in the samples. Equal volume of Griess reagent (1:1 mixture of 1 % sulphanilamide (Sigma-Aldrich) and 0.01 %

naphthyethylenediamine dihydrochloride (Sigma-Aldrich) both made up in 2.5 % phosphoric acid) was added. Subsequently, the plate was read immediately at room temperature at 540 nm (FLUOStar Omega, BMG Labtech).

3.3.6.2 Double Antibody Sandwich Enzyme Linked Immunosorbent Assays (DAS-ELISAs)

Different ELISAs were performed according to manufacturer's instructions. All samples were assayed without prior dilution and 96-well maxisorb plates (Nunc®) were used. All coating occurred overnight at 4 °C while all other steps were performed at room temperature. The optical densities of the colour reaction were read at 450 nm (FLUOStar Omega, BMG Labtech).

For precise human IL-6 detection in the prepared cell supernatants, a human IL-6 cytokine kit (eBioscience®, Ready-Set-Go, Thermo Fisher Scientific) was used as per manufacturer's instructions which provided all reagents required. For accurate quantification of human CXCL8/IL-8, CCL4/MIP-1β, MIF, HSP27/HSPB1 & SOD2 DuoSet ELISA kits were acquired (R&D Systems®) and the manufacturer's instructions followed. As assay diluent a 1 % w/v human serum albumin (HSA; Western Cape Blood Service, South Africa) in 1 X PBS was used.

3.3.7 Statistical analysis

The resulting data was processed in Microsoft Excel (2016) and the statistical analysis of the data using SigmaPlot 14.0 (Systat Software, Inc.) was performed. The one-way analysis of variance (ANOVA) test was done comparing each AgNP concentration with its respective control both for the CPZ inhibited samples and those without. Results are expressed as mean ± SD of the triplicate experiments. Probability ($p \leq 0.001$) was considered statistically significant.

3.4 Results

3.4.1 Characterisation

As reviewed in Chapter 1 *Literature review* the behaviour in biological environments is greatly influenced by the physicochemical properties of NPs. Characterisation therefore is important so that resulting effects in a biological system can be understood in relation to the different properties of the AgNPs.

3.4.1.1 TEM and SEM micrographs and energy dispersive x-ray (EDX) & x-ray diffraction (XRD)

TEM and SEM were performed on the AgNP powder, in additional to the core size and size distribution analysis of the resuspended particles available from Walters *et al.* (2017). The images (a) & (b) (Figure 3.1) show analysis of the AgNP powder with loose agglomeration of the AgNPs.

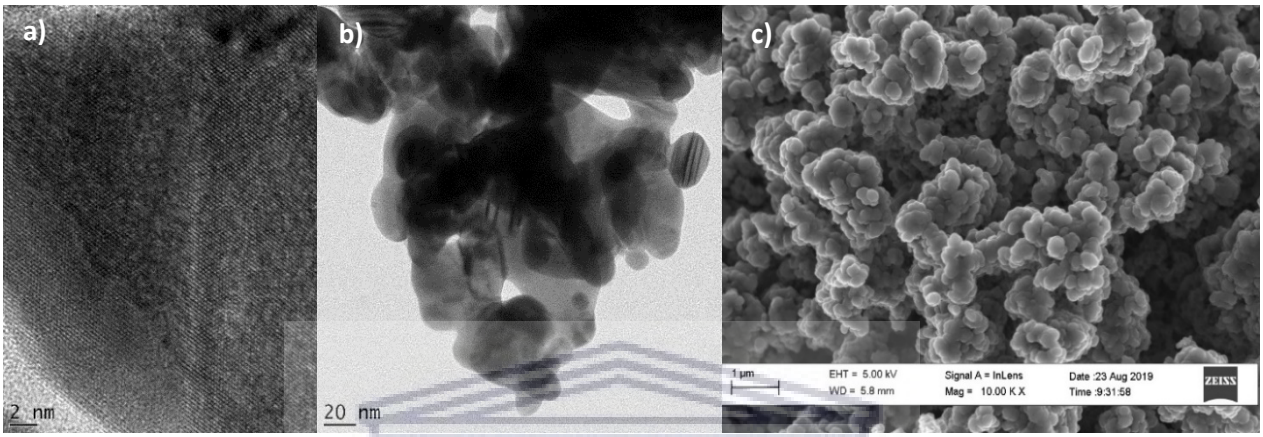

Figure 3.1 TEM (a,b) & SEM (c) micrographs of as-received powder showing agglomerated particles.

The EDX spectrum (Figure 3.2a) confirmed the presence of elemental silver and was performed using a different detector as part of the TEM measurement. The peaks showing carbon (C) and copper (Cu) peaks are expected since the grid the AgNPs were placed on for analysis are coated with both carbon and copper. By using another detector, the selected area diffraction (SAED) (Figure 3.2b) image was produced. It shows the electron diffraction pattern - rings containing bright spots, expected from crystalline AgNPs (Volkov *et al.*, 2009). The XRD pattern of the AgNP (Figure 3.2c) confirm their crystalline nature and lack of impurities.

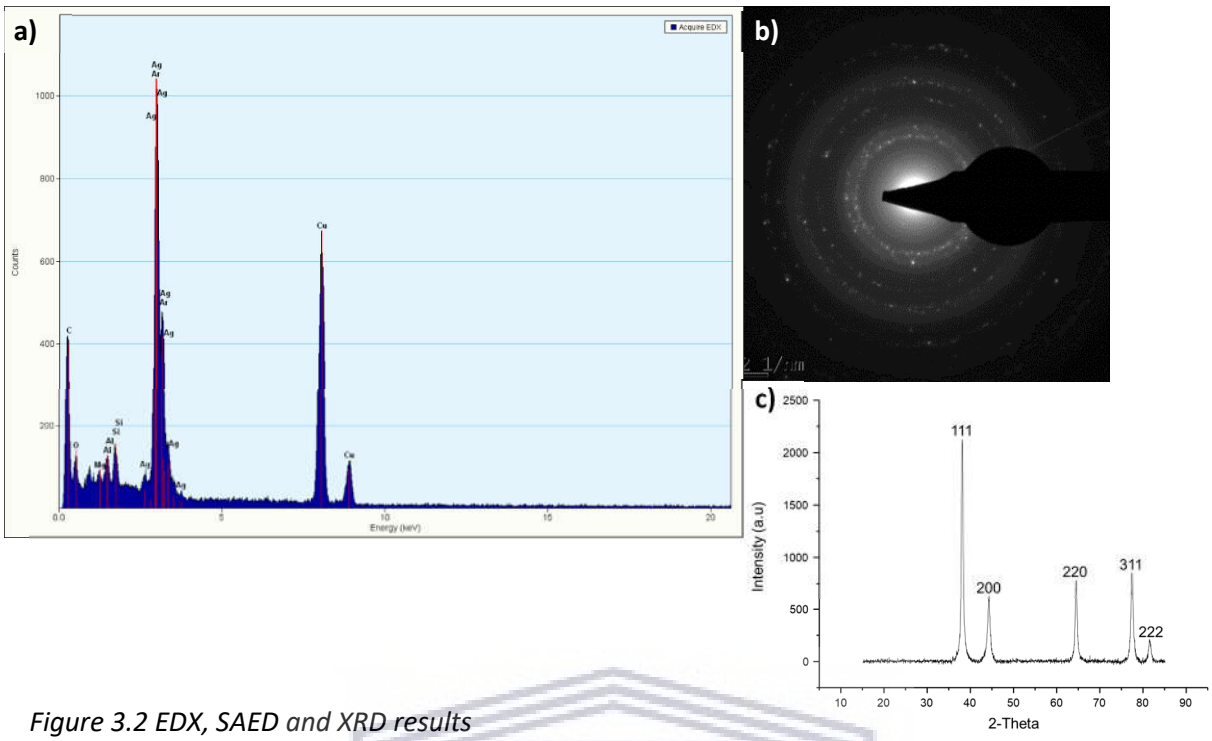

Figure 3.2 EDX, SAED and XRD results
The EDX spectrum (a) and XRD (c) verify the presence of silver atoms. The SAED (b) image confirms the crystalline structure expected for AgNPs.

3.4.1.2 Hydrodynamic size (d_H), Zeta potential (ζ) and polydispersity index (PDI)

Table 3.2 shows the different sizes in solution, PDI and surface charge for the AgNPs in different kinds of media. The d_H of the AgNPs increased from 135 d.nm in diH_2O to more than triple the size in 150 mM NaCl to 416.6 d.nm and further to almost five-fold to 637.9 d.nm in the protein rich 10 % (v/v) FBS in DMEM. Similarly, the ζ decreased, increasing in magnitude, in the: diH_2O to NaCl to 10 % (v/v) FBS in DMEM. A small shift in ζ from -21.7mV to -22.4 mV and a slightly larger move to -26 mV. The PDI did not vary considerably with only a minor increase in the same order as mentioned previously.

Table 3.2 Hydrodynamic size, zeta potential and polydispersity index
Averages of 3 repeats shown ± SD in different dispersion media. cDMEM = 10 % FBS in DMEM

Dispersion media	d_H (d.nm)	Polydispersity index (PDI)	Zeta Potential (mV)
diH_2O	135.3 d.nm ± 15.4	0.497 ± 0.124	-21.7 ± 0.300
150 mM NaCl	416.6 d.nm ± 68.6	0.508 ± 0.051	-22.4 ± 0.436
cDMEM	637.8 d.nm ± 46.2	0.586 ± 0.049	-26.1 ± 0.737

3.4.2 The effect of AgNPs on cell viability and cytotoxicity with and without CPZ pre-exposure

AgNP exposure significantly decreased ($p < 0.001$) cell viability at 6.25-100 µg/mL as compared to the negative control. In the negative (-) CPZ samples, AgNP reduced viability in a concentration dependent manner (Figure 3.3). The (+) CPZ samples showed a significant ($p < 0.001$) reduction only at 50 µg/mL AgNP exposure. Figure 3.3 shows the calculated IC_{50} for the (-) CPZ samples, which was as low as 42 µg/mL AgNP exposure. In contrast, the IC_{50} for the (+) CPZ samples was outside of the AgNP concentrations used (Figure 3.3). The LDH assay was used to assess the toxicity of the AgNPs on the HepG2 human hepatocytes (data not included). None of the concentrations of AgNPs (0-100 µg/mL) in either the (-) or (+) CPZ samples showed cytotoxicity greater than 13 % relative to the percent of the positive control. The (+) CPZ samples did not exceed 9 % cytotoxicity even at the highest AgNP concentration of 100 µg/mL.

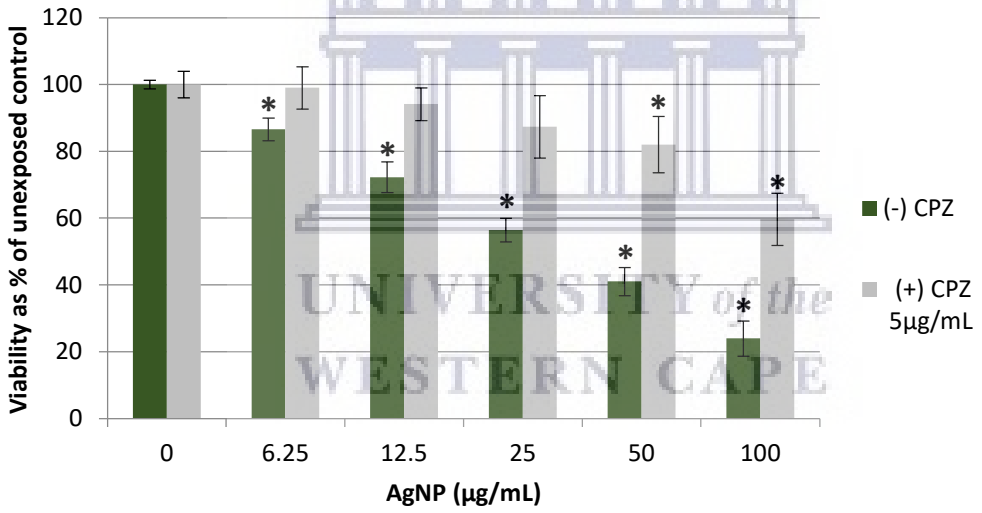

Figure 3.3 XTT - average of triplicate experiments for HepG2 cell viability exposed to 0-100 µg/mL AgNP with and without CPZ.

Cells were pre-exposed to carrying medium or CPZ and exposed to AgNPs for 48hrs and screened thereafter. Data (3 x n = 9) shows mean percentage ±SD.

** Represents a statistically significant ($p<0.001$) difference in comparison to the 0 µg/mL AgNP controls.*

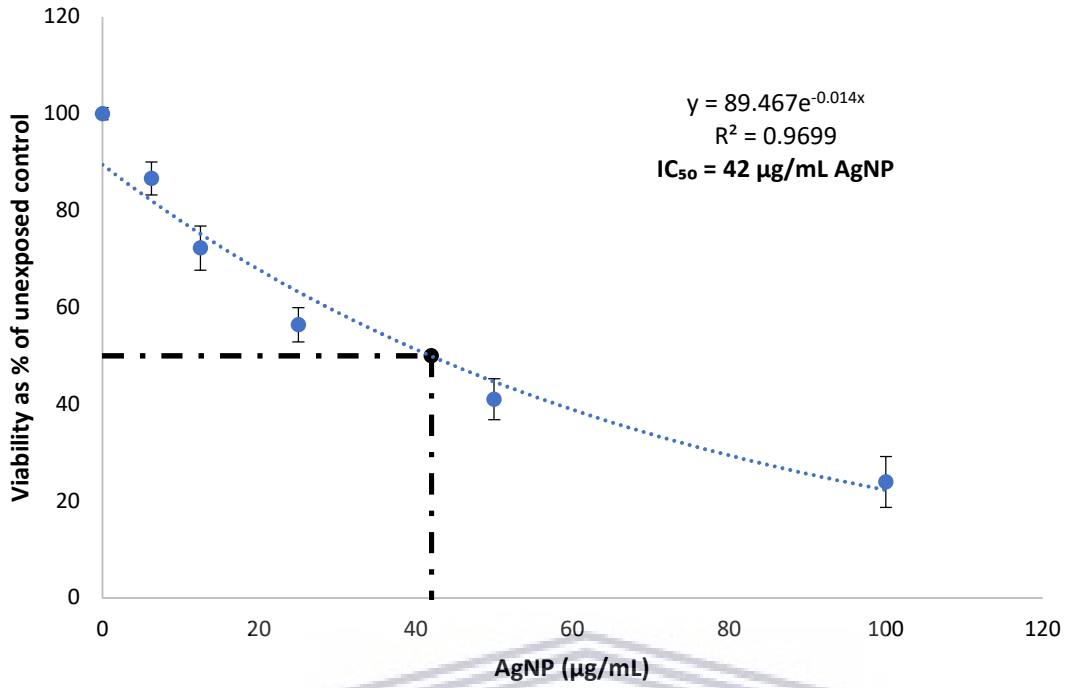

Figure 3.4 IC_{50} determination for (-) CPZ samples of AgNPs on cell viability as percentage of the µg/mL AgNP

3.4.3 The effect of AgNPs on inflammatory biomarkers with and without CPZ pre-exposure

The biomarkers assessed in HepG2 hepatoma cells subsequent to AgNP exposure with and without CPZ pre-exposure included: NO, IL-6, CXCL8/IL-8, CCL4/MIP-1β (data not shown) and MIF. Of these, only MIF stimulation occurred in the 50 µg/mL AgNP exposures in both the (-) and (+) CPZ samples, though significantly less MIF was produced in response to the (+) CPZ 50 µg/mL AgNP sample.

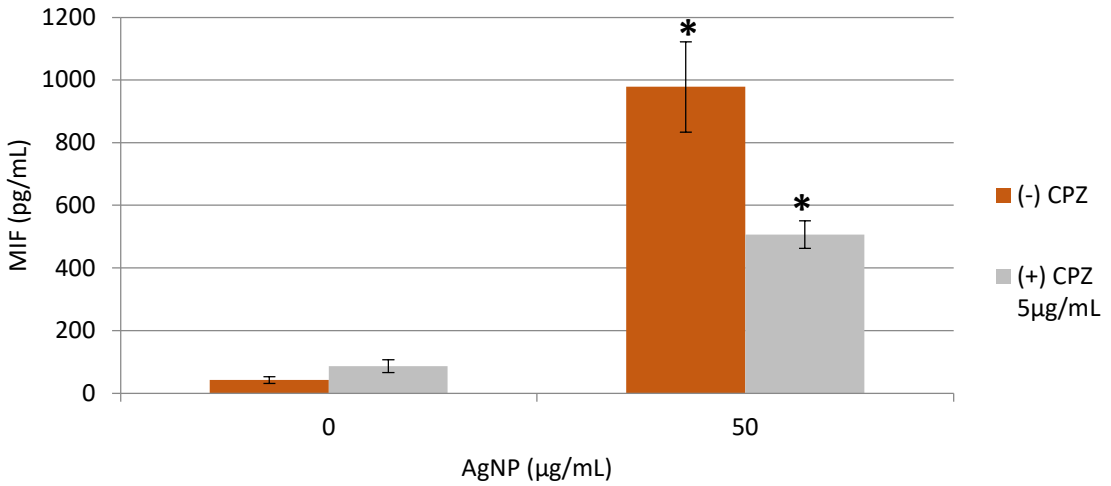

Figure 3.5 MIF levels of HepG2 cell cultures exposed to AgNPs both with and without CPZ treatment. Presented as mean ± SD (n=4)

** Represents a statistically significant (p<0.001) difference in comparison to the 0 µg/mL AgNP controls.*

3.4.4 The effect of AgNPs on cellular stress biomarkers with and without CPZ pre-exposure

The cellular stress biomarkers assessed were SOD2 and HSP27/HSPB1 for which the latter showed increased production in response to 50 µg/mL AgNP exposure in both the (-) and (+) CPZ samples, with no HSP27/HSPB1 production in the negative controls (*Figure 3.6*). Pre-incubation with 5 µg/mL produced a statistically significant ($p<0.05$) lower production of HSP27/HSPB1 when compared to the (-) CPZ 50 µg/mL AgNP samples.

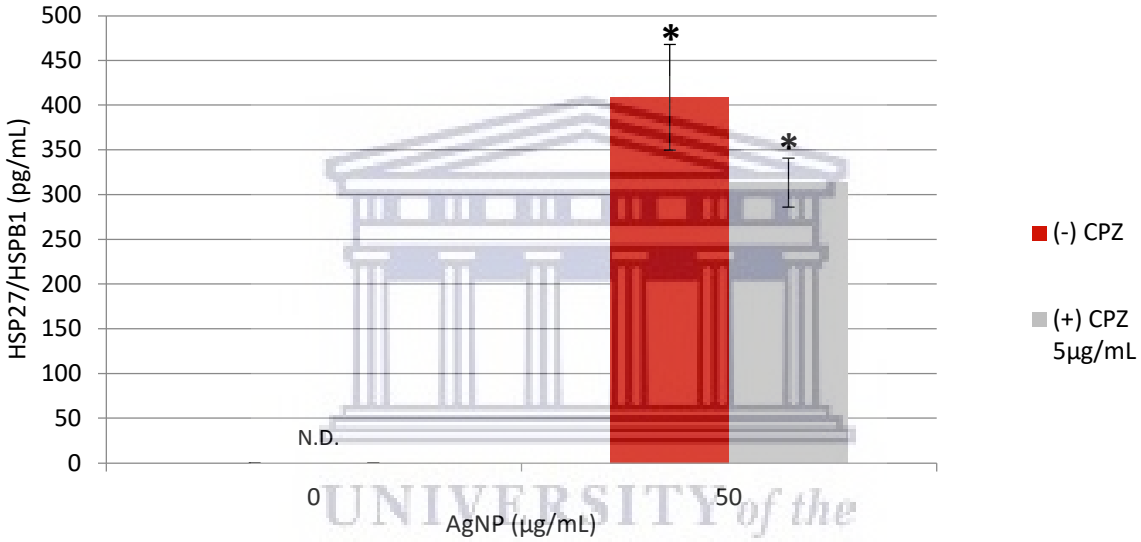

Figure 3.6 HSP27/HSPB1 levels of HepG2 cell cultures exposed to AgNPs both with and without CPZ pre-treatment. Presented as mean ± SD (n=4).
** Represents a statistically significant ($p<0.001$) difference in comparison to the 0 µg/mL AgNP controls.*
N.D. = none detected

3.5 Discussion

3.5.1 Characterisation of AgNPs

3.5.1.1 TEM and SEM micrograph analysis

Both the shape and size of AgNPs have been found to have a significant outcome on different biomarkers as endpoints of potential toxicity (Stoehr *et al.*, 2011; Makama *et al.*, 2018). TEM and SEM (electron micrographs (EMs)) are commonly used to observe objects at the lowest visual scale with high resolution, and therefore are commonly used for the initial characterisation of NPs. It is used to determine the size and shape of the NPs. TEM is especially useful to determine the core size of the particles (Gao and Lowry, 2018).

Both EMs (Figure 3.1) show quasi-spherical AgNPs, with a rather high level of agglomeration, not larger than 100 nm. Considering that the as-received powdered dry AgNP were assessed, this was expected. Therefore, the results corroborate the specification of, <100 nm spherical AgNPs, which was provided by the manufacturer. Further analysis has been published from our laboratory group in Walters *et al.* (2017), investigated TEM and provided size distribution of both the dry and resuspended AgNPs. The dry sample sizes range between 10-40 nm predominantly, with about 65 % around 10 nm. Some aggregation was present in the resuspended samples as the size range lies between 20-40 nm predominantly, with ~5 % at each 70 nm, 200 nm and 1000 nm. The size distribution for the resuspended sample strictly falls outside the definition of a NM; requiring that 50 % of NPs need to have at least one dimension below 100 nm (Gao and Lowry, 2018). Yet, the biological effects are not strictly visible below 100 nm and a toxically relevant definition of NMs is not yet available (Krug and Wick, 2011; Murugadoss *et al.*, 2020). Furthermore, the sample is likely to resemble what would be found in the liver – a variety of sizes, not all strictly within the sub-100 nm range. Drying of the resuspended AgNPs during sample preparation for EMs is prone to artefacts of aggregation and agglomeration; therefore, other methods of characterisation, in solution, were performed (Murdock *et al.*, 2008; Bhattacharjee, 2016).

3.5.1.2 Hydrodynamic size (d_h), zeta-potential (ζ) and polydispersity index (PDI)

In order gain insight into the AgNPs characteristics by the time of exposure, and to determine the effect of the environment on those characteristics, the d_H and PDI were determined in three different media (Table 3.2). Both d_H and PDI, were determined by the non-invasive, dynamic light scattering (DLS) technique. The hydrodynamic size (d_h) is the size of a NP core and adsorbed substances (*e.g.* ions, molecules) that move with it in solution and therefore typically is larger than TEM values (Bhattacharjee, 2016). Together with the d_h, DLS also provides the PDI; a dimensionless measure (0.0-1.0) of the broadness of the size distribution based on intensity, and is therefore not directly comparable to the number-based measurement obtained by TEM (Bhattacharjee, 2016). The PDI is indicative of the size distribution and reliability of the DLS results. A PDI of 0.1 - 0.25 indicates a small size distribution and reliable DLS results. A PDI of ≥ 0.5 is likely very polydisperse and the d_H should be interpreted carefully (Nickel *et al.*, 2014; Ma, 2016). Values ≥ 1 are too polydisperse for measurement by DLS (Spectroscopy, Scattering and Motion, 2011).

The d_H increased in more complex solutions: diH_2O<NaCl<cDMEM (Table 3.2). Initially, the AgNPs were 135 d.nm in diH_2O and more than tripled in 150mM NaCl to 416.6 d.nm, to almost five-fold the diH_2O size in the protein-rich cDMEM to 637.9 d.nm. This is likely due to adsorption of the proteins onto its surface and/or agglomeration as was previously seen (Mahl *et al.*, 2010; El Badawy *et al.*, 2012; Wang *et al.*, 2014). Metal NPs, especially AgNPs, adsorb in the ultraviolet-visual spectrum (UV-vis) at a distinct wavelength (refer to Chapter 1 *Literature review*). Therefore, a UV-vis absorption spectrograph of AgNPs was analysed; yet, without conclusive results (data not shown). PDIs of ~0.5 in all three solutions (Table 3.2), indicate rather polydisperse samples. Explaining the UV-vis results, since for polydisperse samples, it is not recommended as a routine quantitative measure of particle size (Tomaszewska *et al.*, 2013).

The measurement of the zeta-potential (ζ) *i.e.* surface charge of the particles, was used as a possible indication of stability; where a ζ over +25 mV and below -25 mV, is thought of as predictive of colloidal stability (Shnoudeh *et al.*, 2019). Also, surface charge can influence uptake of NPs and therefore their toxicity, hence it is part of a recommended characterization in nanotoxicological studies (Drasler *et al.*, 2017). Here, a slight shift towards a more negative charge from -21.7 mV to -22.4 mV and to -26 mV in diH_2O, NaCl, cDMEM, was observed, respectively (Table 3.2); similar to other's findings (Shannahan *et al.*, 2013). These results, may indicate that the AgNPs are likely more stable in the high protein solution and would not aggregate further over time. This is under investigation by our laboratory group.

3.5.1.3 Energy-dispersive X-ray spectroscopy (EDX), selected area diffraction (SAED) and x-ray diffraction (XRD)

EDX can be performed simultaneously with TEM analysis and is used to confirm the material's elemental specifications or purity. The EDX spectrum (Figure 3.2a) confirmed the presence of elemental silver. By using another detector, the SAED (Figure 3.2b) image containing the diffraction data was produced and shows the electron diffraction pattern - rings containing bright spots, which are as expected from crystalline AgNPs as indicated by the lateral fringes in the 2 nm scale TEM (Figure 3.1a) (Volkov et al., 2009). The XRD pattern of the AgNPs (Figure 3.2c) moreover confirms their crystalline nature. No peaks indicative of impurities were found while the diffraction peak intensities and positions agree with those reported elsewhere for AgNPs (Bindhu and Umadevi, 2015; Walters et al., 2017; Priya, Vijayakumar and Janani, 2020).

3.5.2 The effect of AgNPs on cellular viability and cytotoxicity in HepG2 hepatocytes

XTT is an indicator of metabolic activity and proliferation, yet not specifically of mitochondrial function. The negatively charged XTT cannot enter the cell and therefore is reduced to formazan at and across the plasma membrane; remaining a measure of energetically able and therefore viable cells. Yet, XTT is not as insightful in terms of the toxicological mechanism of AgNPs as is often stated (Berridge, Herst and Tan, 2005; Stepanenko and Dmitrenko, 2015; Stockert *et al.*, 2018; Gu *et al.*, 2019). XTT was used to determine the inhibitory concentration 50 (IC_{50}), which was used in subsequent exposures investigating sub-lethal effects; *i.e.* excluding unspecific effects caused by pronounced cytotoxicity.

Figure 3.3 (green) shows the XTT data of AgNP concentrations (0-100 µg/mL) compared to the measured viability as a percentage of the 0 µg/mL AgNP control, in the (-) CPZ samples, revealing a concentration-dependent reduction in viability from the lowest concentration (6.25 µg/mL). This is close to the 5 µg/mL AgNP (20 and 50 nm; capping agent not mentioned) exposure cytotoxicity threshold Sahu *et al.* (2015) found after 24 hr incubation. Although the authors found the IC_{50} for their study to be in contrast with that found here. The IC_{50} for this current study was calculated as 50 µg/mL AgNPs based on 0-400 µg/mL range initially used (data not shown). The subsequent experiments were performed at the calculated IC_{50}, although it was not the ideal concentration strictly speaking - as was determined later. At concentrations higher than 100 µg/mL AgNPs, the curve flattened and therefore required the exclusion of those concentrations. Therefore, the previously calculated IC_{50} was an overestimation by 8 µg/mL; new IC_{50}=42 µg/mL (Figure 3.4). However, this difference in concentration is not significant in the context of toxicity testing since it falls within the inter-assay variation range common for these type of assays. Moreover, 50 µg/mL AgNPs falls within the real-life exposure values approximately equal to 0.1-100 µg/mL *in* vitro cell culture dose, which was calculated based on the highest amount of silver released from AgNP-enabled medical devices (Sussman *et al.*, 2015; Mishra *et al.*, 2016).

Several viability studies of AgNP exposed HepG2 cells have been investigated and produced varying results. The most used assay investigating PVP coated AgNPs (PVP-AgNPs) and other elements closest to the experimental conditions of this study was MTT, which is a mitochondrial function-based assay and very similar to the XTT used here. In HepG2 cells,

Wang *et al.* (2019) found the IC_{50} to be 160 µg/mL AgNP for PVP-AgNPs (20 nm; 24 hr exposure). This result stands in contrast to the here determined IC_{50} of 42 µg/mL by XTT and could be explained by the larger size of the AgNPs studied here relative to the small 20 nm AgNPs. Although precise comparisons cannot be made due to the lack of d_H measurement (Akter *et al.*, 2018; Gu *et al.*, 2019). Similar to the present results are those found by Priya, Vijayakumar and Janani, (2020), - IC_{50} of 48 µg/mL in HepG2 cells by citrate capped AgNPs (cit-AgNPs) although they also were smaller at 17-50 nm, also no data provided for d_H.

Additionally, the uptake mechanisms of NPs play a definite role in determining the toxicity outcome of NP exposure (Panzarini *et al.*, 2018). AgNPs have been found to be taken up by CME and macropinocytosis by liver cell line, such as HepG2 (AshaRani, Hande and Valiyaveettil, 2009; Ahmed et al., 2017; Wu et al., 2019). Therefore, in addition to determining different toxicity endpoints of AgNPs, the effect of CME of AgNP on these endpoints was also investigated by pre-exposure to CPZ (5 µg/mL), a widely used pharmaceutical CME inhibitor (Greulich et al., 2011; Iversen, Skotland and Sandvig, 2011; Wu et al., 2019). This pre-exposure improved the viability considerably compared to the (-) CPZ samples, showing a statistically significant reduction in viability only at 50 µg/mL AgNP exposure. At the highest dosage of 100 µg/mL AgNP, the IC_{50} was not yet reached (Figure 3.4). Therefore, comparison of the (-) and (+) CPZ results reveals that the inhibition of CME leads to a reduced susceptibility to AgNP induced damage in HepG2 cells. Yet, it did not abolish the viability inhibiting effect completely. Several studies in different *in vivo* or *in vitro* models have found the use of multiple uptake pathways for different NPs, while only one was inhibited in this study, and may explain the lack of total viability inhibition found here (Khan *et al.*, 2014; Ahmed *et al.*, 2017). Furthermore, *Caenorhabditis elegans* (roundworm) cytotoxicity was almost completely removed by inhibition of CME with CPZ pre-incubation in cit-AgNPs; while $AgNO_3$ (as Ag^+ source) cytotoxicity was not significantly reduced by inhibition of CME (Maurer *et al.*, 2016). Therefore, the dissolution of Ag^+ may also play a role, and could explain the remaining viability inhibition present upon CME inhibition.

Impairing the cell's general energy supply would also impair energy-dependent protein synthesis. Since it is not commonly used as a stand-alone viability assay, the cell lysate protein content was determined as supporting assay to viability (Butler, Spearman and Braasch, 2014). It showed a similar trend to the XTT data (data not shown). At 50 µg/mL AgNP viability

decreased to 22 % of the 0 µg/mL AgNP control for (-) CPZ samples occurred, while the CPZ pre-incubation mitigated the reduction, to 77% of the 0 µg/mL AgNP (+) CPZ control.

Cytotoxicity of AgNPs was measured by, the lactate dehydrogenase (LDH) membrane leakage assay (data inconclusive and not shown). Results showed considerably lower cytotoxicity with significantly more variability than the XTT data. This could be an indication of particular membrane stability, or it could be due to interference of the AgNP with the LDH assay. Metabolic assays are prone to interference by NPs, especially AgNPs; leading to false positive or negative results (Han *et al.*, 2011; Oh *et al.*, 2014; Riaz Ahmed *et al.*, 2017). Due to UV-vis spectrograph overlap, in an attempt to mitigate AgNP interference, the AgNPs were centrifuged out of the cell supernatant prior to LDH quantification. This possibly removed LDH which could have adsorbed onto the AgNPs, or the AgNPs could have interfered in another way (Han *et al.*, 2011). While interesting, it is outside of the scope of this study and should be further investigated.

3.5.3 Effects of AgNPs on nitric oxide (NO) production with and without CPZ pre-exposure

NO is constitutively expressed in most cells and upon exposure to toxicants can be produced in large amounts by the synthesis of the inducible nitric oxide synthase (iNOS) enzyme. Therefore, NO concentration quantification is used as an indirect marker of iNOS upregulation. iNOS has been determined to be a critical factor in the development and propagation of liver inflammation; and hence acts as biomarker of an inflammatory response, which can be induced by cellular stress (Diesen and Kuo, 2010). Neither of the AgNP exposed samples (-) and (+) CPZ showed any NO production (data not included). Given the viability data (Figure 3.3) previously produced, this finding is unanticipated since the viability was considerably reduced in (-) CPZ samples from 6.25 µg/mL, indicating cellular stress (metabolic activity reduction), which typically upregulates inflammatory markers such as NO since cellular stress is a trigger for inflammatory processes (Nicholson and Thornberry, 2003; Zhang, 2018).

In contrast to the results here, a significant elevation in NO production was found by the fourth generation of continuous AgNP (5-10 nm) exposure to HepG2 cells at low AgNP concentrations (*e.g.* 8 % IC_{50}=0.24 µg/mL) (Nowrouzi *et al.*, 2010). Similarly, in human osteoblasts, 48 hr exposure to 30 and 50 µg/mL uncoated AgNPs (15nm) produced about 5

and 6 times the NO produced by the control (Zielinska *et al.*, 2016). The complex relationship between the different size, capping agents, cell types or exposure durations and other variables make exact comparisons not always possible and may explain the contrasting results (Caballero-Díaz and Valcárcel Cases, 2016).

Since interference is a concern with AgNPs (Guadagnini *et al.*, 2015); as previously indicated, the NO assay was modified by removal of the AgNPs from the supernatant by centrifugation before assessment. Therefore, the possibility of NO adsorption onto the AgNP surface as interference, was investigated by incubation of the 0 µg/mL AgNP samples with NO (used for the standard concentration range) together with different concentrations of AgNPs. Subsequently the NO/AgNP containing supernatants were centrifuged and analyzed. No interaction was found, the NO concentration could still be accurately detected making it rather unlikely that NO was removed together with the AgNPs (data not included).

3.5.4 Effects of AgNPs on IL-6, CXCL8/IL-8, CCL4/MIP-1β and MIF production with and without CPZ pre-exposure

AgNPs exposure has been reported to induce inflammation both *in vivo* and *in vitro* in cells in homeostatic conditions (Kermanizadeh *et al.*, 2012; Klaper *et al.*, 2014). Yet, several studies report anti-inflammatory effects as well. Those studies investigated wound-healing or cells in which the inflammatory response had been stimulated prior to AgNP exposure - modulating the already induced inflammatory response, or bio-synthesised AgNPs with a biological capping agent often conferring the anti-inflammatory property (Wong *et al.*, 2009; Yilma *et al.*, 2013; Chakraborty *et al.*, 2016; Matysiak-Kucharek *et al.*, 2020). While inflammation is typically beneficial, low level chronic liver inflammation can prime it for liver damage, especially since several liver diseases have an inflammatory basis (Castell *et al.*, 1989; Klaper *et al.*, 2014; Robinson, Harmon and O'Farrelly, 2016). Hence, chemokine/cytokine secretions, known as hallmarks of inflammation, typically released by hepatocytes upon injury or invasion (IL-6, CXCL8/IL-8, CCL4/MIP-1β and MIF) were measured in supernatants of (-) and (+) CPZ pre-treated HepG2 upon 0 and 50 µg/mL AgNP exposure (Rowell *et al.*, 1997; Zhang *et al.*, 2003; Dembic, 2015; Marin *et al.*, 2017).

Only MIF was detected (Figure 3.5) while the cells did not produce detectable levels of IL-6, CXCL8/IL-8 and CCL4/MIP-1β (data not included). These results are surprising, given that the viability data showed considerable reduction at 50 µg/mL AgNP exposure and hence the

induction of inflammation would be possible. Yet, these results are in line with the lack of NO production at this concentration. It is unlikely that a reduction below the detection level of IL-6, CXCL8/IL-8, CCL4/MIP-1β occurred due to induced cell death at 50 µg/mL AgNP exposure since the initial IC_{50} calculation was only slightly higher than the actual IC_{50} of 42 µg/mL.

Whereas, limited data seems to be available for AgNPs so far, specifically looking at the same group of chemokines/cytokines in liver cells, partially similar results have been found or investigated in different NPs. The inverse response to the identical AgNPs (from our laboratory group) was produced in unstimulated whole blood cultures (WBCs) by ≥ 25 µg/mL. The secretion of IL-6 and CCL4/MIP-1β were found to increase, and MIF production was suppressed compared to the control (Lategan, Walters and Pool, 2019). Similarly, in human breast cancer (MDA-MB-436) cells, AgNP (20 and 200 nm) treatment (10 and 50 µg/mL) increased IL-6 and CXCL8/IL-8 protein secretion while only producing low amounts of MIF, with more pronounced effects induced by 20 nm AgNPs (Matysiak-Kucharek *et al.*, 2020). This contrasts the results of the current study and are not fully understood. Yet, this could be related to the difference in cell type or possibly the large difference in d_H size. It is widely accepted that smaller NPs tend to be more toxic, at a d_H of 637.9 d.nm the particles are likely agglomerated and could explain the lack of upregulation in the inflammation biomarkers seen here (Smékalová *et al.*, 2018).

Another possible cause for the low levels of the inflammatory markers is the type of capping agent present. Wang *et al.* (2014) suggested that PVP, the capping agent of the AgNPs, could complex Ag^+ and therefore reduce toxicity in comparison to otherwise capped AgNPs *e.g.* cit-AgNPs. Further information would be required to make more conclusive interpretations of the data. The optimal detection time for measurement of the inflammatory mediators should also be considered as a possible factor in the detection of the different biomarkers since they are produced in a time-dependent manner (Ambrosone *et al.*, 2012; Husain *et al.*, 2013; Klaper *et al.*, 2014). For example, Khan *et al.* (2013) found that inflammatory effects caused by NM exposure lessened after the first 24 hrs.

The MIF upregulation was inhibited by the pre-incubation with CPZ (Figure 3.5). Implying the effect on MIF upregulation induced by AgNPs is due to CME uptake. Similarly, to the effect on viability (Figure 3.3) the inhibition of CME did not fully reduce the MIF production. Given the large d_H and the previous findings that PVP-AgNPs (~60 d.nm) were found to be mainly taken

up by CME and macropinocytosis, the latter of which is commonly used for the uptake of larger molecules, it is likely that another uptake pathway that remains active is macropinocytosis (Ahmed *et al.*, 2017). MIF may induce acute phase protein secretion in hepatocytes, depending on other cytokines present and therefore it is important to investigate other cytokine modulations further (Wheelhouse *et al.*, 2006; Bode *et al.*, 2012).

3.5.5 Effects of AgNPs on SOD2 and HSP27/HSPB1 with and without CPZ pre-incubation

Acute and chronic stresses can cause deleterious effects on cellular infrastructure and homeostasis. Hence, adaptive cellular stress response pathways are initiated by cells and organisms to limit damage and return to homeostasis. Stress response pathways reported to have been induced by AgNPs other than the inflammatory response, include heat shock response and oxidative stress (Brzóska et al., 2015; Xin et al., 2015). Cellular stress upregulates for instance HSP27/HSPB1, which is part of the small heat shock proteins (sHSPs). sHSPs prevent irreversible aggregation of misfolded proteins or promote appropriate folding by binding denatured or incompletely folded proteins (Vidyasagar, Wilson and Djamali, 2012; Sha et al., 2019). Anti-oxidant enzymes are commonly upregulated upon oxidative stress; in liver cells super oxide dismutase (SOD) is one of the common antioxidants. Three types of SOD exist, one of which is found in the mitochondria (SOD2/Mn-SOD) (Culotta, Yang and O'Halloran, 2006).

Therefore, SOD2 and HSP27/HSPB1 protein levels were investigated in the cell lysates. Endogenous levels of HSP27/ HSPB1 in HepG2 cells were below the detection limit of the ELISA for the 0 µg/mL AgNP controls (Figure 3.6) and for all investigated samples for SOD2 (data not shown). Significant upregulation of HSP27 was induced by AgNP exposure (Figure 3.6). The present findings seem to be consistent with other research which investigated mRNA gene expression upon AgNP exposure in HepG2 cells (Sahu *et al.*, 2015b) and Caco2 intestinal cells (Oberemm *et al.*, 2016). Although increase mRNA expression would not strictly lead to upregulation at the protein level, it could play a role in protein level upregulation. In contrast to the current findings, the upregulation of total SOD levels found in HepG2 cells exposed for 24 hrs to 10 µg/mL PVP-AgNPs (~6 and ~29 d.nm mixture) and 10 and 50 µg/mL cit-AgNP (~16 and ~60 d.nm mixture) (Vrček *et al.*, 2016). Interestingly, in human pancreatic ductal adenocarcinoma cells, 24 hrs AgNP (18 nm) exposure induced a reduction of SOD2

protein levels at all exposure concentrations (10, 25 and 50 µg/mL), with an increase in iNOS enzyme and NO levels relative to control (Barcińska *et al.*, 2018). This is different to the current study's data on NO levels while similar in the lack of SOD2 detected, but as has been stated previously, the cellular responses to AgNPs are varied depending on cell line tested and could explain this difference (Kettler *et al.*, 2014). Brzóska *et al.* (2015) found an increase in mRNA expression of HSP27/HSPB1 and CXCL8/IL-8 but the fold change was considerably lower at 24 hrs compared to 6 hrs exposure time – this supports the possibility that transient upregulation of CXCL8/IL-8, rather than lack of CXCL8/IL-8 upregulation, is the reason for the lack of CXCL8/IL-8 detected in the current study. Moreover, 200 nm AgNPs induced a three times less fold change in HSP27/HSPB1 mRNA levels compared to the 20 nm AgNPs; underpinning the effect the NP size has on nanotoxicity (Brzóska et al., 2015).

Similar to the results in MIF levels, the reduction in HSP27/HSPB1 levels in the CPZ pre-incubated samples indicates that CME uptake facilitates the AgNP induced HSP27/HSPB1 increase (Figure 3.6) although likely not exclusively by CME. Since the HSP27/HSPB1 upregulation was not completely diminished by CME inhibition with CPZ. As previously stated, another likely uptake pathway is macropinocytosis especially given the large size of the AgNPs in the cDMEM (Ahmed et al., 2017). Few studies investigated the uptake pathways of AgNPs together with specific potentially toxicological outcomes other than general viability/cytotoxicity and genotoxicity (comet assay), particularly in liver cells. A reduction below the detection level of SOD2 production due to induced cell death at 50 µg/mL AgNP exposure, which was higher than the actual IC_{50} of 42 µg/mL is unlikely due to the small difference but must be considered.

3.5.6 Conclusion

In conclusion, the effect of AgNP exposure in HepG2 human liver carcinoma cell viability, inflammatory and cell stress biomarkers were investigated as well as the effect of CPZ pre-incubation for CME inhibition on the tested biomarkers. The AgNPs significantly reduced viability already at 6.25 µg/mL exposure, without CPZ incubation and reduced viability by half (IC_{50}) at 42 µg/mL AgNP exposure. In CPZ pre-exposed samples the viability was reduced only at 50 µg/mL AgNPs and IC_{50} lying outside the exposure range. This, indicates that CME uptake does facilitate the AgNP induced cytotoxic effects to a large extent, in HepG2 cells.

Several biomarkers for inflammation and cell stress namely NO, IL-6, CXCL8/IL-8, CCL4/MIP-1β and SOD2 were not detected in any of the samples. This could be due to several reasons, such as the non-optimal detection time, the low toxic potential of the likely agglomerated AgNPs, or the presence of the capping agent and therefore requires further study. MIF and HSP27/HSPB1 were upregulated by AgNP exposure, indicating inflammation and cellular stress, despite the lack of upregulation in the other biomarkers. This agrees with the results seen in viability behaviour. Moreover, CPZ pre-incubation reduced the viability limits, MIF and HSP27/HSPB1 response, implying CME plays a significant part in the mechanism of AgNP induced toxicity in HepG2 cells.

Chapter 4 Summary and future recommendations

Despite the insights offered here, further comprehensive future studies are needed. There are a few recommendations that need to be considered for future experimentations. Given the reported interference by AgNPs with metabolic viability/cytotoxicity assays it is recommended to include different mechanisms for viability/cytotoxicity testing, such as dye exclusion assays or fluorescence based flow cytometry (Oh *et al.*, 2014; Stepanenko and Dmitrenko, 2015). Furthermore, in order to contribute to the clarification whether toxicity induction is due to AgNPs or Ag^+ alone or both, the inclusion of $AgNO_3$ as a comparison would be advantageous as was done by (Oh *et al.*, 2014). A strategy to increase the comparability of the results would be to include extensive characterisation subsequent to exposure, include benchmark materials such as used in and include a wider variety of biomarkers such as proteomic analysis and determine the time-dependent changes of these, by measuring at various exposure times (Kermanizadeh *et al.*, 2012; Haase and Lynch, 2018; Langevin *et al.*, 2018; Bahl *et al.*, 2020; Johnston *et al.*, 2020). To provide further insight into the uptake mechanisms and the effects on toxicity, several pharmaceutical inhibitors and other methods of endocytosis inhibition could be employed (Dutta and Donaldson, 2012; Sasso *et al.*, 2018). Lastly, it is recommended to determine endotoxin levels in the NP solution prior to exposure to cells since endotoxin contamination can upregulate several toxicological biomarkers commonly tested (Sahu and Casciano, 2009).

The overall goal in nanotoxicological research is to enable correlation between physicochemical properties of NM with a specific outcome or toxicity behaviour, in order to predict toxicological implications from the NP properties (Gao and Lowry, 2018). Currently

findings are considerably often contradicting, although several reviews have identified trends to varying degrees these do not consider the full picture or have sufficient information at hand to find definitive relationships due to the complex nature of the NP-cell interaction (Francia, Montizaan and Salvati, 2020). This is not surprising given the complex nature in studies involving such a large number of variables each with large impacts. Therefore, it is important to follow protocols as meticulously as possible, given time and availability constraints, and paying close attention to the recommendations and pit-falls highlighted by several reviews and research papers as well as understanding the great effect a nuanced change in laboratory techniques can have on the toxicological outcomes measured (Kettler *et al.*, 2014; Oh *et al.*, 2014; Stepanenko and Dmitrenko, 2015; Vrček *et al.*, 2016; Riaz Ahmed *et al.*, 2017; Francia *et al.*, 2019; Ferdous and Nemmar, 2020; Johnston *et al.*, 2020).

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