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Bauer, Patrik Maximilian

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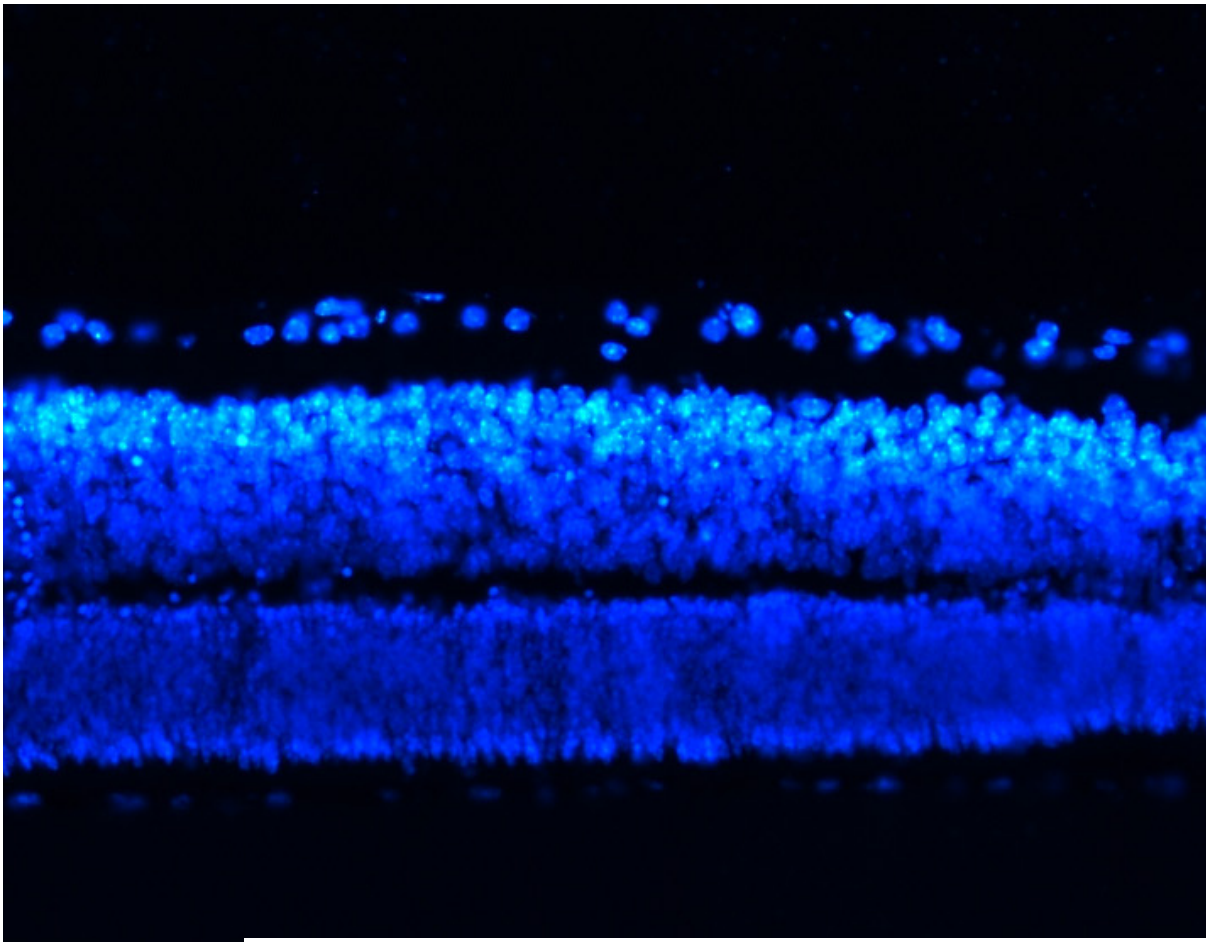

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Abstract

Over the past decade, a massive increase in the use of nanomaterials and nanoparticles (NPs) in both commercial and medical applications has occurred. Medical applications include advanced drug delivery vehicles, imaging and hyper thermic therapies. In retinal research, several nanomaterials have been explored in novel treatment approaches, ranging from metals, carbon, polymers and silica to biological materials such as lipids or lactic acid. NPs, especially, gain much attention as novel drug delivery vehicles due to their ability to cross the barriers of the eye including the cornea, conjunctiva and the blood-retinal barrier (BRB).

This thesis focus on the two most commonly used nanomaterials; gold- and silver nanoparticles (AuNPs and AgNPs, respectively), both commonly used as the active component or as a carrier for a functional agent. AuNPs have desirable properties such as high chemical stability, well-controlled size and are easy to modify with various surface functionalization. AgNPs due to their antibacterial effects are often applied in wound disinfection, coatings of medical devices and prosthesis but also in many commercial products such as textiles, cosmetics and household gods.

However, the literature is yet limited on the effect of AuNPs and AgNPs on the mammalian retina. Therefore, here we investigated the effect of AuNPs and AgNPs on the rodent retina using an ex vivo retina model. The retina is a well-organized laminar neural structure located at the back of the eye bulb. Sensory neurons, i.e. the photoreceptors, located in the outer nuclear layer of the retina, convert light to an electric signal that is transmitted through the bipolar cells and further to the retinal ganglion cells, which axons form the optic nerve that send the information from the retina to the brain for visual processing. All the neurons participating in this process are highly vulnerable to mechanical damage, changed levels of oxygen and nutrients as well as exposure to foreign factors. The immune cells of the central nervous system, also known as microglia cells, are located inside the retina and have the responsibility to sense pathological changes in their microenvironment. Any disturbances in the normal homeostasis will activate these cells which include increased proliferation, migration, phagocytosis and release of bioactive molecules.

Here we characterized 20 nm and 80 nm of Ag- and AuNPs nanoparticles and show that the particles gain a defined protein corona upon entering a biological environment, here the explanted retina model system (Paper 1). With electron transmission microscope we further demonstrated that all NP types are able to translocate into all retinal neuronal layers unhindered. Moreover, we showed that the explanted retina model is a reliable and useful model for testing early prediction of NP-toxicity in the retina and report that low concentrations of 20 nm and 80 nm of Ag- and Au NPs have significant adverse effects on the retina (Paper 3). These effects were compared to the neurotoxicological effects induced by lipopolysaccharide administration, which is the most common way to mimic a bacterial infection (Paper 2). A range of typical pathological hallmarks were included in the analysis; micro- and macro morphological changes, macroglial activation, changes in microglia behavior, apoptosis and oxidative stress (Papers 2 & 3).

Taken together, our results show that exposure to low doses of Au-and AgNPs causes neurotoxicity, similar to a LPS-induced pathological response in the retina. Our results, thus, suggest a careful assessment of candidate nanoparticles of any material to be used in neural systems, for therapeutic or other purposes.

Key words: Microglia, nanoparticles, retina, in vitro

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Contents

List of Papers & Manuscripts

1. Protein corona formation around gold- and silver nanoparticles.
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2. Inflamed *in vitro* retina: Cytotoxic neuroinflammation and galectin-3 expression.
   Bauer P.M, Zalis M.C, Abdshill H, Deierborg T, Johansson F, Englund-Johansson U.
   PLoS ONE. 2016;11(9):e0161723. doi:10.1371/journal.pone.0161723.

3. Silver and gold nanoparticles exposure to *in vitro* cultured retina-studies on nanoparticle internalization, apoptosis, oxidative stress, glial- and microglial activity.
   Söderstjerna E, **Bauer P.M,** Cedervall T, Abdshill H, Johansson F, Englund-Johansson U.
   PLoS ONE. 2014;9(8):e105359. doi:10.1371/journal.pone.0105359.

Articles not included in the thesis

Biocompatibility of a polymer based on Off-Stoichiometry Thiol-Enes + Epoxy (OSTE+) for neural implants.Ejserholm F, Stegmayr J, **Bauer P.M,** Fredrik Johansson, LarsWallman, Martin Bengtsson, and Stina Oredsson.

Biomaterials Research. 2015;19:19. doi:10.1186/s40824-015-0041-3.

Abbreviations

ALS	amyotrophic lateral sclerosis
AMD	age-related macular degeneration
BBB	blood brain barrier
BRB	blood retina barrier
CNS	central nervous system
DCS	differential centrifugal sedimentation
DIV	days *in vitro*
DLS	differential light scattering
FCS	focal calve serum
GCL	ganglion cell layer
GFAP	glial fibrillary acidic protein
HMW	high molecular weight
IFN	interferon
IL	interleukin
INL	inner nuclear layer
IPL	inner plexiform layer
KC/GRO	keratinocyte chemoattractant/growth related oncogene
LMW	low molecular weight
LPS	lipopolysaccharide
MS	multiple sclerosis
NeuN	neuron-specific nuclei
NP	nanoparticle
ON	optic nerve
ONL	outer nuclear layer
OPL	outer plexiform layer
PBST	phosphate buffered saline with tween
PD	Parkinson's disease
PEG	poly ethylene glycol
SEM	standard error of the mean
TEM	transmission electron microscope
TNF	tumor necrosis factor

Abstract

Over the past decade, a massive increase in the use of nanomaterials and nanoparticles (NPs) in both commercial and medical applications has occurred. Medical applications include advanced drug delivery vehicles, imaging and hyper thermic therapies. In retinal research, several nanomaterials have been explored in novel treatment approaches, ranging from metals, carbon, polymers and silica to biological materials such as lipids or lactic acid. NPs, especially, gain much attention as novel drug delivery vehicles due to their ability to cross the barriers of the eye including the cornea, conjunctiva and the blood-retinal barrier (BRB).

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Our results, thus, suggest a careful assessment of candidate nanoparticles of any material to be used in neural systems, for therapeutic or other purposes.

Popular Summary

The antimicrobial properties of silver have been known for many centuries. The history of silver and it's beneficial effect reaches all the way back to at least 4000 B.C.E with the Caldeans as the third metal known to be used by the ancients, after gold and copper. Persian kings preferred drinking water only out of silver cups because of their ability to preserve fresh water for years. Silver was especially important for society in events such as military conflicts, where fresh water was not available but was also used empirically for numerous medical conditions, long before the realization that microbes were the agents of infection.

Once the antibiotics were discovered and implemented around World War II, the use of silver as a bactericidal agent decreased. However, shortly after the discovery of antibiotics it also emerged antibiotic resistant strains of bacteria for example CA-MRSA and HA-MRSA. Today, it is well known that the antibiotic resistance for bacteria is increasing in an alarming rate and there are a numerous additional resistant strains that could be found. Obviously, this made silver regain the attention in the scientific community.

With the recent advances in science in combination with invention of electron microscope, a new world was revealed, the nano world. The relatively incomprehensive definition of nano sized materials "a billion part of a meter" is neither easily comparable nor understandable. An attempt to reflect over the extremely small size of a nanoparticle is to compare a football to earth, where the football is a nanoparticle and earth is the football! Nanotechnology opens up many new and promising abilities within the field of medicine. First of all, their small size gives them the ability to cross barriers and move unhindered throughout the body. It also gives them far greater surface area to volume ratio which favors very high drug loading capacity. Bioavailability, delivering molecules to where they are most needed. Usually chemotherapy is quite invasive and causes a lot of negative and unwanted health effects due to its low precision. An excellent example of this is cancer drugs that bind to tumor sites, where these drugs are extremely cytotoxic and needs very accurate precision with the drug delivery system. Nanoparticles have the potential to give massively increased precision but also gain reduced drug interactivity, meaning less drugs interacting with each other but also potentially less interaction with other drugs taken simultaneously.

The eye is a part of the sensitive central nervous system. The retina, which is located in the back of the eye, is a complex organized structure and contains sensory neurons that are highly responsible for our visual sight. These neurons are very vulnerable for damage and any disturbance can cause visual loss. The ocular research is spending enormous resources to investigate the use of nanomaterials for therapeutic applications, including nanoparticles.

This thesis, focus on two nanoparticles that has gained massive attention in the scientific community. First, we selected silver nanoparticles which are the most common nanomaterial in consumer products. Their simple synthesis and highly antibacterial activity makes them widely used in medical applications. Secondly, gold nanoparticles, they are particularly interesting due to their ability to heat up when they absorb energy from infrared light. This is used in several diagnostic therapeutics such as chemotherapy when attaching antibodies to the surface of the gold nanoparticle and then heat them up after they reached the specific tumor site to destroy the tumor cells, or also potentially to destroy visceral far, a lot more safely than surgery. The advantages are many; however there is a need to investigate for eventual negative effects from this new technology to be able to manufacture safe pharmaceuticals within the field on medicine. The literature is yet quite sparse on assessment of adverse effects of nanoparticles.

In this thesis we characterize and explore the fate of the nanoparticles when entering a physiological environment (Paper 1). A layer with protein is formed around the nanoparticles. We also observe that the particles could translocate anywhere in the retinal tissue and could also be found in many cellular organelles. In Paper 2 we investigate how the immune system responds to a common bacteriological infection with especially focus on the behavior of the microglia cells which are the main immune response cells in the central nervous system, including the retina. We observe that the immune cells initiate an “inflammatory response” where they increase in numbers and also increase in activity. Finally, we expose the retina to gold and silver nanoparticles and observe an elevated immune system activity (Paper 3) and a significant neurotoxic effect.

Taken together, we found that gold and silver nanoparticles cause detrimental negative effects on the retina, and our results therefore strongly suggests careful assessment of novel nanomaterial that are aimed for use in the eye or the retina.

Populärvetenskaplig Sammanfattning

Att silver har en bakteriedödande effekt har varit känt sedan många århundraden. Historien om silver sträcker sig ända tillbaka till 4000 år före Kristus då de antika Kaldeérna använde silver och det var på den tiden en av mest använda metallerna tillsammans med guld och koppar. Persiska kungar föredrog att dricka vatten endast från bägare gjorda av silver för att vattnet kunde bevaras friskt och rent i flera år. Silver var viktigt för samhället, speciellt i krig och konflikter då friskt vatten inte fanns tillgängligt. Man använde även silver i flera medicinska tillstånd, mest på måfå och långt innan man hade en aning om att det faktiskt var mikrober som orsakade infektioner.

När sedan antibiotikan uppfanns och började användas omkring andra världskriget så minskades användningen av silver som ett bakteriedödande ämne. Men, snabbt efter upptäckten av antibiotikan så började man hitta resistensta bakteriestammar som t.ex. CA-MRSA och HA-MRSA. Idag är det välkänt att resistensen hos bakterierna ökar i en okontrollerad takt och det finns en mängd kända resistenta stammar. Detta gjorde emellertid att silver fick uppmärksamheten tillbaka i den vetenskapliga världen.

Med nya vetenskapliga framgångar och upptäckten av elektronmikroskop så öppnade sig en ny värld, ”nanovärlden”. Den lite smått obegripliga definitionen ”en miljarddels meter” är ofta svår att greppa, men man kan föreställa sig att en nanopartikel är stor som en fotboll och jämföra den med jordklotet där själva jordklotet är fotbollen! Nanoteknologin öppnar upp många nya möjligheter inom medicin och läkemedel. Först och främst så kan nanopartiklar ta sig igenom alla barriärer och röra sig obehindrat inne i kroppen. De har också en mycket större yta per volym i förhållande till större partiklar vilket leder till hög biotillgänglighet med hög precision, det vill säga förmågan att leverera läkemedel där det behövs som mest. Detta är ett vanligt problem inom cellgiftsbehandling där den låga precisionen ofta leder till negativa och oönskade bieffekter. Ett bra exempel på detta är cancerläkemedel som binder in till tumörer. Dessa läkemedel är vanligtvis extremt giftiga och då är det extra viktigt med hög precision. Nanopartiklar har potentiellt större precision men även mindre interaktion med själva läkemedlet i sig vilket leder till mindre kontakt med andra läkemedel som tas simultant.

The eye is a part of the sensitive central nervous system. The retina, which is located in the back of the eye, is a complex organized structure and contains sensory neurons that are highly responsible for our visual sight. These neurons are very vulnerable for damage and any disturbance can cause visual loss. The ocular research is spending enormous resources to investigate the use of nanomaterials for therapeutic applications, including nanoparticles.

Ögat är en del av det känsliga centrala nervsystemet. Näthinnan, som finns längst bak i ögat är en komplext organiserad struktur och som innehåller sensoriska nerver som är väldigt viktiga för vår synförmåga. Dessa nerver är mycket känsliga för skada eller annan yttre påverkan och detta kan leda till förlorad syn. Den okulära forskningen spenderar enorma resurser för att ta reda på huruvida nano material kan tillämpas inom ögon terapi och detta inkluderar även nanopartiklar.

I denna studie fokuserar vi på två av de vanligaste metalliska nanopartiklarna som används inom medicinsk forskning, silver och guld. Silver, som också är det vanligaste materialet i kommersiella produkter är även relativt enkelt att tillverka samt har en välkänd antibakteriell förmåga. Guld, har en förmåga att hettas upp av energin från infrarött ljus, vilket kan används inom diagnostik eller som terapi t.ex. kemoterapi när guldnanopartiklar används för att nå tumören och sedan hettas upp för att förstöra tumörcellerna vilket är mycket enklare än dagens kirurgi. Fördelarna med den nya nanoteknologin är många, men det innebär även att man måste titta på eventuella bieffekter för att kunna tillverka säkra läkemedel inom medicinsk forskning.

Ögat och dess näthinna är en väldigt känslig del av kroppen…

Vi karakteriserar och undersöker vad som händer med nanopartiklarna när de exponeras för en biologisk omgivning (Artikel 1). Runt partiklarna bildas det ett lager med protein, så kallad protein corona, från den biologiska omgivningen. Vi fann även att nanopartiklarna kan transporteras genom näthinnevävnaden och kan hittas i alla olika delar i cellerna. I en annan studie så undersöker vi hur immunförsvaret reagerar på en artificiell infektion med fokus på mikroglia cellerna som är de vanligaste immuncellerna i centrala nervsystemet (artikel 2). Immuncellerna visar en tydlig reaktion genom ett ”inflammationssvar” i form av att både föröka sig och bli mer aktiva. Till sist så tillsätter vi nanopartiklar till samma system och undersöker hur immunsystemet reagerar på dessa (artikel 3). Här ser vi en lite starkare reaktion från immunförsvaret och speciellt då vi tillsätter silverjoner som till och med förstör vävnaden i näthinnan. Vi fann även att exponering av silver och guldpartiklar leder till nervcellsdöd i näthinnan.

Sammantaget visar resultaten i denna avhandling att silver- och guldnanopartiklar är väldigt skadliga för näthinnan och detta bevisas genom den ökade aktiviteten i immunförsvaret samt att nervceller dör. Med dessa resultat som grund kan man uppmana till försiktighet, och grundliga undersökningar innan man börjar använda nanopartiklar i produkter som ska appliceras på eller i ögat.

1.Introduction

1.1 Nanotechnology

Over the past two decades the many advantages of using nanomaterials in consumer and medical products have further induced the massive growth in the nanotechnology industry. Nanotechnology advancement has taken a widespread application in many everyday products [1]. As these industries continue to produce products with unique properties the need for evaluating the risks for both human and ecological hazards are increasing [2]. While a vast amount of the publications are focusing on the medical applications, there is a need to increase the knowledge to minimize the risks and hazards by using these nanomaterials. A comparison between the publications containing the word "nanomedicine" and "nanotoxicology" is shown in Figure 1.

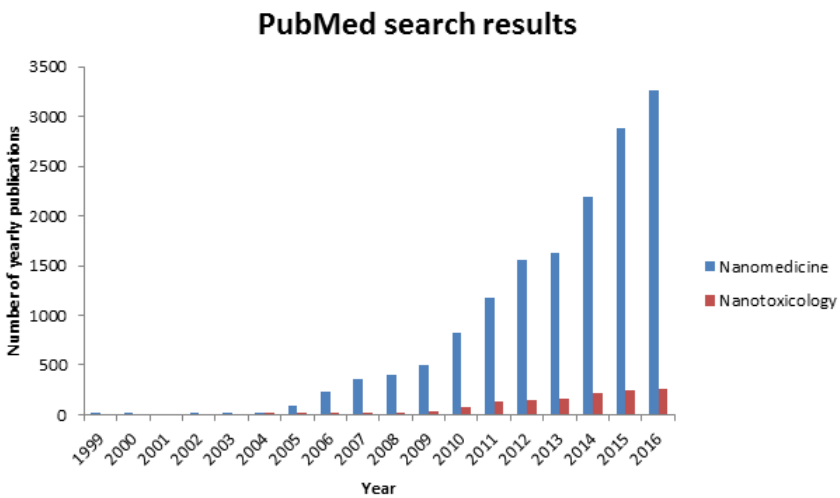

Figure 1: Timeline of PubMed entries
Timeline of PubMed entries matching the search criteria "nanomedicine" and "nanotoxicology". Querying PubMed with the search criteria "nanomedicine" reveals an exploding number of publications over the last ten years. However the seach criteria "nanotoxicology" reveiles that the information about the toxicology remains sparse.

Most publications regarding nanoparticles (NPs) effects to tissues and organs focus mainly on the pulmonary system while other uptake systems are less studied. The complex network of neuronal cells in the retina of the eye in combination with the accessibility to the outer environment makes it a vulnerable system and a potential target of neuronal toxicity, a risk factor of visual loss [3]. Small metal NPs such as gold and silver with the size under 20 nm; gain much of attention in the ophthalmology community due to their potential to cross the barriers of the eye including the cornea, conjunctiva and blood-retinal barriers [4-7]. For medical applications a great range of different NP has been explored including liposomes, different kinds of polymer-based NPs, AgNPs and AuNPs. There is a comprehensive amount of data available on formulation, characterization, targeted, and ocular drug delivery of these nanomaterials [8-11]. However, the information about the safety and toxicity of these system and nanomaterials is sparse. Safety and toxicity are important issues for future approval of ophthalmic products for clinical trials.

Definition of nanomaterials

Nanomaterials are typically defined as "*having internal or surface structures with one or more dimensions in the size range of 1-100 nm*" [12, 13]. Because of their small size they gain different characteristics compared to their bulk material, for example a high surface-area-to-volume ratio, high solubility, high stability in colloidal systems and high drug loading capacity [14]. For example, 1kg of particles of 1 mm^3 has the same surface area as 1 mg of particles of 1 nm^3, which is illustrated in Figure 2. For many medical applications such as drug delivery system these properties are crucial for accuracy and effectiveness since the chemical bonding interactions are occurring at the surface of the particles. Also, a nanometer is one billionth of a meter (10^{-9}) and to understand this extremely small size of a nanoparticle an illustration is shown in figure 3. The small size gives the ability of relatively free transportation inside the body, allowing drug carriers and medicine reaching their target more effectively [9].

Figure 2: Ilustration of the high surface area
Image to illustrate that 1 kg of particles of 1 mm^3 has the same surface area as 1 mg of particles of 1 nm^3. Hence, the particles in the small spoon have the same surface area as the particles in the large beholder.

Figure 3: Ilustration of the minimal size of a nanoparticle
Image illustrating that a nanoparticle is the size of a football as a football is to the earth.

Protein corona

It is well known that the surface of nanomaterials is covered by various proteins which form a corona upon their entrance to a biological environment. Factors including shape, size, surface charge, hydrophilicity/hydrophobicity and functional groups attached to the surface play all an important role in the corona formation [15]. This protein corona dictates the cellular events and interaction with living matter. Upon entrance to a biological environment there is a competition between many different biological molecules to adsorb to the surface of the nanomaterials. In the initial stage, the most abundant proteins (low molecular weight, LMW) are adsorbed to the surface. However, over time these initial proteins will be replaced with larger proteins with higher affinity (high molecular weight, HMW). This is known as the “Vroman’s effect” which shown in Figure 4 using a surface plasma

resonance protein sensor [16]. The protein corona alters the size and the surface composition of the nanomaterial, giving it a new identity to the cells. This can affect the interaction between the cells and the NPs in many different ways [17, 18], which also may reduce the effectiveness in drug delivery systems [19]. Thus, characterization of the protein corona is of utmost importance for understanding how exposure to NPs affects the biological responses of cells and other organisms.

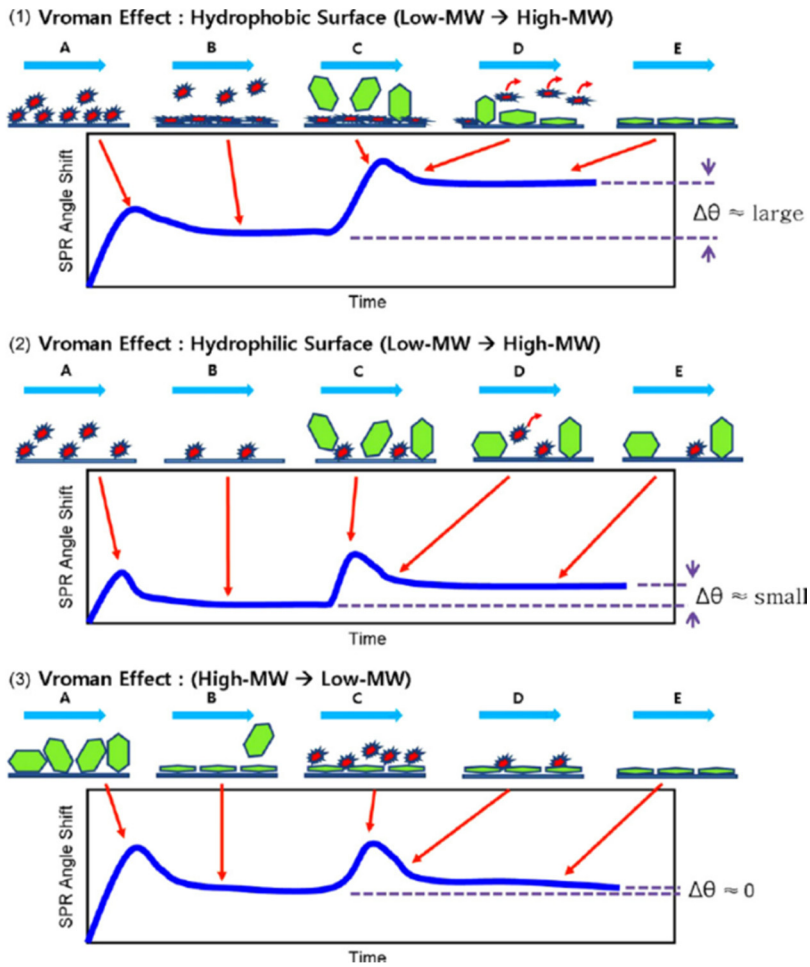

Figure 4: The Vroman effect
A schematic illustration of SPR profiles showing the Vroman effect (1) Initially, LMW proteins adsorb onto the hydrophobic surface and after a certain time these proteins are displaced by HMW proteins with higher affinity. (2) A hydrophilic surface when LMW proteins adsorb first and the HMW protein arrive later. (3) A hydrophilic surface when HMW proteins adsorb first and the LMW protein arrive later. Surface plasmon resonance protein sensor using Vroman effect (PDF Download Available). Available from: https://www.researchgate.net/publication/23233602_Surface_plasmon_resonance_protein_sensor_using_Vroman_effect

1.2 Silver nanoparticles

Silver nanoparticles (AgNPs) are currently the most widely commercialized nanomaterial and is the most commonly used material in consumer products and has been used in fabrics, socks, medicines or disinfectant sprays etc. Silver in various forms has long been recognized for antimicrobial properties. AgNPs have been used clinically for centuries due to its anti-microbial properties, making it a very efficient agent in e.g. wound treatment. In some cases (e.g. gene therapy) it may be desirable to increase the NP-cell interactions and let the NPs be taken up and internalized by cells, whereas for other applications (e.g. vascular imaging agents), it might be necessary to minimize the NP-cell interactions. NPs may cause more toxic effects than larger sized particles and has been reported to translocate within the environment and the body. NPs are also likely to cause different impacts on human health, occupation health and the environment depending on the size, shape and chemical composition of the nanoparticle as described in an increasing numbers of studies. Normally, foreign substances (including NPs) that enter the bloodstream are absorbed by specialized immune cells called phagocytes, which remove the foreign threat from the bloodstream. However, everything smaller than 200 nm is no longer specifically absorbed by these phagocytosing cells and can also be absorbed by other cell types. Furthermore, NPs that are smaller than 20 nm can cross the blood-brain-barrier (BBB) unhindered, meaning they can enter the central nervous system and may cause detrimental damage to the organ controlling all body functions.

Toxicity of silver nanoparticles and silver ions

The cytotoxicity and genotoxicity of silver NPs depends on many factors such as concentration, dispersion, size and surface functionalization. Exposure of the human body to silver NPs can occur through different routes (e.g. inhalation, ingestion, injection and physical contact with cuts or wounds. In the last decade there have been discussions about the mechanism by which AgNPs exert toxicity to living organisms. In this study we focus on the rather unusual uptake route, the eye and the retina. Nowadays, there is no doubt that the release of silver ions from the crystalline core of silver NPs contribute to the toxicity of these nanomaterials. Many toxicity studies that have used different organisms (e.g., bacteria, algae, fungi, zebra fish, human cells etc) have shown that the NPs were more toxic than equivalent concentrations of silver salts. However, ligands such as chloride, sulfide, phosphate, or organic acid in the exposure medium can bind to dissolved silver. This would reduce the toxicity of released silver ions to a greater extent than AgNPs. A recent study, using bacteria under strictly anaerobic conditions

(Figure 5) rules out the direct particle-specific effects and claims that the Ag^+ is the definitive molecular toxicant [20]. Since AgNPs themselves do not significantly exert direct particle-specific toxicity on bacteria, the AgNPs could be engineered with different surface coatings to get a desirable Ag^+ release at a specific target location. Further, they also show that PEG and PVP coating (two very common types of coating) does not protect the particle from the release of Ag^+ and AgNP accurately follows dose-response pattern of E. coli exposed to Ag^+ under these conditions [20]. Silver NPs are oxidized in an aqueous solution exposed to air (equation 1). This oxidation, results in the release of silver ions under acidic conditions (equation 2) [20].

$$4\ Ag\ (0) + O_2 \rightarrow 2\ Ag_2O \qquad (1)$$

$$2\ Ag_2O + 4\ H^+ \rightarrow 4\ Ag^+ + 2\ H_2O \qquad (2)$$

Silver NPs can penetrate into cellular compartments such as endosomes, lysosomes and mitochondria [20]. A possible mechanism of toxicity is proposed which involves disruption of the mitochondrial respiratory chain by Ag-NP leading to production of ROS and interruption of ATP synthesis, which in turn cause DNA damage. This DNA damage caused by AgNPs seems irreversible in contrast to other nanomaterials [21]. It is anticipated that DNA damage is augmented by deposition, followed by interactions of AgNP to the DNA leading to cell cycle arrest in the G_2/M phase [22]. This study also claims that smaller NPs (5-20 nm) enter cells more easy and causes more toxicity. Another important aspect to consider is the shape, which does have an effect on antibacterial activity of NPs. The order of the most antibacterial to least compounds is triangular, spherical, rod-shaped and $AgNO_3$. This is because triangular NPs have more active facets (electron dense facets) than the spherical NPs. However, spherical NPs, which usually aren't perfectly spherical, have more active facets then the rod-shaped NPs [23]. Silver ions are released from AgNPs when oxidized and can penetrate the cell wall but also bind to extra cellular ligands to prevent them from reaching their target. This is shown in figure 5. The silver toxicity on *E.coli* is well-studied and the results may be extrapolated to mammalian cells due to its similarity in the respiratory chain. AgNP contact with cell culture medium or proteins in the cytoplasm liberates Ag^+ ions [24]. Reactions between H_2O_2 and AgNP are considered to be one of the main factors causing the release of Ag^+ ions, shown in the equation (3)

$$2\ Ag + H_2O_2 + 2\ H^+ \rightarrow 2\ Ag^+ + 2\ H_2O \qquad (3)$$

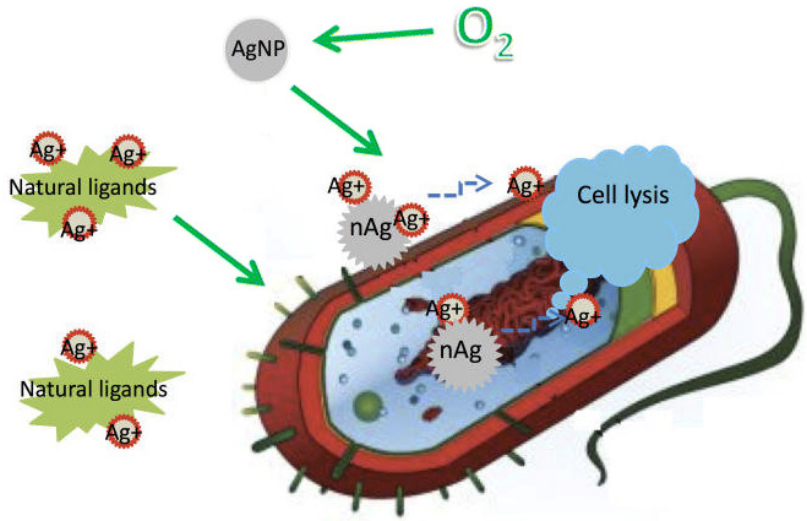

Figure 5: Mechanisms of silver ions toxicity to bacteria
Silver ions are released from AgNPs when oxidized and can penetrate the cell wall but also bind to extra cellular ligands to prevent them from reaching their target. Picture modified from Xiu et al., 2012 [20].

1.3 Gold nanoparticles

Gold NPs (AuNPs) are widely used in biomedical science including imaging, biosensors, diagnostics, drug delivery, thermal therapy, radiation enhancer [25], and immunochromatographic identification of pathogens [26] [27]. It has been argued that AuNPs could be used in almost all medical applications [28, 29]. Among all metallic NPs the AuNPs are considered to be the safest and much less toxic agents for drug delivery and hyperthermic agents for cancer treatment [26, 30]. They are also considered non-cytotoxic to the normal cells [31]. AuNPs are easy to synthesize and to conjugate with various functional groups. They are typically stabilized with surface coatings to enhance the electrostatic, steric or electrosteric repulsive force between the NPs to prevent aggregation or intend other surface functionality, usually with binding to thiol groups [27, 32]. Surface modification of the AuNPs and NPs in general has strong effect on the interaction with cells as it helps to convert toxic materials to non-toxic and vice versa [33]. Today, the most promising and used types of AuNPs is the thiolated derivates of PEG and other molecules which is considered the best stabilizing agents. Further, it's been shown that PEG-coated particles can remain in the blood flow for a longer time and are less susceptible to attacks from the cell components of the immune system [29, 31].

1.4 The eye

Ocular delivery of therapeutic NPs has the potential to improve the pharmacokinetics of traditional ophthalmic drugs. This includes slow release and localizing the drug where it is needed with high accuracy and precision. The eye is a complex and sensitive organ and consists of multiple tissue types which have very different structures. It is composed of three main barriers. The primary outer layer consists of the cornea and its surrounding sclera. The sclera is highly vascularized and is composed of connective tissue which includes a fibrous layer of collagen and elastic fibers. This provides mechanical stability to the eye. The cornea is a clear, transparent, avascular tissue consisting of several layers such as epithelium, stroma, Descemet's membrane, Bowman's layer and endothelium. Importantly, the latter cells are a monolayer of cells that regulate the fluid and transport through the cornea. These cells do not regenerate in contrast to the epithelium. Instead, they stretch to compensate for dead cells. Only about 5% or less of the topically administrated drugs is absorbed through the corneal epithelium [34]. Generally, the lipophilic molecules are transported transcellular, while the hydrophilic molecules and ions are transported by a paracellular route. The molecular cut-off for a paracellular route is considered to be below 400-500 kDa [35].

The middle layer of the eye includes the choroid, ciliary body, and iris. He choroid is a densely vascular layer between the retina and the sclera. The vitreous humor is a transparent, colorless, gelatinous mass that is located between the lens and the retina. It does not adhere to the retina, except at the optic nerve disc and the ora serrata (the end-point of the retina anteriorly). It also contains phagocytic hyalocytes that can remove nanomaterials and initiate an immune response. Hence, the half-life of drugs inside the vitreous cavity is relatively short. Usually, the ocular diseases affect many of these tissues at once, making the introduction of new therapeutic NPs and mechanisms of toxicity very challenging [4].

Figure 4 shows the pathways for administration of ocular drugs in the eye. Which all are ends up in the retina. However, only a fraction of the topically administered drug reaches the retina or the vitreous body following systemic administration [36]. A well-placed intravitreal injection is a convenient way of target NPs to the surface of the retina [4, 11].

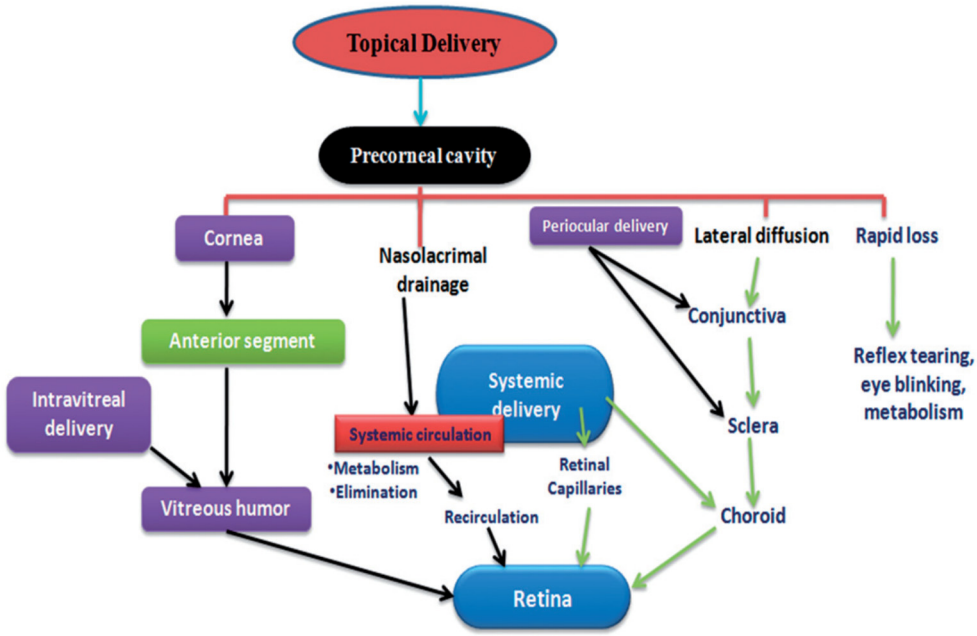

Figure 4: Pathways for administration of ocular drugs
A well-placed intravitreal injection is a convenient way of target NPs to the surface of the retina.

The retina

The most important part of the eye is no more than 0.5 mm thick and lines the back and inside of the eyeball. The tissue develops from the embryonic forebrain and therefore is considered a part of the brain and the central nervous system (CNS). It is consisting of 3 nuclear layers and 2 synaptic layers. The first nuclear layer, closest to the surface is called the *ganglion cell layer* (GCL) and contains 20 types of ganglion cells. Impulses from these cells travel to the brain via more than a million optic nerve fibers. The adjacent area is a synaptic layer known as *inner plexiform layer* (IPL), which is the area where the bipolar and amacrine cells connect to the ganglion cells. The very middle layer is called *inner nuclear layer* (INL) and contains horizontal cells, amacrine cells and bipolar cells. The horizontal and amacrine cells send signals using various excitatory and inhibitory molecules such as amino acids, catecholamines, peptides and nitric oxide. The next region of cells contains synapses linking the photoreceptors to the bipolar and horizontal cells and is known as the *outer plexiform layer* (OPL). The last but most important layer of the retina contains the photoreceptors and is located in the back of the eyeball. Hence, light must pass through the entire retina before reaching pigment molecules to excite. When exposed to light, ions channels in the cell

membranes close. The human eye have two different photoreceptors (Rods and Cones) that convert light to an electric signal and is transmitted through the bi-polar cells and to the ganglion cells that forms the optic nerve and further to the brain for visual processing and perception of the signal. Rods and Cones respond quite differently to light. Rods, detecting dim light and is usually responding to slow changes. Cones, dealing with bright signals and can detect rapid fluctuations. Each horizontal cell receives input from many cones and can either signal the bipolar cells or feed information back to the cones. This complicated circuit is still a debatable subject in the community of retina scientists. A schematic drawing of the eye and the retina is shown in Figure 5 [37].

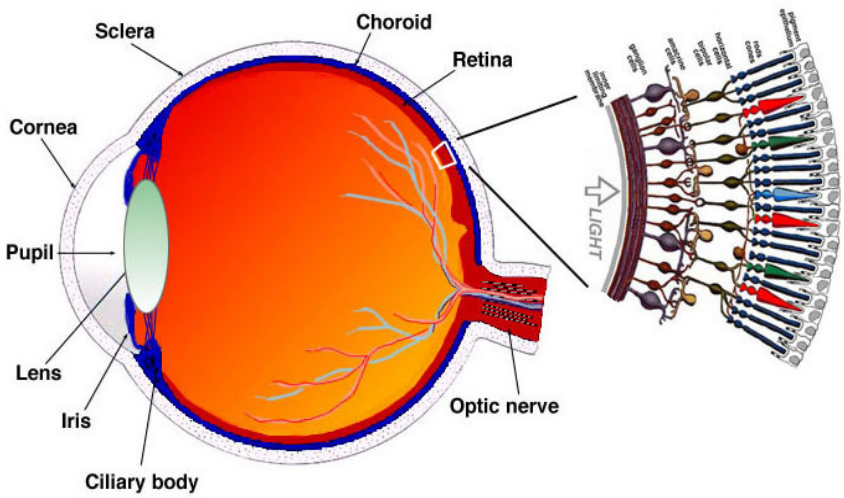

Figure 5: Illustrative picture of the eye and its retina
The retina is located in the back of the eyeball, highlighted in the picture. Picture taken from Kolb H - *The neural organization of the human retina*. In: Heckenlively JR, Arden GB, editors. Principles and practices of clinical electrophysiology of vision. St. Louis: Mosby Year Book Inc.; 1991. p. 25-52 [37].

1.5 Inflammation

Inflammation is a host defense response to injury, tissue ischemia, autoimmune responses or infectious agents. Inflammation within tissues outside the brain includes classical features such as swelling, redness, heat and often pain. Today a more mechanistic definition of inflammation has been established, including invasion of circulating immune cells (lymphocytes and macrophages). Many inflammatory mediators are produced locally and have proven to be involved in tissue inflammation and are therefore therapeutically key targets for a wide range

of diseases. Much of today's key evidence demonstrates that inflammation and inflammatory mediators contribute to acute, chronic and psychiatric CNS disorders. However, these inflammatory mediators may have dual roles with detrimental acute effects in the initial phase but beneficial effects in a long-term perspective [38].

Inflammation is tightly regulated in the body. Too weak inflammation could lead to tissue destruction by harmful stimulus (e.g. bacteria) and compromise the survival of the organism. In contrast, chronic inflammation may lead to a vast amount of diseases such as atherosclerosis, hay fever and even cancer. Acute inflammation can limit proliferation of invading pathogens. It includes production of acute phase proteins by the liver, activation of the sympathetic nervous system, changes in cardiovascular function, altered neuroendocrine status. In addition behavioral changes which lead to energy conservation such as increased sleep, lethargy, reduced appetite and the most common feature of infection, fever, which can reduce bacterial proliferation.

There is no doubt that the brain differs significantly from other tissues in its responses to a pathological threat. Leukocyte invasion may be delayed as a response to acute insults however activation of brain microglia and release of inflammatory mediators are rapid, occurring within minutes or hours [38]. Most importantly, there is now extensive evidence that inflammation within the CNS contributes to many acute and chronic degenerative disorders. Neurons, astrocytes, microglia and oligodendrocytes can produce inflammatory mediators, and cytokine receptors are expressed throughout the CNS. Proinflammatory cytokines and other mediators play an essential role in CNS inflammation. Thus, the CNS can be affected not only by inflammatory mediators produced within the brain, but also through the actions of mediators originating from the periphery. IL-1 is the most widely studied proinflammatory cytokine and it has been implicated in several neurodegenerative conditions and is generally believed to have neurotoxic actions, however the mechanisms behind these effects are unclear.

1.6 Microglia cells

Microglia cells represent the resident macrophages of the central nervous system and the microglia population is approximately 10-20% of all cells found within the brain. Today it is generally known that microglia cells are located in the mononuclear system in the parenchyma of the CNS, including the retina. The origin of microglia starts from the bone marrow and the hematopoietic stem cells that are the progenitors of all blood cells. Some of these stem cells differentiate into monocytes and migrate from the bone marrow via the blood stream and enter

the brain at this early development. While settled inside the CNS they further differentiate into microglia [39-41]. Further studies show indications that microglia originate in the yolk sac during a very restricted period [42]. Despite that all the immune cells in the body have the same origin, i.e. the myeloid progenitor cells, there are many differences compared to the microglia.

A series of endothelial cells, also known as the BBB, prevents circulating infectious agents from reaching the vulnerable nervous tissue. Deficiency or damaged BBB can enable circulating macrophages from other tissue to enter the CNS and cause severe autoimmune diseases [43, 44]. Other phagocytosing cells like macrophages and dendritic cells are constantly being depleted and replaced by myeloid progenitor cells which differentiate into the needed type. However, due to the BBB, the possibility to replenish microglia is sparse and there is a huge difference in the turnover rate compared to other immune cells. Therefore, in contrast to other tissue macrophages, microglia persists throughout the entire life of the organism due to their longevity and high capacity of self-renewal [45].

Because of the highly vulnerable neural tissue, the protection that the immune cells provide must be both rapid and efficient to prevent potentially fatal damage. Under neuronal homeostatic conditions, microglia is highly regulated and also restricted to occupy a defined area. In this state the microglia is characterized by a branched morphology with a small round cell body also known as “ramified” morphology. Resting microglia does not possess MHC class-I or MHC class-II receptors in their inactive form, nor expressing signaling molecules for inflammation or recruitment. Although this state of microglia is considered “resting” the microglia is very active in surveying the surrounding environment for any potential threat. The branches reaching out from the cell body are extremely sensitive to any physiological disturbance. A large part of an inactivated microglial cell’s role is maintaining homeostasis [46].

Infection, trauma, ischemia, neurodegenerative diseases or loss of brain homeostasis or any other danger to the CNS evokes rapid changes in the microglia behavior and is defined as “microglia activation”. Upon activation, microglia changes morphology (Figure 5) and undergo rapid proliferation to increase their numbers to provide improved defense against the invading germs. Activation of microglia includes anabolic and catabolic processes that causes reduced microglia lifetime [47, 48].

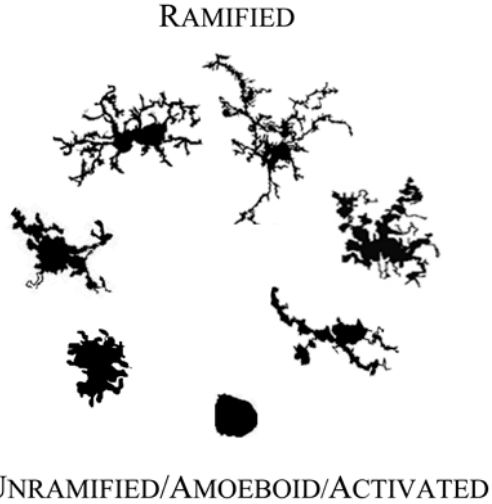

Figure 5 Morphologies of microglia cells, from resting state "ramified" to activated state "amoeboid" and reverse.
The picture shows round and amoeboid classification of microglia cells corresponding to an active and mobile cell while the branched morphology with a small cellular core is classified as "resting" stationary cell. Picture taken from *quantitating the subtleties of microglial morphology with fractal* analysis [Karperien 2013] [49].

However, the terms "activated" and "inactive" could be misleading. They indicating that ramified microglia are always inactive and round shaped are always considered active which is not entirely correct. Hence, there could be different levels of activation. At maximum activation, microglia takes on a large amoeboid shape and enables free movement throughout the neural tissue. In addition, they also have antigen presenting, cytotoxic and inflammatory mediating signals, displays the resulting immunomolecules for T-cell activation and phagocytosis of cellular debris. During inflammation, T-cells are able to cross the BBB with special surface markers and directly bind to microglia to receive antigens. Activated phagocytic microglia also interacts with astrocytes and neural cells to minimize the damage to the healthy cells in the CNS. This is accomplished through an extremely complicated series of extracellular signaling molecules that allows communication with other microglia, astrocytes, nerves, T-cells and myeloid progenitor cells. For example, cytokine IFN-γ could be used to activate microglial cells and once activated, the microglia releases more IFN-γ into the extracellular space rapidly activating even more microglia in cytokine cascade activation. TNF-α produced by microglia is generally known to increase inflammation and causes neural tissue to undergo apoptosis. However, several reports claim that TNF-α could also have neuroprotective abilities [50, 51]. IL-8 recruits B-cells, T-cells and dendritic cells to aid in fighting the infection. IL-1 inhibits IL-10 and TGF-β, which downregulate antigen presentation and pro-inflammatory signaling.

The main role of microglia is phagocytosis, which includes engulfing materials consisting of cellular debris, lipids, bacteria, virus and any cells or foreign material in the inflamed state. The process of phagocytosis is often via direct cell to cell contact to infectious organisms but also release of large amounts of cytotoxic substances. Hydrogen peroxide and nitric oxide causes directly cell damage and will lead to neuronal cell death. Proteases catabolize specific proteins causes direct cell damage. Cytokines (IL-1) promote demyelination of neuronal axons. Glutamate, aspartate and quinolinic acid injure neurons through NMDA receptor-mediated processes. The cytotoxic secretion from the microglia is aimed towards infected neurons, virus and bacteria but can cause vast amounts of collateral damage to adjacent neurons. Hence, many comprehensive articles [52, 53] show information that the microglia activation leading to chronic inflammation is indeed considered "ravaging" and could have detrimental effects on the retina.

Eventually, after engulfing a certain amount of debris, the microglia becomes unable to phagocytose any further materials. Post-inflammation microglial cells undergo several steps to promote regrowth of neural tissue. This includes "synaptic stripping" [42, 52], secretion of anti-inflammatory cytokines, recruitment of neurons and astrocytes to the damaged area. The eventual successful result of a microglia cells phagocytosis is known as a "Gitter cell", named after its grainy appearance. These cells are considered relative non-active as visualized by post-infection areas that have healed [52, 54, 55]. The post-activated microglia may remain undistinguishable by morphology from the "resting cells" in nearby populations while still having acquired long-lasting adjustments. Also, the experienced microglia cells may behave differently when being challenged again [40]. Lately, a vast amount of experimental evidence suggests a link between chronically activated microglia and various neurodegenerative diseases. Many outstanding review articles provide comprehensive information about the complex microglia physiology and pathology in neurodegenerative diseases such as Alzheimer's disease [56, 57], Parkinson's disease [58, 59], multiple sclerosis [60], amyotrophic lateral sclerosis [61], stroke [62], neurotrauma [63], prion disease [64] and radiation-induced brain injury [65].

Galactin-3

Galectin-3 is a relative newly discovered protein which is involved in the signaling of the inflammation process. It can be secreted by microglial cells upon inflammatory events and can often be found in the cytoplasm, mitochondria and near the cell walls. Galectin-3 is only expressed in activated microglial cells and could have value in early disease diagnosis, where microglial response may alter disease progression.

2 Objectives

The overall aim of this thesis is to study how metallic NPs affect the retina, with special emphasis on the immune response.

The specific aims are:

- To investigate the protein corona formation, that forms around the NPs when introduced to a biological system (Paper 1).
- To investigate how lipopolysaccharide (LPS) affects the retina (Paper 2).
- To investigate how Ag- and AuNPs affect the retina (Paper 3).

3 Material and Methods

3.1 Particle characterization

The diameters of the respective particles were measured using four different methods, i.e. manually from TEM images, DLS, DCS, and by absorption spectra (Paper 1 & 3). The particle sizes were measured from transmission electron microscopy images and presented as mean +/- standard deviation (n=50). The AuNPs, diluted 10 times in ultrapure water, and AgNPs were characterized in water. The particle size was determined in triplicates by Dynamic Light Scattering (DLS) using a Dynapro Plate Reader II (Wyatt Technology, USA) and in duplicates by Differential Centrifugal Sedimentation (DCS) in a 24% to 8% gradient using a DC-24000 Disc Centrifuge (CPS Instrument Inc., USA). The reported hydrodynamic diameter by DLS is from cumulates analysis and the reported diameter from DCS is the peak value from the absorbance size distribution. The absorption spectra for the particles were recorded using a UV-800 spectrophotometer (Shimadzu, Japan). Prior to dilutions of the NPs the NP stock solutions were vortexed.

3.2 Protein corona analysis

For analysis of the protein corona (Paper 1), several different techniques were used. The particle/media sample was kept tightly sealed at 37^{o} C between all time-points. Analysis of the protein corona was made after 30 min, 1 hour, 6 hours and 24 hours. A complete list of different media ingredients is found in appendix 2.

Particle preparation

Commercially available citrate stabilized colloidal Ag- and AuNPs of both 20 and 80 nm in diameter, respectively, in water were purchased from BBI International (Cardiff, UK).

Differential centrifugal sedimentation

Differential centrifugal sedimentation measurements were performed at each time point in a disc centrifuge (CPS Instruments, Inc. Model DC240000 UHR). 100 µL of the incubated sample was injected into 24-8% sucrose gradient at 24,000 and 23,094 RPM for Ag- and AuNPs, respectively. Stämmer det?

Absorbance spectra

Absorbance spectra between 270 and 600 nm (Shimadzu UV-1800) was used to evaluate aggregation and changes in refractive index on the surface of the NPs prior to corona experiment.

Dynamic light scattering

Hydrodynamic radius of the NP-protein complex was measured by DLS (Wyatt Technologies DynaPro Platereader-II) in duplicates at 37°C, with acquisition times between 1 and 10 seconds in 96-well plates (Corning, Costar® Assay Plate) sealed with Corning plate sealer.

Chemometry

A chemometry analysis was performed including all the media components to evaluate the differences between the media. Coefficient of Variation (CoV) values above 1.4 where considered of interest.

3.3 Electrophoreses

SDS-PAGE

The gold media mixture with the different protein sources were loaded on top of a 500 uL 20% sucrose gel. Then the mixture was centrifuged at 18000 RPM for 20 minutes. The supernatant on top was removed and the pellet from the bottom was removed and washed with 500 uL of water. The washed pellet was then centrifuged for 20 minutes. The supernatant was removed and the remaining pellet was mixed with SDS-PAGE sample buffer and then added to the corresponding wells in a electrophorese plate.

3.4 Animals

In-house bred C3H/HeA wild-type mice were used for the study. Animals were kept under conditions with standard white 12 hours cycling lightning, free access to food and water and were used irrespectively of gender. Mouse retinal tissues were taken from postnatal day 7 (PN7). Animal handling was performed in accordance with approved guidelines of the Ethics Committee of Lund University, the Institute for Laboratory Animal Research (Guide for the Care and Use of Laboratory Animals, Malmö-Lund Ethical Committee in Sweden), and the ARVO statement for the use of animals in ophthalmic and vision research.

3.5 *In vitro* retina culturing

Animals were sacrificed by an overdose of CO_2. The eyes were enucleated; thereafter the anterior segment, the vitreous body and the sclera were removed. The neural retina with pigmented epithelium was explanted onto a Millicell-PCF 0.4 µm culture plate inserts (Millipore) with the vitreal side oriented upwards. The retinal explants were cultured in serum-free conditions in R16 culture medium (Invitrogen, Paisley, UK; 07490743A). Explants were allowed to adjust to culture conditions for two days *in vitro*, before receiving fresh R16 medium and to selected groups addition of NPs for 72 hours or LPS (100 ng/ml) for 24 hours. Conditioned media was collected from LPS-exposed retinas and corresponding controls at, 2, 3, 4 and 7 days *in vitro* (DIV). Four independent culture experiments were performed on different days, rendering in total n= 5-8 explants / group.

3.6 Tissue handling

For histological staining the retinas were fixed in 4% paraformaldehyde and then embedded in Yazulla medium (30% egg albumin and 3% gelatin in distilled water). Sections of 12-16 µm were cut with a cryostat, mounted onto chrome-alum coated glass slides and stored at -20^{o} C until further processing.

For transmission electron microscopy TEM the retinas were fixed in 2.5% glutaraldehyde in 0.15 M Na-cacodylate buffer (pH 7.2) for 4 hours at 4^{o} C. After rinsing in 0.1 M Na-cacodylate buffer and dehydration, the retinas were post-fixed in 1% osmiumtetraoxide in 0.1 M Na-cacodylate buffer at 4^{o} C for 1 hour. After dehydration, the samples were embedded in Epon. An ultramicrotome (Leica

ultracut, Leica Microsystems GmbH, Germany) was used to cut ultrathin sections. The sections were stained with 2% uranyl acetate in Pb-citrate.

3.7 Lipopolysaccharide administration

One selected group received fresh R16 medium mixed with LPS (100 ng/ml, from salmonella enterica, Sigma-Aldrich Sweden) for 24 hours (Paper 2). Conditioned media was collected from LPS-exposed retinas and corresponding controls at 2, 3, 4 and 7 DIV days.

3.8 Nanoparticle administration

After 2 days of culturing (Paper 3), AuNPs, AgNPs and $AgNO_3$ were added with fresh R16 medium for 72 hours. Au- and AgNPs, respectively, of either 20 nm or 80 nm were added to the R16 medium to give the final concentrations; 0.0065 µg/ml 20 nm AuNPs, 0.4 µg/ml 80 nm AuNPs, 0.0035 µg/ml 20 nm AgNPs, 0.22 µg/ml 80 nm AgNPs.

Silver nitrate ($AgNO_3$) (VWR International Radnor, PA, USA) was dissolved in deionized water to give a stock solution of 1 mg/ml. The stock solution was sterile-filtered and stored at 4^o C until use. Further dilutions were made in fresh R16 Medium. $AgNO_3$ was added to the R16 medium to give final concentrations of 0.5, 1.0 and 5.0 µg/ml.

For analysis within protein corona (Paper 1) the particles were shaken/vortexed before mixing with different media (Table 3). The total concentration of each sample were; $3.5*10^{10}$ NPs per ml (20 nm particles) and $5.5*10^9$ NP per ml (80 nm particles).

Tabel 1: Protein sources

Tabel is showing the protein sources used for analysis in Paper 1. A complete list of chemicals included in the medias is shown in appendix.

Culture media	Note
Glutamax complete with 10% FCS	For culturing BV-2 microglia cells
RPMI-1640 complete with 10% FCS	For culturing primary macrophages
HNPC basic with mitogens	For expansion of HNPC cells
HNPC complete with 1% FCS	For differentation of HNPC cells
Porcine vitreous	*In vivo* intraocular studies in the mouse
R16 without serum	For *in vitro* retina culturing

3.9 Immunohistochemistry & analysis

Hematoxylin-eosin staining

For gross morphological analysis, every tenth section throughout all specimens was stained with hematoxylin-eosin (Htx-eosin). Sections were cover-slipped, using Pertex mounting media (Histolab, Sweden). Gross as well as detailed morphological analysis was performed using light microscopy (Nikon, Tokyo, Japan). Eight to ten sections per specimen representing the entire retina were included (n = 4-6 retinas/group). Evaluation of gross morphology was made with a ranking system divided into five different categories:

- Layering (0 = normal layering, 0.5 = minor deformation, 1 = major deformation)
- Fold formation (0 = no folds, 0.5 = few folds, 1 = many folds)
- Rosette formation (0 = no rosettes, 0.5 few rosettes, 1 = many rosettes)
- Nuclear layer tissue architecture (0 = normal, 0.5 = small and few disseminated regions, 1 = large and many disseminated regions)
- Pyknotic nuclei (0 = <10, 0.5 10-50, 1 = >50)

Fluorescent immunostaining

For immunostaining 6-8 sections per specimen (together with the whole specimen) were rinsed and then pre-incubated in phosphate buffered saline containing 0.1% Triton X-100 (PBST), 1% bovine serum albumin (BSA), and 5% normal donkey serum (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) for 1 h at room temperature. Sections were then incubated with primary antibodies, rabbit anti-glial fibrillary acidic protein (GFAP, 1:1500 DAKO Cytomation, Glostrup Denmark), rabbit anti-Iba1 (1:200, WAKO, Japan) and rat anti-mouse ED1 (CD68, 1:1000, Nordic Biosite, Sweden), 1:1000 overnight at 4ºC, and thereafter incubation on secondary antibodies for 2 h. Secondary antibodies included were Texas Red-conjugated donkey anti-rabbit antibody (1:200; Abcam, Cambridge, UK), Alexa 488 goat anti-rabbit IgG (Molecular Probes) and Alexa 564 goat anti-rat (Molecular Probes). Both primary and secondary antibodies were diluted in PBST containing 1% BSA. For counterstaining of nuclei, the sections were cover-slipped using 4'6-diamidino-2-2phenylindole (DAPI)-containing Vectashield mouting medium (Vector Laboratories, Burlingame, CA, USA).

TUNEL staining for apoptotic cells

Eight sections per specimen (together representing the whole specimen) were stained with a fluorescein-conjugated terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling (TUNEL) assay according to the manufacturer 8Roche, Mannheim, Germany). For counterstaining of nuclei, the sections were cover-slipped using DAPI-containing Vectashield mounting medium.

AvidinD staining for oxidative stress

Six sections per specimen (together representing the whole specimen) were stained with AvidinD (1:400, Texas Red-conjugated avidin; Molecular Probes Inc., Eugene, OR, USA; A-820). AvidinD was diluted in PBST containing 1% BSA and sections were incubated for 45 min at RT. For counterstaining of nuclei, the sections were cover-slipped using DAPI-containing Vectashield mounting medium. Initial analysis of the AvidinD staining revealed only positive stained cells in the ONL. Hence, quantification of AvidinD-positive cells was limited to this region of the retina (n = 4 per group).

Microglial activation

Microglial cells were double immune-labeled using the microglial-specific markers Iba1 and ED1 (n = 4 per group and sex sections per specimen). Total numbers of cells were quantified throughout the entire retina without subdividing it into specific nuclear layers. At first, the total number of microglial cells was enumerated by the expression of Iba1/ED1 or ED1. Secondly, the number of Iba1-positive cells (expressed in all stages of microglial activation) expressing the marker for activated microglia cells, i.e. ED1, was quantified. Thereafter the morphological change occurring in response to activation of microglia was used to assess the level of activation. The following morphological classification for activation stage was used:

Ramified (round cell body, long branched processes) = resting stage

Intermediate (elongated cell body, with short thick non branched processes) = mid-activated stage.

Round (round cell body, no processes) = active stage.

Amoeboid (irregular/ellipsoid cell body, no process) = active stage.

Glial activation

Analysis of changes of GFAP staining intensity and staining pattern was performed (n = 5-8 per group and eight sections per specimen). In addition, gross- and detailed structural morphologies of the GFAP-labeled cells were performed.

Tabel 2 List of primary antibodies used for immunohistrochemistry
Primary antibodies with information about the host species.

Antigen	Host	Source
Glial fibrillary acidic protein (GFAP)	Rabbit	WAKO, Tokyo, Japan
Iba1	Rabbit	WAKO, Tokyo, Japan
ED1	Mouse	Nordic Biosite, Täby, Sweden
Ki-67	Goat	Millipore, Temecula, CA, USA
NeuN	Mouse	Chemicon Int., Temecula, CA, USA
Galectin-3	Rat	WAKO, Tokyo, Japan

3.10 Microscopy and image analysis

Transmission Electron Microscopy

Detailed analysis was performed throughout the entire retina (from inner to outer regions). A minimum of 50 cells per nuclear layer was analyzed for investigation of intracellular localization of the respective NP. Sections were imaged using a JEOL JEM 1230 electron microscope (JEOL, Japan).

Fluorescence microscopy

Gross as well as detailed morphological analysis with counter-stained and immune-stained sections of every tenth section throughout the retinal explants (n=5-8/group) was performed using light-and fluorescent microscopy (Nikon Eclipse E800, Tokyo, Japan), equipped with appropriate filters. Images were captured with digital acquisition system (DCP Controller).

3.11 Enzyme-linked immunosorbent assay

Conditioned media was collected at 2, 3, 4 and 7 DIV (n = 3 samples / group). A electrochemiluminescence ELISA was performed using the V-PLEX Plus Proinflammatory Panel 1 (mouse) kit (Mesoscale discoveries, Rockville, Maryland

USA). The following immune mediators were analyzed (Table 3). A pre-coated plate was used with capture antibodies on independent and well-defined spots in a 10-spot MULTI-SPOT® plate. All CV-values (coefficient of variation) above 35 were closer investigated and the values beneath detection level or/and out of calibration range were removed.

Tabel 3 List of cytokines used in the ELISA analysis
List of cytokines used in the V-PLEX Plus Proinflammatory Panel 1 (mouse) kit with alternative common name.

Cytokine	Aliases
Mouse interferon gamma (IFN-γ)	
Mouse interleukin-1beta (IL-1β)	IL-1F2
Mouse interleukin-2 (IL-2)	T-cell growth factor (TCGF)
Mouse interleukin-4 (IL-4)	B-cell stimulatory factor 1 (BSF-1), Lymphocyte stimulatory factor 1
Mouse interleukin-5 (IL-5)	B-cell growth factor II (BCGF-II), T-cell replacing factor (TRF)
Mouse interleukin-6 (IL-6)	
Mouse KC/GRO	CXCL1, GROα, Neutrophil-activating protein 3 (NAP-3)
Mouse interleukin-10 (IL-10)	Cytokine synthesis inhibitory factor (CSIF)
Mouse interleukin-12p70 (IL-12p70)	
Mouse tumor necrosis factor alpha (TNF-α)	Tumor necrosis factor ligand superfamily member 2 (TNFSF2), Cachectin

3.12 Statistical analysis

All data are expressed as mean ± standard deviation unless stated otherwise with n signifying the number of used animals. Quantifications were performed using ImageJ or Photoshop Creative Cloud (Adobe systems, CA, USA) and all quantifications data was normalized to cells per mm^2. The area was measured using the DAPI staining, when applied. Statistical analysis was performed using SPSS 22 (IBM, NY, USA) software. Student's *t-test* was used when comparing 2 groups, or one- or two- way analysis of variance (ANOVA). A chi-test was used to analyze the ranking results in the gross morphological analysis. A chemometry analysis was performed including the culture media. Coefficient of variation (CoV) values above 1.4 was considered of interest. Correlation analysis was also performed where stated. Differences were considered statistically significant at $p<0.05$ and p-values are given as $*p<0.05$, $**p<0.01$ and $***p<0.001$.

4 Results

4.1 Nanoparticle characterization (Papers 1 & 3)

We characterized the particles (BBI, UK) with regard to size and material (Papers 1 & 3). The maxima obtained from the spectrophotometer showed very similar data compared with the data given from the manufacturer. The size distribution data from DCS and manually taken data from TEM pictures were also similar to the values provided by BBI, UK. However, TEM images with the larger particles (80 nm Ag and 80 nm Au) revealed a fraction of non-spherical particles. These abnormalities were included in the size measurement and influenced the standard deviation, i.e. larger than for the smaller particles. However, we conclude that the size and surface properties of the particles used in the study are in line with those described for similar particles.

4.2 Protein corona formation around nanoparticles (Paper 1)

Upon entrance to a biological environment (here culture media) the particle gains a protein corona. This corona can alter the size and surface composition and give the NP a new identity to the cells. Therefore, we wanted to investigate the relation between the culture media and the NPs. We chose four commonly used and available media from our laboratory. The result shows that a complete corona is formed rapidly within minutes and will reach equilibrium after approximately 24 hours of incubation. Increased concentration of protein in the media, results in a faster corona formation. R16 media, used in the retina explant model, was partially tested (data not shown), however, the results showed similar trend compared to the other serum free media. The complete list of media recipes can be found in the appendix of Paper 1.

4.3 Experimental design with organotypic retina (Papers 2 & 3)

We use post-natal mouse retina from day 7 in our study, as at this time point all retinal layers are developed [66]. As we wanted to do a detailed histological study, we utilized an organotypic culture model extensively used in our laboratory, which provides well-preserved laminar retinal architecture over a long period of time in culture. The model comprises whole mount retinal culture in defined, serum-free media [67]. Importantly, post-natal rodent retinas possess a microglial population that has spread throughout the entire retina (from the central part to the ora serrata) resembling that of an adult. These cells have formed quiescent, ramified morphology at the age used here as well as in other papers [68, 69].

Several end-points were studied; gross morphological organization, level of apoptosis and oxidative stress, glial- and microglial activity. These are common signs of insult to the retina.

All the control retinas displayed normal retinal layering with all the three nuclear layers intact as well as the synaptic layers, *i.e.* the inner- and outer plexiform layer (IPL, OPL), respectively and no obvious sign of toxic insult to the overall tissue or cells. Only occasional pyknotic cells could be found from 1 DIV up to 7 DIV.

At 0 DIV abundant number of apoptotic cells (TUNEL labeled cells) could be found in the INL but only occasionally detected in the GCL and ONL. This was followed by a consistent and relatively small loss up to 7 DIV.

The control retinas displayed macroglial activation very similar to the *in vivo* expression pattern of GFAP. At 3, 4 and 7 DIV, GFAP up-regulation was seen in Müller cell processes spanning over the entire retina.

4.4 Nanoparticle uptake (Paper 3)

In general, all NPs were taken up and found distributed in all neuronal layers of the retina, the ganglion cell layer (GCL), the inner nuclear layer (INL) and the outer nuclear layer (ONL). The small sized particles (20 nm of both Au and AgNPs) could be found in the nucleus, the nucleolus, the mitochondria and in the cytosol as well as in the extracellular space. The large sized particles (80 nm of both Au- and AgNPs) could also be found in the nucleus, but in lesser amount than compared to the smaller counter-parts. However the 80 nm NPs were not detected in the nucleolus and within the mitochondria. All particles could be found both as single particles and clustered.

4.5 Alterations in the retina after exposure of lipopolysaccharide (Paper 2)

Gross morphological change

Immediately after the exposure and removal of LPS (acute response) we observed a larger fraction of pyknotic nuclei especially in the inner nuclear layer (INL) and outer plexiform layer (OPL) for up to 7 DIV. We also observed disseminated nuclear layers mainly in INL, however only significantly difference compared to control up to 4 DIV.

Apoptotic cells

LPS-treated retinas at DIV 3 showed an increase in TUNEL-labeled cells in all nuclear layers. For cells in GCL and INL this increase disappeared at later time points. However, in the ONL the number of TUNEL-positive cells remained significantly higher at all time-points observed. In selected specimen from all LPS-treated groups an attempt of finding double-stained cells with ED1 and TUNEL was made, however it was not successful, indicating that the TUNEL-positive cells in the nuclear layers are most likely of a neuronal origin and not microglial.

Macroglial cell activation

The GFAP expression after LPS-treatment was only slightly intensified over all time-points compared to the control groups.

4.6 Alterations in the retina after exposure of gold and silver nanoparticles (Paper 3)

Several end-points were studied; gross morphological organization, glial activity, microglial activity, level of apoptosis and oxidative stress. These are common signs of insult to the retina.

Gross morphological change

Exposure to 20 and 80 nm Au- or AgNPs caused no disruption of the retinal layering e.g. fold- or rosette formation up to 7 DIV. However, large vacuoles could be found in the tissue, especially in the INL. This was accompanied by large fraction of pyknotic cells, especially in the same region (INL) and much more overall compared to the control retinas. The findings were in line with the findings from LPS treated retinas where pyknotic cells could be found mainly in ONL with overlapping cell-free areas. All concentrations of silver ions ($AgNO_3$, *i.e.* 0.5, 1.0 and 5.0 µg/ml) caused severe insult to the retinal tissue and cells.

Ag- and AuNPs and LPS induce apoptotsis

At PN7 in the control retinas apoptotic cells could be located in the INL but also occasionally in the GCL and ONL. In the GCL, an acute and massive loss of RGCs caused by axotomy, was found in the controls. Quantification using the marker NeuN for neurons (RGC and amacrine cells, revealed a loss of 50% at 2 DIV (data now shown). However, TUNEL-labeled cells in the GCL were studied and revealed no further loss up to 14 DIV (data not shown).

Macroglial cell activation

The control retinas displayed macroglial activation very similar to the *in vivo* expression pattern of GFAP. Between 3 and 7 DIV, GFAP up-regulation was seen in Muller cell processes spanning over the entire retina.

The GFAP expression after all NP administration showed a slightly higher up-regulation compared to the controls which was even further with the 80 nm particles, but no difference in level between the Au- and AgNP groups.

All $AgNO_3$-treated retinas, at all concentrations (0.5-5.0 µg/ml) caused severe insult to the retinal tissue and resulted in an intensive GFAP-staining throughout the entire retina.

Oxidative stress

AvidinD is commonly used in studies of retinal degeneration to identify oxidative damage in DNA. In all NP-treated groups, the vast majority of the AvidinD-positive cells were located in the ONL. Only scattered or no AvidinD-positive cells could be found in INL and GCL.

4.7 Microglia response and behavior (Papers 2 & 3)

In a healthy retina the microglial cells are located primarily in the IPL and OPL. First we investigated the microglia response induced by the cultivation process. At 0 DIV, which corresponds to the *in vivo* situation at PN7, microglia could occasionally be found in the GCL and after DIV 3, microglia could also be located inside INL. The total number of microglia was significantly increased at all time points after seeding. At 0 DIV no gal-3 expression could be found and only about 50% of the microglia cells expressed ED1 and were primarily found in the INL and GCL. At 0 DIV, the majority of the cells displayed a ramified inactive cell profile and about 35% demonstrated round or amoeboid. At 3, 4 and 7 DIV the majority of the cells were amoeboid and over time the ramified cells decreased. The amoeboid cells were only found within the GCL. For the cytokine profile, only at 7 DIV, very low levels of TNF-α, IL-2, IL-6 and KC/GRO could be found.

Exposure to LPS did only slightly increase the total numbers of microglia cells compared to the control for 3 and 4 DIV. At 7 DIV the difference was larger but still not significantly. The round morphology was strongly significantly higher compared to the control group at all time-points after LPS administration. These cells could be located in the IPL, INL and OPL. Galectin-3 expressed by microglia cells upon inflammatory event, was significantly higher at 4 and 4 DIV but diminished at 7 DIV. The vast majority of the Galectin-3 expressing cell bodies and processes co-localized with Iba-1 staining and could mainly be found inside the GCL. For the cytokine release profile, IL-2, IL-6, KC/GRO and TNF-α were significantly increased at all time-points after LPS administration.

Exposure to all types of NPs increased the total number of microglia cells. AgNPs sized 20 nm and 80 nm gave a 15% and a 12% increase, respectively. AuNPs sized 20 nm and 80 nm gave a 35% and a significant 53% increase, respectively. All Iba1 positive cells also expressed ED1, hence the attempt to assess the microglial activity based on the expression of ED1 was not successful. The round and amoeboid morphological cell profile, here described as an activated microglia cells, was increased by a significant 30% for 80 nm AgNPs, but no increase for the group exposed to 20 nm AgNPs. The 20 and 80 nm AuNPs were increased with 15% and 11%, respectively.

5 Discussion

The organotypic retina model

The main objective of this thesis is to understand the interactions between the NPs and the inflammation response in the retina of the eye, which is a part of the highly vulnerable CNS. We used an *ex vivo* assay, comprising organotypic cultured mouse whole retina as a model for our investigations. The model is well-characterized, stable and reliable for initial testing of the effects of innovative therapies [70-72], here represented by NPs on a neural system [67], and evaluating microglia activation [69]. The model provides many advantages such as a long-term maintained complex architecture, neurons that closely resemble the neuronal assembly present in the adult retina [73, 74], microglia cells that are homogenously populated throughout the entire retina and finally the similarity to other neural systems in the CNS is excellent. It's been suggested that the human eye is the "gold standard" for studying potential ocular hazards [75], however, such experimentation is regarded as both unrealistic and unethical [76], therefore this *in vitro* model serves as an excellent first step, before using full animal models. Moreover, our culture protocol included a serum-free culture media that allows for very accurate and reproducible experimental data. With this model, we tested both an LPS induced retina and a retina exposed to nanoparticles for interesting comparison purposes.

Protein corona

We show that NPs causes unwanted cytotoxic effects at very low concentrations. The concentrations were chosen at the lowest possible provided from the manufacturer (BBI). A comparison with other studies revealed that we are far below the very lowest region of the standardized NP-concentrations used in toxicity testing [77, 78]. This shows the high sensitivity that this novel model provides. Neuronal systems, including the retina, are especially vulnerable to metal intoxication which is linked to major degenerative diseases such as Alzheimer's and Parkinson's diseases [79, 80]. The protein corona surrounding the nanoparticles alters the size and surface composition of the nanomaterial. This will dictate the cellular events and interactions with living matter, hence, affecting the

cytotoxicity [17, 18]. However, several of the articles describing the cytotoxicity of the nanoparticles present a huge variation in the results. One aspect that could influence this behavior is the time-point at when the nanoparticles are administrated to the system. We show that the protein corona, which is clearly affecting the cells [81, 82], is progressively building up and reaches a maximum size after approximately 24h. This makes the time of administration a critical aspect to consider when using nanoparticles in cytotoxicity studies.

Nanoparticle internalization

There is little doubt that the NPs are internalized by cells. An ample amount of TEM pictures reveals NPs inside the nucleus, nucleolus, mitochondria, the cytosol and the extracellular space. However, the mechanism behind this seems elusive. Despite the remarkable advances in nanoscience, the understanding about the uptake mechanism, intracellular fate and function of NPs is still lacking. Although it's been suggested that particle sizes of less than 120 nm favors endocytic uptake, particles around 200 nm involves clathrin-mediated internalization and larger particles around 500 nm caveoale-mediated uptake [83, 84]. In contrast, some researchers claim that there are no nanomaterials in biological environment due to the agglomeration and protein corona, while others show some comprehensive papers that a well-designed coating can prevent both agglomeration and corona formation problems [9, 85]. Further, attachment with PEG or similar polymer coatings camouflages the surface properties, which the cell uses for receptor- and non-receptor-mediated uptake hence prevents phagocytosis [86, 87]. Bannunah et al showed remarkably increased NP transport across an epithelial cell monolayer, where negatively charged NPs had 16-24% efficiency compared to the <5% of positive charged NPs (50-100 nm sized). Same paper claims that the cell internalization was caveoale-dependent endocytosis for the particles used in this system [88]. Caveoale are abundant in many mammalian cells (endothelial, smooth muscle cells and fibroblasts etc) but rare in others. In conclusion, the complexity involving protein corona, cellular contact, particle wrapping at cell surfaces, endocytosis makes the questions about uptake and intracellular transport a remaining issue.

Microglia proliferation

The data containing the turnover rate, i.e. the number of microglia cells, arises many questions. One theoretical and logical scenario would be to expect that the generally, due to the release of ions, more toxic AgNPs would trigger a stronger immune response, including a higher microglia proliferation rate than the

corresponding AuNPs. However, data shows a significant 35-53% increase of microglia population for AuNPs compared to a non-significant 12-15% for the AgNPs. It could be possible that the ion-release from AgNPs causing some of the microglia cells to undergo apoptosis, or at least stopping the cells in the G_2-phase of mitosis which would hinder the proliferation rate. Ions are known to interact with the Ca_2^+ pumps inside of the mitochondria and lower the ATP production, causing irreparable DNA damage or stopping the cells in the G2-phase of mitosis, all which will generate a negative impact on the turnover number [20, 77]. AuNP administration showed the proliferating potential for activated microglial cells, especially for the 80nm AuNPs (53%). Interestingly, the proliferation rate for LPS administration was not increased at all. Increased proliferation is a typical immunological response for microglia upon an inflammatory event and LPS is considered to be the strongest inducer of microglia cells [53]. However, this is not seen in our mouse model.

Microglia morphology

Another interesting observation was a strong and significant increase of rapidly activated microglia cells with round morphology (30%), but only for 80 nm sized AgNPs. TEM pictures revealed an ample amount of irregular shapes which was not found in any of the other particles included in the experiments. Irregular shaped particles have more electron dense sites aka “critical sites” compared to spherical particles [23] which could lead to a stronger immunological response, hence promote a more activated morphology of microglia to compensate for the stronger threat to the vulnerable neurons.

Microglia migration and behavior

The loss of RGCs combined with the observation of amoeboid microglia cells that migrated into the GCL both in controls and in treated retinas shows that the axotomy have detrimental effects on the RGCs. However, amoeboid microglia cells could be found in a vast amount in GCL at all time-points. The typical properties of an amoeboid microglia cell are migration trough the neural tissue and phagocytosis of apoptotic cell debris [53].

Usually, microglia recruits neurons and astrocytes to support in the immunological response. After a successful phagocytosis and repair of neurons, the remaining cells are known as “Gitter cells”. In this study, we don’t have any evidence that recruitment of neurons and astrocytes is occurring, nor the formation of Gitter cells. However, we show a number of anti-inflammatory cytokines released, most likely from the microglia [69], which may be involved in the repair and remapping

of the neural tissue. The TNF-α cytokine, classified as a pro-inflammatory cytokine is exclusively generated by microglia and not by astrocytes or any other cell type [89] and could be involved in the repair and recovery of damaged neurons [50, 51]. This indicates that the microglia after 7 days of seeding may be undergoing “synaptic stripping” which is a step in the regrown and remapping of damaged neurons in the retina [40, 46, 90]. It has been shown that IL-6 could also be excreted by Muller cells [91], and we can clearly see with our GFAP-staining that they are activated after seeding. Combined with the cytokine release data, it is reasonable to believe that activated microglia could possibly further activate adjacent Muller cells and neurons [53, 90, 92].

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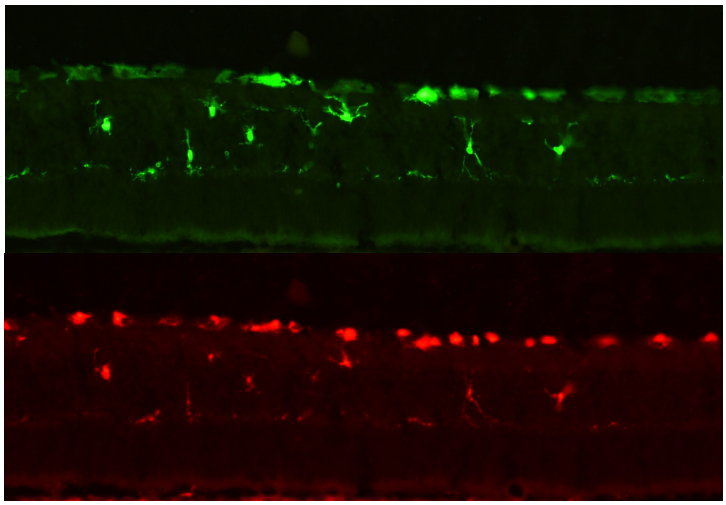

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