Nanofeatures of Biomaterials and their Impact on the Inflammatory Response

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Dissertation presented at Uppsala University to be publicly examined in Å2001, Lägerhyddsvägen 1, Uppsala, Thursday, 9 June 2016 at 09:30 for the degree of Doctor of Philosophy. The examination will be conducted in English. Faculty examiner: Professor Matteo Santin (University of Brighton).

Abstract

Nanomaterials offer an advantage over traditional biomaterials since cells naturally communicate via nanoscale interactions. The extracellular matrix, for example, modulates adhesion and cellular functions via nanoscale features. Thus incorporating nanofeatures into biomaterials may promote tissue regeneration, however in certain forms and doses nanomaterials can also cause harm. A thorough understanding of cell-nanomaterial interactions is therefore necessary to better design functional biomaterials. This thesis focuses on evaluating the effect of nanofeatures on inflammation using two different models: nanoporous alumina and hydroxyapatite nanoparticles (HANPs).

The inflammatory response caused by in vitro exposure of macrophages to nanoporous alumina, with pore diameters of 20nm and 200nm, was investigated. In addition in vivo studies were performed by implantation of nanoporous membranes in mice. In both cases the 200nm pore diameter elicited a stronger inflammatory response.

Nanoporous alumina with 20, 100 and 200nm pores were loaded with Trolox, a vitamin E analogue, in order to scavenge ROS produced by primary human polymorphonuclear (PMNC) and mononuclear (MNCs) leukocytes. Unloaded alumina membranes stimulated greater ROS production from PMNCs cultured on 20nm versus 100nm pores. This trend reversed when PMNCs were cultured on Trolox loaded membranes since Trolox eluted slower from 20nm than 100nm and 200nm pores. ROS production from PMNCs was reduced between 8-30% when cultured on Trolox loaded membranes. For MNCs, ROS production was not affected by pore size. However when the alumina was loaded with Trolox ROS production was quenched by 95%.

HANPs with distinct morphologies (long rods, sheets, dots, and fibers) were synthesized via hydrothermal and precipitation methods. The HANPs were then exposed to PMNCs, MNCs, and the human dermal fibroblast (hDF) cell line. Changes in cell viability, ROS, morphology, and apoptotic behavior were evaluated. PMNC and hDF viability decreased following exposure to fibers, while the dot particles reduced MNC viability. Fibers stimulated greater ROS production from PMNCs and MNCs, and caused apoptotic behavior in all cell types. Furthermore, they also stimulated greater capsule thickness in vivo, suggesting that nanoparticle morphology can significantly influence acute inflammation.

The outcome of this thesis, confirms the importance of understanding how nanofeatures influence inflammation.

Keywords: Nanofeatures, alumina, hydroxyapatite, inflammation

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ISSN 1651-6214
urn:nbn:se:uu:diva-284402 (http://urn.kb.se/resolve?urn=nbn:se:uu:diva-284402)
“If you can meet with triumph and disaster
and treat those two impostors just the same.
Yours is the Earth and everything that’s in it,
and which is more- you’ll be a Man, my son.’’

Rudyard Kipling

To my loving family
Ma, Papa, Ratki, Michael, and Michael
This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I  

II  

III  

IV  

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Summary of my contributions to the papers included in this thesis:

Paper I: I participated in the experimental planning and performed all the *in vitro* experiments. I also contributed to the writing of this article.

Paper II: I participated in the experimental planning and performed all the *in vitro* experiments. I also contributed to the writing of this article.

Paper III: I participated in the experimental planning, and material synthesis of the nanoparticles. I also contributed to the writing of this article.

Paper IV: I participated in the experimental planning, contributed to the material synthesis and characterization of the nanoparticles. I also performed all the *in vitro* experiments and took part in the *in vivo* experiment. I contributed to the writing of this article.
Articles not included in this thesis:

**Journal articles**


**Conference proceedings:**


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<td>BET</td>
<td>Brunauer Emmett Teller</td>
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<td>CL</td>
<td>Chemiluminescence</td>
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<td>DLS</td>
<td>Dynamic light scattering</td>
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<td>HANP</td>
<td>Hydroxyapatite nanoparticles</td>
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<td>H&amp;E</td>
<td>Hematoxylin and Eosin</td>
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<td>LDH</td>
<td>Lactate dehydrogenase</td>
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<td>Luminol</td>
<td>5-amino-2,3-dihydro-1,4-phthalazinedione</td>
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<td>NADH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
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<td>NET</td>
<td>Neutrophil extracellular trap</td>
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<td>OCT</td>
<td>Optimal cutting temperature</td>
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<td>PBS</td>
<td>Phosphate buffered saline</td>
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<td>PMNC</td>
<td>Polymorphonuclear cells</td>
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<td>ROS</td>
<td>Reactive oxygen species</td>
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<td>SEM</td>
<td>Scanning electron microscopy</td>
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Introduction

Biomaterials

Materials designed to interact with the biological environment are classified as biomaterials. Biomaterials are not only used *in vivo*, but also *in vitro* for applications such as cell culture, gene array analysis, and biotechnology processes. There are many different materials that are used as biomaterials including metals, ceramics, polymers, natural materials and composites, comprised of two or more of these materials. The application for which a material is best suited is determined by its physical and chemical properties. Metals are often used for hard tissue replacements because they possess desirable mechanical properties, biocompatibility, and osteoinductivity [1]. Polymers are often used for soft tissue applications because the mechanical properties are closer to those of natural tissue, thus minimizing compliance mismatch. Ceramics are primarily used for hard tissue applications, particularly bone and calcified tissue because bone and ceramics are both made primarily of calcium and phosphate. Biomaterials can be bioactive, as in the case of hydroxyapatite-coated hip implants that facilitate more mineral deposition and bone integration, or be inert, such as heart valve implants, which elicit no chemical or biological response [2].

Biomaterials are used in many diverse applications, from joint replacements to bone fillers, dental implants, or skin repair devices. The discipline of biomaterial science focuses on creating and developing materials that are biocompatible. Williams describes biocompatibility as “the ability of a material to perform with an appropriate host response in a specific application.” Some examples of an appropriate host response are resistance to blood clotting, resistance to bacterial colonization, and resolution of inflammation in a timely manner. Materials that are biocompatible do not diminish the immune response, rather they trigger normal wound healing and promote good tissue integration while minimizing excessive and undesirable host reactions [3].

One of the largest determinants of the biocompatibility of a material is how plasma proteins bind to the material surface. Proteins from blood and tissue surrounding the biomaterial adsorb to the surface; the amount, speed, and orientation (folding) of the protein layer all affect how the body responds to the biomaterial. Biomaterial scientists have attempted to improve material biocompatibility by creating materials that inhibit and minimize
non-specific protein interactions. The material surface can also be modified by attaching biologically active molecules such as heparin, molecules from the extracellular matrix (ECM), or to modify the surface by utilizing different nanostructured topographies that mimic the biological environment. As a result, the surrounding cells and tissue are less likely to recognize the material as foreign and instead promote tissue-material integration [4, 5].

Nanomaterials:
Nanomaterials are materials that have components smaller than 100nm in at least 1 dimension. These materials include nanoparticles, nanotubes, nanofibers, nanocrystals etc. When the size of a material decreases to the nanoscale, physical properties, such as strength, charge, surface area, shape, etc., become overwhelmingly important. For example, the ratio of surface area to volume is quite large for nanomaterials [6, 7]. The field of biomedical nanotechnology is multidisciplinary and includes researchers from biology, materials science, physics, medicine, and manufacturing. There are many nanomedical products on the market, such as self-assembled nanoparticles, nanocomposites, and nanohydroxyapatite based products. A few examples of nanomedical products include nanohydroxyapatite based bone fillers, such as Vitoss, Perossal, and Ostim [6].

How do nanomaterials relate to biomaterials
The extracellular matrix (ECM) found in the body is a naturally produced nanoscale material [8]. Hydroxyapatite in the bone is made of plate like crystals ranging from 20-80nm in length. Connective tissue is also a nanoscale material; collagen chains are 10nm, with fibrils measuring up to 500nm in length [9, 10]. Nanomaterials can provide external stimuli and topographical cues similar to the physiological environment. There is often poor integration between the surrounding tissue and the surface of a biomedical device because, without appropriate surface properties, the body recognizes the device as foreign. Implants with nanofeatures could therefore, play a similar role as the native ECM when interacting with the surrounding tissue [7].

The physical properties of a nanomaterial typically differ greatly from the bulk material. Nanomaterials possess much greater surface area per unit of volume, novel features such as nanoscale surface roughness, and altered chemical properties resulting from unique electron distributions, compared to the bulk material. These nanoscale properties affect the protein interactions that occur at the nanomaterial-tissue interface. Nanofeatures on the surface of an implanted material can stimulate new biochemical behavior such as protein misfolding and denaturation, which interfere with the normal physiological behavior of proteins. The increased surface area of nano-
materials can also provide greater number of binding sites for proteins to interact with which in turn, alters cell-nanomaterial interactions, as the initial protein layer dictates the cellular response. For example the adsorption of fibronectin, an ECM protein involved in cell adhesion, is affected by the size of the nanoscale feature on the surface of a material. Fibronectin has little to no interconnectivity when adsorbed to surfaces with 500nm spherical features, while fibronectin adsorbed on 200nm spherical features was well-spread and had a high degree of interconnectivity, thereby showing how the host interaction with nanomaterials can be altered [11]. Nanoparticles can also cause unfolding of fibrinogen, promoting interactions with the integrin receptor, Mac-1, upregulating the NFkB pathway, leading to inflammatory cytokine production and release [12].

Potential mechanism of toxicity:
Nanomaterials are used in many different industries such as in cosmetics, food, clothing, and electronics. The use of nanoparticles (NPs) in many common household items has increased the likelihood and intensity of exposure e.g silver nanoparticles found in clothing and sheets, titanium dioxide nanoparticles found in cosmetics and sunscreens, and carbon nanoparticles found in bikes and other transportation vehicles. There are four main routes of exposure: inhalation, injection, ingestion, and transdermal delivery [13-15]. Inhalation is the most common in humans. Once nanoparticles (NPs) enter the body, toxicity arises mostly from excessive production of reactive oxygen species (ROS), which results in excessive oxidative stress. Increased oxidative stress causes DNA damage, lipid and protein oxidation, protein denaturation, and eventual cell death [16]. Some NPs are capable of inducing increased intracellular ROS and toxic oxidative stress. This toxicity arises from mitochondrial injury resulting from NP accumulation in mitochondrial vacuoles, swelling, and loss of mitochondrial cristae [17]. Nanoparticles in the liver inhibits superoxide dismutase (SOD) and glutathione (GSH), antioxidative enzymes that normally catalyze the dismutation of free radicals, thereby increasing oxidative stress. Oxidative stress caused by NPs can enhance transcription of inflammatory factors and cause the activation of apoptotic and necrotic pathways [18]. Studies have shown that cobalt, titanium, and iron NPs cause monocyte/macrophages to secrete high levels of the pro-inflammatory cytokine, TNF-α [19]. Nuclear as well as mitochondrial DNA can also be damaged by oxidative stress [20]. Mitochondrial DNA damage has been associated with clinical syndromes such as neurogenic muscle weakness, stroke like episodes, ataxia, and retinitis pigmentosa [21]. In vivo, smaller sized NPs (20nm in diameter) cause a greater inflammatory response and impair alveolar macrophage function more than larger particles do (250nm in diameter), as shown in models of lung exposure [22]. Collectively, nanomaterials can induce toxicity by a number of different mechanisms.
It is paramount that researchers continue to investigate the effects and mechanisms underlying nanomaterial toxicity. This thesis focuses on evaluating the effect of nanofeatures on inflammation using two different models: nanoporous alumina and hydroxyapatite nanoparticles (HANPs).

Nanoporous Alumina

Nanoporous alumina (Fig 1) is formed when aluminum is anodized using acidic electrolytes such as sulphuric acid, oxalic acid, and phosphoric acid. When anodized, a thin film of aluminium oxide is deposited which grows into hexagonally packed array of cylindrical pores. Large pore diameters are formed by anodizing at high voltages in phosphoric acid while anodizing with sulphuric acid results in smaller pores. Depending on the applied voltage, the pore size distribution can range from 10 to 450nm. The pores form in a parallel fashion and grow perpendicular to the surface. The pore distribution is narrow and interpore spacings can be controlled and range from 10 to 100nm [23-26]. Due to its orderly nanostructured pores, nanoporous alumina has been widely used in many applications such as filtration, drug delivery, biosensors, immunoisolation devices, and tissue engineering applications [26-29]. Nanoporous alumina has also been evaluated as a potential bone implant coating [30, 31]. Anodized alumina on machined titania can support enhanced osteogenic differentiation in vitro and in vivo [32].

![Figure 1. Schematic of nanoporous alumina](image)

Hydroxyapatite Nanoparticles

Hydroxyapatite nanoparticles (HANPs) have gained significant interest in the field of biomaterials, specifically for bone tissue engineering, drug delivery, and gene therapy [33]. HANPs are nanoscale hydroxyapatite crystals
(Fig 2), chemically similar to the hydroxyapatite found in bone [6, 34]. HANPs are stable and reversibly adsorb many chemicals and proteins, thus acting as effective drug delivery vehicles [35]. HANPs are also able to pass through the cell membrane to deliver drugs and have therefore been investigated for biomedical applications. However, NPs circulating in the blood can cause increased oxidative stress, inflammatory cytokine production, lipid peroxidation, and a fibrotic reaction when in contact with immune cells [36, 37]. For this reason many researchers are interested in whether HANPs are safe or cause pathological inflammation.

![Figure 2. HANP morphology of (A) dots, (B) sheets, (C) long rods, (D) fibers](image)

**Host response towards biomaterials**

**Protein Adsorption on the Surface of a Biomaterial**

Once a biomaterial is implanted into the body, proteins immediately begin interacting with the biomaterial surface. Initially, plasma proteins adsorb to the implant surface. The shape, identity and conformation of the adsorbed protein layer will greatly affect the subsequent inflammatory response. Following protein binding, there is an acute phase of coagulation and clot formation at the injury site, recruitment and activation of phagocytes, and production of inflammatory signals that will orchestrate wound healing and later (chronic) inflammation. Whether the body successfully creates an inert matrix around the material, or continues to try and degrade or remove the im-
planted biomaterial is largely determined by the protein adsorption and the cell and inflammatory cytokine response [38].

Protein adsorption is the first event following implantation of a biomaterial. Albumin, fibronectin, vitronectin, gamma globulin, fibrinogen, and complement are a few of the many proteins that adsorb to biomaterials and modulate how the host immune system subsequently will react. Protein adsorption is affected by the surface properties of the material (charge, hydrophobicity, roughness, etc.) the solution conditions, and the nature of the proteins [39]. The biomaterial surface energy is a significant factor influencing protein adsorption. Hydrophobic surfaces adsorb more proteins than hydrophilic ones, the “driving force” largely due to an increase in entropy, caused by conformational changes of the protein structure as it refolds. The way the protein conforms to the surface is important since binding sites that normally are hidden can become available to cells [40]. Protein adsorption is not static; over time proteins on the surface of a material will be exchanged for other blood and plasma proteins. This replacement process, known as the Vroman effect, allows for abundant low molecular weight proteins to first adsorb to the surface and later be replaced with high molecular weight proteins that are present at low concentrations [40, 41].

Cells depend on proteins to adhere, migrate, and proliferate [42]. The composition of the protein layer formed on the surface of a material, therefore, dictates cell behavior. Aberrant protein folding stimulates the immune system to treat the implant as foreign and attempt to remove or destroy it. In contrast, proteins that adsorb to the implant surface and retain a folding conformation similar to the native state are less likely to trigger an inflammatory response. [43].

Immune response cascade after biomaterial implantation: Stages of Inflammation

The inflammatory response begins once there is an injury to the vascularized tissue (Fig 3). The acute inflammatory response involves exudation of plasma proteins and fluid to the implant site, and the recruitment of leukocytes and platelets. The recruited cells secrete a provisional matrix as the first step in wound healing. This matrix is composed of plasma proteins, such as fibrin, that can adsorb to the biomaterial surface. Fibrin is also produced during thrombosis and coagulation, and acts as a chemoattractant to recruit inflammatory cells to the implant site. Once the plasma proteins have adsorbed to the surface of the material, the coagulation pathway, complement system and platelets become activated. Platelets help to form the initial clot, and release many cytokines (i.e., TGF-β, PDGF) that recruit other inflammatory cells to the site of injury. Neutrophils (polymorphonuclear leukocytes,
PMNCs) extravasate from the blood vessels and tissues to the implant site within the first 24 hours of an injury. The main function of neutrophils is to phagocytose and destroy foreign material. Neutrophils do this by producing many different types of reactive oxygen species (ROS) and secrete their granular content in order to degrade foreign material. Acute inflammation normally lasts less than 1 week and resolves quickly [44-46].

After the acute phase, the chronic inflammatory phase begins and is dictated by the presence of mononuclear cells such as lymphocytes, monocytes, macrophages, and foreign body giant cells. Monocytes/macrophages are recruited to the area of implantation by a gradient of chemokines and growth factors such as transforming growth factor beta, interleukins, and platelet derived growth factor [44, 47]. Once monocytes arrive to the implant site, they start to differentiate into macrophages. Macrophages, like neutrophils, are involved in phagocytosing foreign material and produce and secrete a variety of cytokines and growth factors that will influence the inflammation. Activated macrophages produce growth factors that include tumor necrosis factor (TNF, recruits and activates neutrophils), IL-1, TGF-β (promotes extracellular matrix synthesis), PDGF (initiates chemotaxis of neutrophils, macrophages, and fibroblasts as well as mitogenesis of fibroblasts) and cytokines that recruit more macrophages to the injury site. The macrophages also bind to the surface protein layer on the material surface. This binding sends intracellular signals that will dictate the macrophage behavior. The macrophage cytoskeleton remodels and spreads over the surface, sending signals intracellularly to downstream effectors [48].

When multiple macrophages come into contact with each other, they can fuse and form foreign body giant cells (FBGCs) [49]. The protein layer found on the surface dictates how and if the macrophages will fuse [50]. Macrophages and FBGCs produce reactive oxygen species and try to phagocytose the material. Macrophages become activated once they come into contact with chemical chemoattractants, immune complexes, and microbial products and release different inflammatory cytokines in response. Macrophages that adhere to a biomaterial become activated and attempt to phagocytose the biomaterial. When unable to do so, they will remain activated in a frustrated state, commonly called “frustrated phagocytosis”. Frustrated phagocytes will continue to produce increased levels of ROS and proinflammatory cytokines in a continued attempt to degrade the material and recruit additional inflammatory cells, lengthening inflammation, from acute to chronic, and stimulating fibrosis [51, 52]. FBGCs are also able to take part in chronic inflammation through the production of cytokines and ROS. The formation of FBGCs at the surface of the implanted material is considered to be the hallmark of the foreign body response.

Wound healing commences with the infiltration of fibroblasts and macrophages at the implant site. Activated macrophages produce growth factors (PDGF and TGF-β) that promote fibroblasts and vascular endothelial cells to
proliferate and form granulation tissue, remodeling the wound. Granulation tissue formation can begin as early as 3 to 5 days following biomaterial implantation. Granulation tissue is a specialized type of tissue that is characteristic of healing inflammation and consists of the proliferation of small blood vessels and fibroblasts. Fibroblasts are responsible for restoring anatomic structure and function in tissues by synthesizing extracellular matrix (ECM) proteins such as collagen and proteoglycans [44].

Parenchymal cells found around the implanted area eventually replace granulation tissue. However, in most cases granulation tissue is not able to regenerate into normal tissue and is instead remodeled into nonfunctional scar tissue. Furthermore, if the inflammation around the implant is not resolved, a foreign body reaction (FBR), consisting of FBGCs and a fibrous capsule around the implant forms, giving rise to fibrosis. This fibrous capsule can eventually interfere with the function of the biomaterial. The aim of biomaterial science is, therefore, to create a material that encourages tissue to implant integration, while minimizing the fibrotic response [44].

Figure 3. Time line for the predominant cell types present at the site of inflammation. Adapted from [53].

Neutrophils

Neutrophils make up half of the circulating white blood cell population. They have a distinctive polymorphic nucleus, segregated into 3-5 lobuled nuclei and are therefore termed, polymorphonuclear (PMNs) cells. An adult normally releases about $10^{11}$ neutrophils everyday from the bone marrow. These cells are terminally differentiated and do not proliferate. Neutrophils have the shortest lifespan of leukocytes, with a half-life of 12 hours in circulation. Once they enter a tissue, neutrophils can live up to 5 days, but in most cases undergo apoptosis after 1 to 2 days [54, 55].

Circulating neutrophils migrate through the tissues via interactions with endothelial cells. This is mediated by chemoattractant molecules, that change
CD11/CD18 (Mac-1) from a non-adhesive to an adhesive conformation. Neutrophils are able to adhere to endothelial cells via ICAM-1, -2 ligand receptor interactions. This process is known as “rolling” or “diapedesis,” and allow neutrophils to flatten and pass through the endothelium (extravasation) and make their way to the inflammatory site [56].

The interaction of neutrophils with pathogens to initiate phagocytosis occurs via pattern recognition receptors (PRRs) or via opsonins. Neutrophils have many PRRs, the most notable groups being the N-formyl peptide receptor (FPR) and the Toll-like receptor (TLR) family. Once the PRRs are activated by sensing pathogens, or tissue damage, neutrophils react by using oxygen independent and dependent mechanisms [57]. Oxygen independent mechanisms involve releasing stored granules that contain many lytic and antimicrobial peptides to disrupt the integrity of the bacterial membrane. Oxygen dependent mechanisms involve producing toxic reactive oxygen species (ROS) such as superoxide anion (O$_2^-$), hydroxyl radical (OH$^*$), hypochlorite (OCl$^-$) and hydrogen peroxide (H$_2$O$_2$), known as the respiratory burst [58].

Once neutrophils engulf a pathogen or another foreign material, they become entrapped in a vesicle, called a phagosome, which forms from the fusion of the cell membrane around the foreign material. Phagosomal maturation occurs when stored granules in neutrophils fuse to the phagosome. Antimicrobial molecules are delivered into the phagosome in order to degrade the pathogens. Assembly of the NADPH Oxidase system on the phagosomal membrane creates a toxic environment for the pathogens [59]. Neutrophils also store granules in order to destroy microbes. There are three types of granules, primary, secondary, and tertiary, containing enzymes and antimicrobial peptides that are released during degranulation [60].

As they near the end of their life, neutrophils produce an extracellular matrix that consists of DNA, histones, chromatin, granules, metalloproteinasases and pattern recognition molecules. This matrix, known as neutrophil extracellular traps (NETs) is a cell death dependent pathway. Neutrophils secrete these NETs in a process called NETosis before they undergo apoptosis, in order to trap themselves and pathogens for macrophages to phagocytose [61, 62].

Monocytes and Macrophages
Unlike neutrophils, monocytes and macrophages are mononuclear cells, containing a single nucleus. Monocytes develop from hematopoietic stem cells in the bone marrow and are the progenitor cells for macrophages and dendritic cells. They travel to the site of injury, phagocytose pathogens, and produce ROS and a variety of inflammatory cytokines such as TNF-α, IL-1β, IL-6, and IL-10 [63]. Monocytes differentiate into M1 like macrophages (proinflammatory macrophages) after exposure to macrophage colony stimu-
lating factor (M-CSF), IFN\(_\text{n}\), or lipopolysaccharide (LPS). M1, are found to defend against foreign objects invading the host, such as bacteria and viruses. They secrete pro-inflammatory cytokines such as tumor necrosis factor (TNF), IL-1, ROS and nitrogen intermediates that are toxic for pathogens, but can also be toxic to the surrounding tissue. If monocytes are exposed to IL-4, they instead differentiate into M2 like macrophages, which possess anti-inflammatory functions and regulate wound healing. M2 exhibit macrophage fusion, decreased phagocytosis, and ROS production. They also secrete growth factors such as vascular endothelial growth factor (VEGF) and transforming growth factor beta (TGF-\(\beta\)), assist in the resolution of inflammation, and promote tissue formation and remodeling [64-66]. Monocytes are thus, affected by the specific type of biochemical stimuli they encounter [67, 68].

Macrophages stem from differentiated monocytes and can be very long lived (up to months) in the tissue. Macrophages are found in every organ in the body, and are distinct and tissue specific. Some specialized macrophages in tissue specific areas include osteoclasts in bone, Kupffer cells in the liver, histiocytes in interstitial connective tissue, and alveolar macrophages in the lungs.

Macrophages are activated in response to many different biomaterial implants in vivo, including ceramics, cements, metals, and polymers. This sensitivity, therefore, is a wide concern in the field of biomaterials. Macrophages are the most dominant infiltrating cell type in the initial stages of inflammation, and are involved in mediating the host innate immune response by recognizing and later internalizing foreign materials through phagocytosis, as well as by participating in the biodegradation of extracellular matrices. Macrophages are also needed to remove dead and apoptotic cells as well as pathogens. Lysosomal hydrolytic enzymes, and reactive oxygen species (ROS) are produced by macrophages to kill and degrade the internalized pathogens. When particles and pathogens are too large for a single macrophage to engulf, multiple macrophages fuse into multinucleated foreign body giant cells. Macrophages further secrete different cytokines to increase cell recruitment to the damaged tissue to promote tissue regeneration [69].

**Fibroblasts**

Fibroblasts are connective tissue cells that provide tissue mechanical strength by synthesizing and secreting extracellular matrix proteins, such as collagen, glycosaminoglycans, and glycoproteins. Fibroblasts can differentiate into cartilage, bone, adipocyte and smooth muscle cells. During an inflammatory response, fibroblast activation leads to increased production of cytokines, chemokines, and prostanoids. Over activation of fibroblasts eventually leads to persistent inflammation, tissue damage, and fibrosis [45].
Reactive Oxygen Species Production

Neutrophils and macrophages are “professional” phagocytes, undergoing a powerful respiratory burst during phagocytosis. The respiratory burst is a biochemical reaction that consumes oxygen and produces ROS (Fig 4). When foreign material comes into contact with the plasma membrane of the phagocyte the nicotinamide adenine dinucleotide phosphate reduced (NADPH) oxidase (an enzyme, located in the plasma membrane) is activated. Normally, components of the NADPH Oxidase complex lie dormant within the plasma membrane, cytosol, and granules. Once activated, the enzyme, comprised of many different protein subunits, redistributes itself in the plasma membrane. NADPH Oxidase takes electrons from NADPH in the cytosol and reduces molecular O₂ into superoxide anion, \( O_2^- \) (NADPH + 2O₂ → NADP⁺ + 2O₂⁻ + H⁺). Superoxide anions then get converted into hydrogen peroxide (H₂O₂) via superoxide dismutase (SOD) (O₂⁻ + O₂⁻ + 2H⁺ → H₂O₂ + O₂).

Figure 4. ROS production from a phagocyte

These byproducts can then be broken down into oxygen and water through a catalase dependent reaction. Superoxide anion and hydrogen peroxide can react with other microbicidal products and produce varied types of ROS such as the hydroxyl radicals, OH*, singlet oxygen \( ^1O_2 \), and ozone O₃. Furthermore, superoxide can react with nitric oxide (NO*) to produce OH* radicals. These ROS are released to the outside of the cell or inside the phagosome to degrade the foreign material. Increased production of free radicals can however, damage lipids that comprise the cell membrane, DNA, proteins and fatty acids, inducing degeneration and necrosis [70, 71].

Priming agents such as phorbol esters, chemoattractants, and proinflammatory cytokines cause a “priming” state, where cells are no longer in the
resting state, but are also not yet fully “activated”. Phorbol 12-myristate 13-acetate (PMA) is an ester that causes indirect phosphorylation of the NADPH Oxidase complex by activating Protein Kinase C (PKC). In vitro, the process of priming and activating phagocytes with synthetic stimulants is intended to mimic the native activation process in vivo [72].

**Antioxidants**

The body uses antioxidants to quench excessive ROS. There are two major groups of antioxidants: antioxidative enzymes and antioxidant chemicals. Antioxidative enzymes are produced in the cell and act as a rapid line of defense to protect the cell from ROS. Superoxide dismutase (SOD) for example, converts the superoxide radical into hydrogen peroxide:

\[ \text{O}_2^- + \text{O}_2^- \rightarrow \text{via SOD} \rightarrow \text{H}_2\text{O}_2 + \text{O}_2 \]

Hydrogen peroxide is then remediated by catalase and other peroxidase proteins such as glutathione peroxidase. This removal process yields water and O₂ as byproducts.

Antioxidant chemicals (scavengers) have many advantages over enzymatic antioxidants. Scavengers are generally small molecules that are able to pass through the cell membrane and localize close to the source of ROS. Scavengers directly interact with the radical and neutralize it by donating electrons to the reactive species. The scavenger is then either further oxidized, or regenerated to a reduced form, and then recycled to an active form. Some examples of scavenger antioxidants are Vitamin C and Vitamin E [73, 74].

Vitamin E is a physiological antioxidant that can interrupt lipid and fat radical peroxidation. Vitamin E can also inhibit thrombin generation, platelet adhesion, and reduce monocyte adhesion. While vitamin E is a hydrophobic antioxidant that requires plasma membrane transporters to enter the cell, the water soluble analog form of vitamin E, Trolox, can freely enter cells. The inflammatory response that occurs after a biomaterial is implanted in the body can determine whether the biomaterial is eventually rejected, isolated or accepted by the body. Antioxidants, such as Trolox, can reduce inflammation and help control the inflammatory response in vivo [75]. Thus, incorporating antioxidants into biomaterials may help to control the inflammation that occurs following implantation [76-78].

**Trolox:**

Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) is a water soluble analogue of vitamin E (Fig 5). It is able to localize to the aqueous and lipophilic compartments of the cell due to its amphiphilic structure. Trolox is capable of scavenging many different free radicals such as *OH, *-OCH₃, *OOH, and *OOCHCH₂. It scavenges radicals via many different
mechanisms including hydrogen transfer (HT), single electron transfer (SET), and radical adduct formation (RAF) [79].

\[
\text{HT: } \text{HT}^x + \text{*R} \rightarrow \text{Tx}^* + \text{HR} \\
\text{SET: } \text{HT}^x + \text{*R} \rightarrow \text{HT}^x \text{*} + \text{R}^- \\
\text{RAF: } \text{HT}^x + \text{*R} \rightarrow (\text{HT}x\text{-R})^*
\]

![Chemical structure of Trolox](image)

**Figure 5.** Chemical structure of Trolox

### Death mechanisms

#### Apoptosis vs. Necrosis:

Apoptosis normally occurs during development and aging, to keep a homeostatic balance of cell population in tissues. However, apoptosis can also occur if a cell is damaged, or triggered by different death signaling stimuli such as irradiation or drugs that cause DNA damage. Certain cell types also express Fas or TNF receptors that lead to apoptosis in the presence of ligand binding. Apoptosis is known to be an active, programmed cell process that is tightly regulated. Nuclear condensation, DNA damage, formation of apoptotic bodies are all effects of apoptosis. There is a morphological change in the cells once they start to undergo apoptosis. Cells shrink, the cytoplasm becomes dense, and extensive plasma membrane blebbing occurs leading the cell to fragment into apoptotic bodies (Fig 6). This process is known as budding. Phagocytic cells such as macrophages later phagocytose these apoptotic bodies. Apoptosis is controlled and coordinated by cysteine proteases called caspases. Caspase proteins are normally in an inactive state, but once activated they are responsible for orchestrating the biochemical mechanisms of apoptosis. There are 2 pathways that lead to apoptosis: the extrinsic pathway and the intrinsic pathway. The extrinsic pathway occurs via the activa-
tion of the death receptors, Fas or TNF. The intrinsic pathway occurs through changes in the inner mitochondrial membrane. This results in the opening of the mitochondrial permeability transition pore, releasing pro-apoptotic proteins such as cytochrome C, into the cytosol. Both pathways can activate caspase proteins, which then lead to the execution pathway of apoptosis. Caspase-3, caspase-6, and caspase-7 are the proteases that are known as the effector/executioner caspases. These caspases cause activation of endonucleases in the cell that eventually degrade DNA, cause chromatin condensation, cytoskeletal reorganization, and fragmentation of cells into apoptotic bodies. Phosphatidylserine (PS), a phospholipid that is normally found on the inner leaflet of the plasma membrane, is then externalized, facilitating uptake and disposal of apoptotic cells [80-82].

Necrosis occurs passively and typically results from environmental perturbations, such as trauma or injury. Some of the morphological changes in necrotic cells include cell swelling, formation of cytoplasmic vacuoles, and disruption of the cell membrane. Once the cell loses its cell membrane integrity, the contents within the cell are released, sending chemotactic signals that cause the recruitment of inflammatory cells [80, 83, 84].

Figure 6. Apotosis vs. Necrosis
Aims of investigation

Nanomaterials are materials with at least one dimension in the nanoscale (<100nm). Since cells naturally communicate via nanoscale interactions, nanomaterials offer many advantages over traditional biomaterials. The extracellular matrix, for example, modulates adhesion and cellular functions via nanoscale features. Nanofeatures, in the form of particles, crystals, pores, etc., can promote tissue regeneration by mimicking nanoscale features of the native ECM, a promising approach for use in biomedical applications.

Although beneficial in many respects, excessive exposure to nanomaterials can cause toxicity via increased ROS production, DNA damage, and cell death. A thorough understanding of cell-nanomaterial interactions is necessary to better design functional biomaterials. The present work focuses on investigating the effect of nanotopography (alumina) and nanoparticle morphology (HANP) on inflammation, *in vitro* and *in vivo*.

The specific aims of the appended papers were as follows:

- To investigate the effect of macrophage behavior, *in vitro*, and fibrotic response *in vivo* when exposed to alumina membranes with different nanoporosities. (Paper I)
- To investigate the effect of Trolox on primary leukocyte (PMNC, MNC) ROS production when delivered conventionally or by release from nanoporous alumina. (Paper II)
- To synthesize and characterize four distinct HANPs (Paper III)
- To investigate primary leukocyte (PMNC, MNC) and fibroblast behavior *in vitro* as well as *in vivo* acute inflammation, following exposure to HANPs (Paper IV)
Methods

Material Preparation and Characterization

Trolox Loading and Release from Nanoporous Alumina (Paper II)

A 20 µl solution of 60mM Trolox (suspended in 99% ethanol) was added to each of the nanoporous alumina membranes (20nm, 100nm, and 200nm pores) and evaporated at room temperature for 30 minutes. Released Trolox was measured for up to 24 hours, using a UV Spectrophotometer at 290nm.

HANP synthesis (Paper III, IV)

There are many different ways of synthesizing HANPs can be synthesized, e.g. wet chemical processes. Altered nanoparticle morphology can be synthesized by means of the emulsion technique, sol-gel, hydrothermal, precipitation method, etc [85]. The hydrothermal and precipitation methods were used in this thesis, to synthesize HANPs with different morphologies (long rods, sheets, dots, and fibers). The hydrothermal method can induce crystal growth under high temperature and pressure, and produce HANPs with high crystallinity, relatively narrow particle size distribution, and distinct morphological features [86]. For large-scale hydrothermal processes, repeatability and expense can however be an issue. The precipitation method instead uses the precipitation from salt solutions to give rise to HANPs, which in contrast to the hydrothermal method, is a more simple and economical approach when synthesizing HANPs.

Xray Diffraction (XRD) (Paper III and IV)

XRD is a technique used to characterize crystalline materials. Based on the scattering principle and Brag’s law, x-rays are used to generate diffraction patterns in which the resulting peak position and intensity are related to the structure of the material. The average bulk composition of the HANPs was determined using this method.
Brunauer-Emmett-Teller (BET) Surface Area (Paper IV)

BET analysis is a technique used to measure the specific surface area of a material by calculating the volume of gas physically adsorbed to the material. The surface area of the HANPs was measured using \( \text{N}_2 \) adsorption.

Dynamic Light Scattering (DLS) (Paper IV)

DLS analysis is used to measure particle size distributions while dispersed in a liquid. The Brownian motion of particles causes a laser light to be scattered at different intensities, generating the velocity of the Brownian motion, and thereby yielding the particle size. HANP particle size distribution was measured after 1 hour of incubation in medium.

Calcium ion concentration (Paper IV)

Calcium ion content was measured using the calcium colorimetric assay. This assay uses the chromogenic complex that forms between calcium ions and O-cresolphthaleine in order to measure free calcium colorimetrically at \( \lambda_{575} \). Calcium ion content in HANP suspensions was measured after 1 hour of incubation in medium.

pH of particle suspensions (Paper IV)

HANPs were incubated in medium for 1 hour. The pH of the HANP suspensions was then measured using a pH meter.

Transmission Electron Microscopy (TEM) (Paper III and IV)

The TEM technique uses transmission of a beam of electrons through an ultra-thin specimen in order to develop an image. HANP morphology and size dimensions were determined using this technique.

\textit{In vitro} studies

Cell models (Paper I, II, III, IV)

In this work, the \textit{in vitro} response caused by exposure to different nanofeatures was evaluated using cell lines and primary cell models. RAW264.7 murine macrophages, NIH3T3 mouse embryonic fibroblasts, and human dermal fibroblasts (hDFs) were the three cell lines used. The primary cells, human polymorphonuclear (PMNC) and human mononuclear (MNC) leukocytes were isolated from heparinized blood.
Primary Cell Isolation

**Human MNC isolation (Paper II, IV)**

Blood buffy coats were obtained from anonymous blood donors from the Uppsala University Hospital. MNCs were isolated using the Ficoll-Paque Plus density gradient centrifugation solution.

**Human PMNC isolation (Paper II, IV)**

For PMNC isolation two separation procedures were performed, the Ficoll-Paque Plus density gradient separation followed by the Dextran separation.

Cell Viability Assays

**Alamar Blue (Paper I, IV)**

Alamar Blue is a non-toxic metabolic indicator. The oxidized form of Alamar Blue is introduced into the culture medium, where it converts to its reduced form through mitochondrial enzyme activity ($\text{NAD}^+ + 2\text{H}^+ + 2\text{e}^- \rightarrow \text{NADH} + \text{H}^+$) by viable cells present. This reduction causes a color change in the medium and a shift in fluorescence which can be detected colorimetrically and fluorimetrically $[87, 88]$. The extent of reduction correlates to the number of viable cells present.

**Lactate Dehydrogenase (LDH) (Paper IV)**

LDH is a stable enzyme that is found in all cell types. It is found inside the cell and catalyzes the interconversion of lactate to pyruvate. There are two methods in which LDH activity can be evaluated and correlated to cell viability. Cells release LDH into the culture medium, upon damage to the plasma membrane. Analyzing LDH activity in the cell culture medium will therefore relate to cell toxicity. In order to determine viable cells, attached cells are lysed with lysis buffer to disrupt the cell membrane, allowing the LDH sequestered in the cell to be released and measured. This assay is based on measuring the reduction of NAD to its reduced form, NADH, by the LDH enzyme. The reduction of NAD to NADH can be quantitated colorimetrically at 490nm.

**Live/Dead staining (Paper IV)**

The live dead fluorescence stain (calcein-AM and propidium iodide) was used in order to visualize the number of viable and dead cells. Calcein-AM is a highly lipophilic and cell membrane permeable compound. Calcein-AM itself is not fluorescent. However, once calcein-AM gets cleaved by an ester-
ase in viable cells, calcein gets generated and emits a very strong green fluorescence ($\lambda_{\text{ex}}$ 490nm $\lambda_{\text{em}}$ 515nm). Propidium iodide cannot pass through the cell membrane of viable cells. However, if the cell membrane is damaged, PI can pass through and intercalate with DNA, emitting red fluorescence ($\lambda_{\text{ex}}$ 535nm, $\lambda_{\text{em}}$ 617nm) and thus pinpointing dead cells.

**Reactive Oxygen Species Quantification:**

**Nitrotetrazolium Blue Assay (NBT) (Paper I)**

To determine intracellular ROS production, the NBT assay was used. The NBT assay is commonly used in clinics to detect chronic granulomatous disease (CGD), a disease that causes reduced respiratory burst. In this assay, the cells are first incubated with yellow-colored nitroblue tetrazolium (Y-NBT), a membrane permeable compound that gets absorbed by the cells and reduces in the presence of $\text{O}_2^-$ species. This produces insoluble formazan crystals that can be detected microscopically and dissolved to quantitate the amount of $\text{O}_2^-$ species formed in the phagocytes [89].

**Luminol amplified Chemiluminescence (CL) (Paper II, IV)**

The luminol amplified chemiluminescence method measures light emission generated from different reactive oxygen species, such as $\text{O}_2^-$ and $\text{H}_2\text{O}_2$, produced intracellularly and extracellularly by activated phagocytes. This approach offers the ability to measure ROS production over time, generating kinetic measurements. Total ROS was further quantified by integrating the total area under the chemiluminescence kinetic curve (AUC) using the Origin Software.

**Apoptosis Quantification**

**Caspase 3/7 Activity (Paper IV)**

Caspase-3 and caspase-7 are known as the effector/executioner caspases in the apoptosis pathway. These caspases cause activation of endonucleases in the cell that eventually degrade DNA, cause chromatin condensation, cytoskeletal reorganization, and fragmentation of cells into apoptotic bodies. Caspase 3/7 activity in cells exposed to HANPs, was measured using the caspase-glo 3/7 assay. This assay provides a luminogenic caspase 3/7 substrate, that contains a tetrapeptide sequence. If caspase 3/7 is present in the sample, the luminogenic caspase 3/7 substrate will be cleaved, providing a luminescent signal that can be quantified.
Morphology Analysis

Light Microscopy (Paper IV)

An inverted light microscope (Nikon) at 600X magnification was used to observe changes in PMNC morphology after HANP exposure.

Scanning Electron Microscopy (SEM) analysis (Paper I, II)

SEM generates images by scanning the sample with focused electron beams. The electrons interact with the atoms of the sample providing information of the sample’s surface and composition. The morphology of HANPs alone as well as the cell morphology when exposed to HANPs and nanoporous alumina was studied using SEM analysis.

In vivo studies

In vivo implantation (Paper I, IV)

Nanoporous alumina membranes with pore sizes of 20 and 200nm were implanted into the subcutaneous cavity of female (8-10 weeks old) Balb/C mice. The mice were anesthetized with isoflurane and the incision mark was disinfected with 70% ethanol. The alumina membranes were implanted at least 1 cm away from the incision site, with the 20nm membrane placed on one side of the incision and the 200nm membrane on the other side. The implants and the surrounding tissue were collected after 2 weeks, and histological, immunohistochemical staining, and protein array analysis was done.

HANPs (1 mg particles mixed with 0.5 ml of sterilized saline) were subcutaneously implanted in 4 sections dorsally in Balb/C mice. Mice were sacrificed three days after implantation, and the surrounding tissue around the HANPs were excised and frozen in optimal cutting temperature (OCT) embedding media at -80°C for histology.

Histology (Paper I, IV)

Hematoxylin and Eosin (H&E) staining was done to determine capsule cell density. Hematoxylin stains nucleic acids in a deep purple color thereby staining cell nuclei, while eosin is pink and stains proteins in the cytoplasm and extracellular matrix. The Masson Trichrome stain was used to evaluate fibrotic capsule thickness and collagen production (Paper I). The trichrome stain includes three separate stains. Aniline blue which stains collagen, nuclei are stained with Weigert’s iron hematoxylin stain, and the muscle is stained by Beibrich scarlet-acid fuchsin. Images of the stained sections were taken using a Leica microscope with a CCD camera. ImageJ software was
used to quantitate capsule thickness, collagen content, and capsule cell density.

**Inflammatory protein array (Paper I)**

Protein antibody microarray analysis is a powerful technique that is used to quantify multiple protein expression levels at once [90, 91]. Inflammatory protein production by cells surrounding the alumina membranes were determined using a RayBio Mouse Inflammation Antibody Array G series I (Raybiotech, Norcros, GA). 30 tissue section slices from the central part of the membrane implants were obtained, lysed, and extracted for protein content. Protein concentrations were then determined using the BCA assay. 50 µg of each protein sample was then used to perform the mouse cytokine antibody array. Protein samples were placed on top of glass slides that had targeted cytokines spotted on it. The slides were then subjected to image analysis by an Axon GenePix 4000B microarray scanner (Molecular Devices, Sunnyvale, CA, USA), using the Cy3 channel. In order to identify changes in cytokine and growth factor expression, fluorescence ratios for each spot was compared between the 20nm and 200nm alumina membranes. A ratio of 2.0 or above was considered up-regulated, while a ratio of 0.5 or below was considered down-regulated expression levels.
Results and Discussion

Effects of nanoporous alumina on inflammatory cell response (Paper I)

**Nanopore size affects macrophage ROS production and morphology**

Macrophages cultured on alumina with 200nm pores produced increased levels of intracellular ROS at all time points as compared to the cells exposed to 20nm pores (Fig 7A). SEM analysis also showed that macrophages cultured on the 20nm alumina (Figure 7B) adopted a rounded morphology, while cells on the 200nm membrane (Figure 7B) were flattened and elongated, showing typical signs of activated macrophages.

![Figure 7. Macrophage ROS production (A) and morphology (B) when cultured on nanoporous alumina with pore diameters of 20 and 200nm. * indicates significance (p<0.05) between the 20 and 200nm pores.](image)

ROS plays an important role in inflammation, partly by acting as a signaling molecule by recruiting inflammatory cells and as an activator of the inflammasome, a cytosolic complex that is involved in the production of proinflammatory cytokines [92, 93]. Nanosurface topography can induce ROS and have an effect on leukocyte behavior including migration, cell orientation, and phagocytic activity [94-97] Studies have shown that monocytes/macrophages cultured on 200nm alumina membranes as compared to 20nm alumina membranes, secreted increased levels of proinflammatory
cytokines such as, IL-1β and TNF-α, and also adopted a flattened morphol-
y with increased filopodial extensions [95]. Collectively, the data indicates 
that macrophages cultured on 200nm pores, as compared to 20nm pores, 
resulted in increased activation, in vitro.

**Nanopore Size affects in vivo cell recruitment and inflammatory 
cytokine production**

The fibrotic capsule thickness was not affected by pore size (Fig 8A, 8C). 
Collagen production, evaluated with the Mason Trichrome stain, was how-
ever lower around the 20nm membrane compared to the 200nm membrane, 
although this difference was not statistically significant (Fig 8A, 8D). Cap-

tule cell density however, was higher on the 200nm membrane compared to 
the 20nm membrane, suggesting that more cells were recruited to the 200nm 
pores than to the 20nm pores (Fig 8B, 8E).

*Figure 8. Foreign body response analysis to nanoporous alumina 2 weeks after sub-
cutaneous implantation in Balb/C mice. Masson trichrome (A) and H&E staining 
(B) was performed to assess the extent of implant-associated fibrotic tissue for-
mation. Capsule thickness (C), collagen content (D) and capsule cell density (E) 
were evaluated. * indicates significance (p<0.05) between the 20 and 200nm pores.*

Previous studies assessed the effect of nanoporous alumina on the acute in-
flammatory response, by implanting the membranes in the peritoneal cavity 
for 16 hours. The 200nm alumina membrane caused higher immune cell 
recruitment to the peritoneal cavity compared to the 20nm membrane im-
plant [98]. When in contact with whole blood in vitro, the 200nm mem-
branes also caused higher levels of soluble complement activation products 
to form compared to the 20nm membranes [99]. Complement activation is
one of the first events that occur in inflammation, and cause the subsequent activation and recruitment of platelets and leukocytes to the injured area.

In the present study the inflammatory protein array analysis confirmed that the 200nm membranes induced an increased inflammatory response compared to the 20nm membranes. Many potent proinflammatory cytokines such as GM-CSF, IL-9, IL-2, IL-1β, IL-13, leptin, and fas ligand were up-regulated in response to 200nm membranes (Table 1). Upregulation of these cytokines directs the innate response by recruiting and activating immune cells, and subsequently initiating fibrotic tissue response.

Table 1. Production of inflammatory cytokines in tissue sections around the nanoporous alumina membranes. Data represents the ratio of the expression between the 200nm and the 20nm membranes. Protein expression is considered significantly lowered or elevated if the ratio of expression (200nm/20nm) is <0.5 or >2, respectively.

<table>
<thead>
<tr>
<th>Elevated Cytokines</th>
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<tbody>
<tr>
<td>BLC</td>
<td>2.524</td>
</tr>
<tr>
<td>CD30 L</td>
<td>2.293</td>
</tr>
<tr>
<td>Fas Ligand</td>
<td>2.291</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>2.437</td>
</tr>
<tr>
<td>IL-13</td>
<td>2.289</td>
</tr>
<tr>
<td>IL-1β</td>
<td>2.415</td>
</tr>
<tr>
<td>IL-6</td>
<td>2.095</td>
</tr>
<tr>
<td>IL-9</td>
<td>2.131</td>
</tr>
<tr>
<td>KC</td>
<td>2.113</td>
</tr>
<tr>
<td>Leptin</td>
<td>2.269</td>
</tr>
<tr>
<td>Lymphotactin</td>
<td>2.029</td>
</tr>
<tr>
<td>RANTES</td>
<td>2.095</td>
</tr>
<tr>
<td>SDF-1</td>
<td>2.182</td>
</tr>
<tr>
<td>TCA-3</td>
<td>2.230</td>
</tr>
<tr>
<td>TECK</td>
<td>2.137</td>
</tr>
<tr>
<td>IL-2</td>
<td>3.029</td>
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<table>
<thead>
<tr>
<th>Cytokines with no significant change</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1α, IL-3, IL-4, IL-17, LIX, MCSF, sTNF RII, TIMP-2, sTNF RI, MIP-1γ, Eotaxin-2</td>
</tr>
</tbody>
</table>
The increased level of IL-1β and GM-CSF indicates higher macrophage activation [100, 101] on the 200nm membrane, which is in agreement with the elevated ROS produced in vitro. IL-13 is involved in fibroblast proliferation and collagen production, while Fas ligand production is involved in biomaterial-induced fibrosis and apoptosis [102]. The elevated levels of these cytokines is likely the cause for the increased cell recruitment seen in the histology, around the 200nm alumina.

Reduced oxidative stress in primary human cells by antioxidant released from nanoporous alumina (Paper II)

**ROS production and morphology of PMNC and MNC on nanoporous alumina**

MNCs cultured on alumina membranes all produced equivalent amounts of ROS, independent of pore size (Fig 9A). PMNCs cultured on alumina with 20nm pores however, produced more ROS than PMNCs on 100nm pores (Fig 9B). When exposed to the 200nm pores, PMNCs also readily released more ROS compared to the 100nm pores, although this was not statistically significant. This trend is in agreement with previous studies showing greater ROS production from PMNCs cultured on the 20nm versus 200nm alumina [103].

![Graph A](image)

![Graph B](image)

*Figure 9. ROS production from PMNCs cultured on 20, 100, and 200nm pores on nanoporous alumina. * indicates significance (p<0.05) between the 20 and 100nm pores.*

PMNC morphology was evaluated after being exposed to the alumina membranes for 2 hours (Fig 10). PMNCs cultured on the 20nm pores exhibited a more flattened morphology, with visible lamellopodial extensions, while
cells on the 100nm and 200nm pores were rounded. The flattened morphology seen on the 20 nm alumina is typical of an activated PMNC phenotype. MNC morphology was identical on all alumina membranes (figure not shown).

Figure 10. PMNC morphology micrographs cultured on (A) 20nm, (B) 100nm, (C) 200nm pores of nanoporous alumina. Scale bar represents 2 and 10 µm.

NADPH Oxidase, the enzyme that produces ROS, is not fully active until cells adhere to a surface [104, 105]. ROS production in activated PMNCs begins once NADPH oxidase is translocated to the plasma membrane via the cytoskeleton [106, 107]. It is not surprising, given the intimate association between cytoskeletal arrangement and ROS production, that ROS production in activated PMNCs is affected by the shape of the cell [108]. Studies have shown that when macrophages are cultured on 50nm nanodot arrays, cell adhesion and cytoskeleton organization is promoted in contrast to when they are cultured on 200nm nanodots hindered cell adhesion and cytoskeleton organization [109]. PMNCs seeded onto roughened polystyrene also produced greater ROS as compared to smooth polystyrene, showing that surface topography has an effect on ROS production [94]. The observed flattened morphology, as well as the increase in ROS production of PMNCs on 20nm pores, is thus in agreement with the present findings.

Scavenging effects of Trolox on polystyrene
Trolox, a hydrophilic analogue of Vitamin E, is capable of scavenging various free radicals. MNCs and PMNCs seeded on tissue culture polystyrene (TCPS) showed a dose dependent reduction in ROS release, with increasing concentrations of Trolox (Figure 11). PMNCs (Fig 11B) required a 10-fold higher Trolox concentration in order to quench ROS, compared to MNCs (Fig 11A), likely due to the fact that PMNCs typically undergo greater respiratory bursts [110]. 100 µM of Trolox was the most effective concentration for quenching ROS for both cell types and was therefore chosen for the successive experiments.
Figure 11. ROS produced from MNCs (A) and PMNCs (B) when cultured on polystyrene, with increasing concentrations of Trolox.

**Trolox Released from Nanoporous Alumina:**
Trolox eluted rapidly from nanoporous alumina. 80% of the loaded Trolox had eluted from the 20nm alumina pores within 15 minutes, while a significantly greater amount (90%) was released from the 100nm and 200nm pores during the same time (Figure 12).

Figure 12. Trolox released from 20, 100, and 200nm alumina pores. * indicates significance (p<0.05) between the 20nm pores to the 100 and 200nm pores after 20 and 40 minutes of release.
The release profile observed in studies using nanoporous alumina,[111,112] nanoporous titanium [113] mesoporous silica [114], and in the present study, was predominantly burst release. Diffusion models, such Peppas-Korsmeyer [115] or Higuchi [116], cannot be applied to the present study since greater than 60% of the loaded drug is released within 20 min.

**Scavenging effects of Trolox on Nanoporous Alumina**

Trolox was delivered via two methods: external addition directly to the media (Fig 13A), or by preloading Trolox into the pores of nanoporous alumina (Fig 13B).

**Figure 13.** Principle sketches detailing the experimental set up when introducing Trolox externally (A) and preloading Trolox into nanoporous alumina(B).

External addition of Trolox (Fig 13A):

ROS produced from MNCs was quenched by 97% on all pore sizes when adding 100µM of Trolox (Fig 14A). PMNCs seeded on the 20nm pores however only exhibited a 20% reduction in ROS release (Fig 14B), and no change in ROS was observed for PMNCs cultured on the 100nm and 200nm pores. Interestingly, the observed difference in Trolox capability of scavenging ROS produced by PMNCs is dependent on the material and its nanotopography, since our results show that Trolox is more efficient in scavenging ROS on polystyrene than on alumina.
Figure 14. ROS production after the external addition of 100 µM of Trolox to MNCs (A) and PMNCs (B and C).

Preloaded Trolox (Fig 13B):

When Trolox was preloaded into the membranes, MNC (Fig 15A, 15C) and PMNC (Fig 15B, 15D) ROS production was significantly reduced on all pore sizes, compared to the unloaded controls. For MNCs, ROS production was reduced to approximately 95% on all pore sizes, while with PMNCs, there was a 30% reduction in ROS for cells cultured on the 20nm pore sized alumina, 8% for 100nm pores, and 15% for 200nm pores.
Figure 15. ROS production of MNCs (A,C) and PMNCs (B,D) following exposure of alumina preloaded with Trolox.

**Trolox caused a delay in Chemiluminescence peak time in PMNCs**

Trolox caused a delay in the peak time of chemiluminescence (CL), for PMNCs, which was dose dependent, on all pore sizes and on polystyrene (Fig 11B, 14C, 15D). A delay in CL peak time was however not observed for MNCs. One significant difference between MNCs and PMNCs lies in the signaling cascade of the NADPH oxidase complex. Rac is a rho GTPase protein involved in transducing cytoskeletal changes, NADPH oxidase activity, and lamelopodia activation [117-120]. In human MNCs, 95% of the Rac is found in the Rac1 isoform, while for PMNCs, 95% of Rac is in the Rac2 isoform in PMNCs. While Rac1 is required in actin cytoskeleton regulation, and migration [120], Rac2 is needed in regulating the NADPH oxidase complex. Rac1 knockouts do not inhibit ROS production, while Rac2 knockouts display delayed NADPH oxidase kinetics and impaired ROS production in PMNCs [121]. This delay in ROS production is similar to what was observed when PMNCs were treated with Trolox in the present investigation. Trolox may therefore be interacting with Rac2 in PMNCs, but not in MNCs. This is consistent with the observed reduction in lamellopodia and spreading as well as the impaired ROS production seen in PMNCs treated with Trolox.
In vivo and in vitro evaluation of hydroxyapatite nanoparticle morphology on the acute inflammatory response (Paper III and IV)

**Hydroxyapatite Nanoparticle Characterization:**
All synthesized HANPs were composed of HA and were crystalline, as indicated by the XRD spectrum (Fig 16).

![Figure 16. XRD pattern of the HANPs.](image)

**HANP Morphological Analysis:**
HANP morphology was determined using TEM and SEM techniques. The TEM micrographs showed that the dots were approximately 15 nm in diameter (Fig 17A). Below a pH of 7.4, sheet-like particles formed (Fig 17B) with a slight contamination of long rod-like particles. The sheet particles were 75 nm in length and 30 nm in diameter. Below pH 6, the resultant particles formed long rods (Fig 17C) and were 200 nm in length and 20 nm in diameter. Below pH 4, fiber-like NPs formed, with a diameter of 60 nm and lengths between 1-4 µm (Fig 17D). The aspect ratio was similar independent of synthesis method i.e. hydrothermal or precipitation methods. As reported previously, aspect ratio increases with decreasing pH. The dots and sheets had an aspect ratio between 1-3, the long rods between 2.5-5, while the fibers had the largest aspect ratio, ranging from 20-50. The aspect ratio is determined kinetically by the surface energy of each lattice plane and the mobility of surface ions. Initial calcium concentration, calcium/phosphate ratio, and pH, also have an affect on aspect ratio [122].
Figure 17. HANP TEM micrographs of dots (A), sheets (B), long rods (C), and fibers (D).

SEM analysis further displayed that HANP morphology did not change after 1 hour of suspension in serum free medium (Fig 18), expect for the fiber particles where the length decreased slightly, likely due to the use of sonication.
Figure 18. SEM micrographs of the HANPs before and after 1h suspension in serum free medium.

HANP particle size distribution after 1 hour of incubation in serum free medium demonstrated that the HANP sizes ranged from 25nm to 85nm (Fig 19). The sheets had a median size of 68nm, the dots were 33nm, the long rods were 37nm, and the fibers were approximately 50nm. The size distribution was similar to what was observed in the TEM and SEM analysis.
Figure 19. Particle size distribution of fibers (A), long rods (B), sheets (C), and dots (D) after 1 hour of suspension in serum free medium.

**HANP surface area analysis:**
The fibers had the smallest surface area (52.7 g/m²) compared to the other particle morphologies (Table 2). The sheets had the second smallest surface area (57 g/m²), followed by the long rod particles (71 g/m²). The dot particles had the greatest surface area with 91.5 g/m².

Table 2. HANP surface area

<table>
<thead>
<tr>
<th>HANP Morphology</th>
<th>BET surface area (g/m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sheets</td>
<td>58.6</td>
</tr>
<tr>
<td>Long Rods</td>
<td>71.6</td>
</tr>
<tr>
<td>Dots</td>
<td>91.5</td>
</tr>
<tr>
<td>Fibers</td>
<td>52.7</td>
</tr>
</tbody>
</table>

**Calcium ion content and pH of HANP suspensions:**
The amount of free calcium found after incubating HANPs in serum free medium was significantly reduced by 59% with the addition of long rods, 43% with the sheets, and 45% with the dots (Fig 20A). The fiber particles caused a 16% decrease in free calcium, although this difference was not statistically significant. Calcium is usually drawn to the surface of HANPs, thus this result is expected [123, 124]. The pH of the HANP suspensions was found to be comparable to the serum free medium control group (Fig 20B).
Figure 20. Calcium ion content (A) and pH (B) of HANPs 1 hour after suspension in serum free medium. * indicates significance (p<0.05) between control and long rods, dots, and sheets.

**Hydroxyapatite nanoparticle shape affects cell viability**

Fibers induced greater toxicity as shown by LDH and Alamar blue than the sheet particles when cultured with PMNCs (Fig 21A, 21B) and hDFs(Fig 21A, 21D). Live dead imaging revealed the same effect, with less viable PMNCs and hDFs when in contact with the fiber particles. MNCs however, experienced greater toxicity when exposed to the dot particles (Fig 21A, 21C).
Figure 21. Live cell viability images of PMNCs, MNCs, and hDFs (A). Cell viability of PMNC, measured by the LDH assay (B), * indicates significance (p<0.05) between the sheets and fibers, MNC viability as determined by the LDH assay (C), * indicates significance (p<0.05) between the dots and all other particles. hDF viability evaluated by the Alamar blue assay (D), *,#, and + indicates significance (p<0.05) between the fibers and all other particles on 1, 2, and 3 days.
Nanoparticle morphology affects ROS production in PMNCs and MNCs

Fibers stimulated the greatest amount of ROS production from PMNCs, 45% higher than the positive control as compared to the other particle morphologies (Fig 22A). This difference was however, was not statistically significant. MNCs also released more ROS when exposed to the fibers than sheets or long rods (Fig 22B). The fibers caused a 40% increase in ROS, while the sheets and the long rods only caused a 22% increase, compared to the positive control.

Figure 22. ROS production from PMNCs (A) and MNCs (B) following exposure of HANPs. * indicates significance (p<0.05) between the sheets and fibers, and # indicates significance (p<0.05) between the long rods and fibers.

Previous studies have shown that macrophages were unable to fully phagocytose asbestos fiber particles, causing super oxide anions and other peroxide radicals to be released to the outside of the cell, leading macrophages into the state of frustrated phagocytosis. Fiber particles can exhibit differing morphologies, such as straight, curved, entangled, etc. Straight fibers, produce more ROS and are incompletely taken up, leading to frustrated phagocytosis [125]. In the current study, straight fibers were used, which may account for the elevated ROS production for both cell types.

Nanoparticle shape induces a change in neutrophil morphology

After one hour of exposure to fibers PMNC cell membranes appeared to undergo blebbing, as seen in the light microscopy images (Fig 23D). The short rod particles also caused slight blebbing of the cell membrane. Membrane blebbing is a characteristic morphological feature that occurs when cells undergo apoptosis.
During apoptosis, phospholipids, specifically phosphatidylserine (PS), which is kept intracellularly in the plasma membrane, is translocated to the outer surface of the cell membrane. This exposes PS to the extracellular space and further marks the cell for scavenger phagocytes, such as macrophages, to come and engulf the apoptotic cells [126]. Blebbing also occurs when the cytoskeleton is weakened, causing the cytosol of the cell to put pressure on unsupported parts of the plasma membrane [127]. Previous studies have shown that long and straight fibers cause incomplete uptake, resulting in disruption of the cytoskeleton [125].

**Nanoparticle morphology affects apoptosis**

PMNCs, MNCs, and hDFs all responded with increased levels of caspase 3/7 activity after exposure to fibers as compared to the other particles (Fig 24). PMNCs (Fig 24A) cultured with the fibers showed a 20% increase in caspase 3/7 activity compared to the other particle morphologies, although this difference was not statistically significant. MNCs (Fig 24B) exhibited a 50% increase, while hDFs (Fig 24C) exhibited a 200% increase in caspase 3/7 activity when in contact with fibers, compared to all other particle morphologies. Furthermore, the long rod particles caused a 30% decrease in MNC caspase 3/7 activity compared to the sheet particles.
Figure 24. Caspase 3/7 activation of (A) PMNCs (B) MNCs * indicates significance (p<0.05) between the fibers and all other particles, + indicates significance (p<0.05) between sheets and long rods (C) hDFs * indicates significance (p<0.05) between fibers and all other particles.

Caspase 3/7 activity is an indicator of cell commitment towards the apoptotic pathway. These caspases are the effector caspases and play a critical role in apoptosis by causing chromatin condensation, DNA fragmentation, and apoptotic body formation [128]. Asbestos fiber particles have been extensively studied in fibroblasts and phagocytes [129, 130], however this is the first study on hydroxyapatite fiber particles. Fiber particles can also cause increased ROS production and activation of the inflammasome [15] as well as induce cell cycle delays, DNA damage, and apoptosis [131]. ROS acts as a secondary messenger in the apoptosis pathway [92, 132]. It has also been shown that caspase-3 is not only involved in ROS-induced apoptosis, but may also be activated by ROS [133-135]. Due to its high specific surface area, nanoparticles are able to have increased amount of contact with cells, making them highly reactive [136]. Studies have shown that particles with a higher specific surface area, such as the dots, induce increased levels of apoptosis compared to particles with a smaller surface area [137]. The observed toxicity induced by the dot particles is thus in agreement with prior studies. Interestingly, the increase in toxicity induced by the dots did not occur via apoptosis, since caspase 3/7 activity was not elevated when MNCs were exposed to the dot particles. It is thus likely, that the dots instead initiate toxicity via necrosis instead.

**Nanoparticle shape affects acute inflammation, in vivo**

H&E staining of tissues after HANP implantation showed slightly increased cell recruitment around fibers compared to the other particles (Fig 25A-E). Fibrotic capsule formation (Fig 25F) was also significantly thicker around the fiber particles (Fig 25D) compared to the long rods (Fig 25A). This thicker capsule indicated that the fibers caused the highest degree of inflammation in the mice.
Figure 25. H&E staining evaluated 3 days after subcutaneous implantation of sheets (A), long rods (B), dots (C), and fibers (D) in Blab/C mice. Cell recruitment (E) and capsule thickness (F) was determined using ImageJ analysis. Scale bar represents 100 µm.

Fiber length and diameter are two important parameters that can modulate acute inflammation [138]. The dimensions of the fibers used in the present study, are similar to other fiber particles that have been reported to prolong inflammation. Fibers with a diameter below 100 nm, and a length of at least 4 µm were shown to stimulate inflammation in the peritoneal cavity. The morphology of the fibers cause macrophages to undergo frustrated phagocytosis, which can subsequently promote chronic inflammation. It is most likely that the length of the HA fiber particles is the instigator of the observed inflammation in vitro and in vivo.
Conclusions and Future Work

The present work emphasizes the role that nanostructures play in modulating the inflammatory response.

The present results show that macrophages adopt an elongated and flattened morphology as well as produce greater amounts of intracellular ROS \textit{in vitro} when cultured on alumina with 200nm pores versus membranes with 20nm pores. When implanted \textit{in vivo}, the 200nm membrane also stimulated greater production of proinflammatory cytokines and increased cell recruitment to the implantation site, thereby suggesting that the larger pore size induces a more proinflammatory behavior.

Primary human PMNCs produced higher total ROS when in contact with the 20nm alumina compared to the 100nm alumina membranes. Trolox, a Vitamin E derivative, was loaded into nanoporous alumina in order to scavenge ROS produced by PMNCs and MNCs. The release of Trolox occurred via burst release. After exposure to Trolox, PMNC ROS production was reduced by 8-30%, while MNC ROS was scavenged by 95%. Unlike MNCs, PMNCs exposed to Trolox exhibited a delay in ROS CL peak time when cultured on both polystyrene and nanoporous alumina. The antioxidant therefore seems to be working in a manner distinct from its antioxidative activity. Furthermore, nanoporous alumina membranes preloaded with Trolox were able to scavenge ROS produced from PMNCs and MNCs successfully.

Four distinct HANP morphologies (sheets, long rods, dots, and fibers) were synthensized and characterized. The fiber particles caused the highest amount of toxicity in PMNCs and hDFs. MNCs however experienced the greatest amount of toxicity following exposure to the dot particles. PMNCs and MNCs both produced the greatest amounts of ROS when treated with the fibers. Caspase 3/7 activity, a marker for apoptotic behavior, was also increased for all cell types following fiber exposure. In addition, when implanted subcutaneously \textit{in vivo}, the fiber particles caused an increase in fibrotic capsule thickness compared to the long rod particles.

Future work will focus on improving the drug release profile of Trolox from nanoporous alumina by chemically modifying or immobilizing Trolox to the membrane. Additionally, we will evaluate the inflammatory response to Trolox-conjugated nanoporous alumina not only with primary leukocytes, but also with platelets and the complement system, \textit{in vitro} and \textit{in vivo}. 

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In recent unpublished work we have observed interesting PMNC behavior following exposure to HANPs: a matrix, resembling NETs, was formed after exposure to fibers (Figure 26).

*Figure 26. PMNC NET formation following exposure to the fiber particles.*

NETs are released during PMNC induced cell death in order to trap extracellular foreign material [61, 139]. Future work will focus on understanding the mechanism of how HANP morphology affects NETosis.

There is increasing concern that exposure to nanoparticles during pregnancy can adversely affect fetal development [140]. Upcoming experiments will therefore also include a zebrafish exposure model to evaluate the effects of HANP morphology on embryological development.
Svensk sammanfattning

Nanomaterial är material som består av komponenter mindre än 100 nm i åtminstone en dimension. Under de senaste två decennierna har intresset ökat för att undersöka användningen av nanomaterial i biomedicinska tillämpningar som till exempel i biosensorer, klinisk diagnostik, och vävnadsregenerering. Nanostrukturer är inte bara konstgjorda, utan kan också hittas i naturen. Till exempel, innehåller vaxkristallerna som täcker ett lotusblad, spindelsilke och den extracellulära matrisen (ECM) som finns i kroppen komponenter av nanostorlek. Alla material, inklusive nanomaterial, som är avsedda att användas i kroppen defineras som biomaterial. Nanostrukturer i form av partiklar, kristaller, eller porer, kan främja regenerering av vävnad genom att ”härma” funktioner hos nanostrukturerna i den extracellulära matrisen. Att använda nanomaterial är därför en lovande strategi för biomedicinska tillämpningar.

Även om nanomaterial är fördelaktiga i många avseenden, kan överdriven exponering ge toxiska effekter och orsaka ökad produktion av reaktiva syreradikaler (ROS), DNA-skador och slutligen celldöd. En grundlig förståelse för interaktioner mellan celler och nanomaterial är nödvändig för att bättre kunna designa funktionella biomaterial. I det här avhandlingsarbetet utvärderas vilken effekt nanostrukturer har på inflammation med hjälp av två olika modeller: nanoporös aluminiumoxid och hydroxyapatitnanopartiklar (HANP).

Nanoporös aluminiumoxid har utvärderats för olika biomedicinska tillämpningar såsom en potentiell beläggning av benimplantat och beläggning av stentar för läkemedelstillförsel. Den första halvan av avhandlingen fokuserar på hur storleken av nanoporer påverkar inflammation in vitro och in vivo. Makrofager odlades på nanoporös aluminiumoxid med två olika porstorlekar, 20 nm och 200 nm. ROS-produktion och makrofagernas morfologi utvärderades i 7 dagar. Makrofager på material med 200 nm stora porer producerade genomgående högre nivåer av ROS jämfört med makrofager på aluminiumoxid med 20 nm porer. Cellerna visade även en ”aktiverad” morfologi (långsträckta och platta) när de odlades på aluminiumoxid med 200 nm porer, jämfört med en rund morfologi som kunde ses på aluminiumoxid med 20 nm porer. Aluminiumoxidmembran implanterades även subkutan i möss i två veckor för att utvärdera fibrotisk respons och produktion av proinflammatoriska cytokiner runt implantaten. Membranen med 200 nm porer gav upphov till en större produktion av cytokiner samt gav ökad cellrekryte-
ring till området kring implantatet, vilket tyder på att den större porstorleken inducerar ett mer proinflammatorisk beteende.

För att minska ROS-produktionen laddades nanoporösa aluminiumoxidmembran (20-, 100-, och 200 nm i por diameter) med Trolox. Antioxidanten Trolox är ett hydrofilt E-vitaminderivat som effektivt neutraliserar ett flertal fria radikaler. Primära humana polymorfonukleära celler (PMNC) och mononukleära celler (MNC) med och utan tillsats av Trolox utvärderades. PMNCs producerade högre total ROS mängd när de odlades på aluminiumoxidmembran med 20 nm stora porer jämfört med 100 nm porer. Produktionsen av ROS hos MNC påverkades dock inte av porstorleken. Aluminiumoxidmembranen laddades sedan med Trolox och frisättningen utvärderades. ROS-produktionen för PMNC minskade med 8-30%, medan ROS för MNC minskade med hela 95%.

Nanopartiklar används i en ökad omfattning i många olika produkter såsom i kosmetika, livsmedel, kläder och elektronik, därav ökar även sannolikheten och intensiteten för exponering. Partiklar gjorda av hydroxyapatit (HANP) har tex utvärderats som potentiella läkemedelstransportörer och benersättningsmaterial. Dessa partiklars effekt på immunförsvaret har tidigare inte utvärderats i någon större omfattning.


Sammanfattningsvis bekräftar denna avhandling nanostrukturens betydelse vid modulering av inflammation, och kommer förhoppningsvis att stimulera till fortsatta studier om hur nanomaterial samverkar med de biologiska systemen.
Acknowledgements

There have been so many people that have helped me over the course of my career, and have had a positive influence on my life.

I would first and foremost like to thank my supervisors, Marjam and Håkan for their unconditional support these past 4 years. Marjam, words cannot begin to express how thankful I am to you. Little did I know, six years ago almost to the day we met at Arlanda airport, how much you would change my life. You have not only been my supervisor, but family to me here in Sweden, and made the process of living away from home so much easier. Through your optimism, motivation, and true love for science you taught me to never give up and to always see a positive in every negative result. You always believed in me when I didn’t believe in myself. Working with you has been the highlight of my life. Even in my wildest dreams, I can only hope to be half as good of a mentor as you were to me.

Håkan, thank you so much for all the opportunities and support you have given me. I have really cherished every discussion we have had together, whether it be from scientific discussions, to sports, or Seinfeld episodes that we have both liked. You have an incredible ability to encourage and inspire in a way that is rare to find in supervisors, and I am so thankful that I have been able to experience that. I hope like you, I am able to cultivate an environment where people have the freedom to develop their own ideas.

Wei, thank you so much for helping me with so many different experimental techniques in the lab, for the long hours in the SEM room, for always believing that we will get to the finish line with the HA paper, and for always making me feel that every question has value. Cecilia, thank you so much for all the discussions we have shared together, for being my walking encyclopedia in statistics, and for all the helpful advice you have given me over the years. Caroline, your friendship is something I am grateful to have. Thank you so much for all the adventures we have had in and outside of the lab, be it from microCTing zebrafish to running for that last train back home! You will always be the Neal to my Peter!

Thank you to everyone, past and present, in the MIM group for making it such a fun environment to work in. I will really miss seeing your faces everyday! Xi, I am so happy that you decided to move to Uppsala, so we could finally be in the same place and not have a long distance friendship like we have had for pretty much the whole time we have known each other! Thank you so much for putting up with me before my morning tea, for help-
ing me with all my images and figures in my papers and thesis, and for al-
ways making me smile, even in the saddest and most frustrating of times.
Charlotte, I am so lucky to have you as my office mate! We have had such a
great adventure with all our crazy side experiments that we have going on in
our office! Thank you for being there for me when I needed someone to talk
to, for helping me with the Swedish summary, and for accepting the fact that
I will unintentionally kill every plant that is ever placed in our office! 😊
Celine, your ability to change someone’s mood with just your smile is amaz-
ing! Thank you so much for all the times we have spent together and for the
laughs that we have shared. You are such a strong person, and that has in-
spired me to find strength in myself. Ale, you have been one of my closest
friends here in Sweden from the beginning, and understood what it felt like
to be homesick and miss the sunshine and warmth from our home countries.
Thank you for always supporting me in every decision I made, for your hon-
esty and trust in me, for our shared love in 80s music and Felicity. I am so
happy that you are back here in the lab! Song, it has been so much fun to
work with you! Thank you so much for your patience and determination
every step of the way on our HA project, for all the countless number of
SEM hours we spent together in hopes to see something interesting, and for
always helping me whenever I needed it no matter the circumstance. Tor-
björn, you are such a funny person, thank you so much for always making
me laugh, for taking me to buy my very own pair of ice skates, and for al-
ways emphasizing the importance of celebrating every win, even if it is with
Balsam! Satwik, my chocolate hero 😊, I am so happy that our paths crossed.
Thank you for all the times we have shared in and outside of the lab, its been
great fun to get to know you. Kathryn, you were the best flatmate I could
have ever asked for. Thank you for helping me transition into life in Upps-
lä, and for your friendship! I promise to visit you soon! Andreas, thanks for
all the fun times we have had together and for all the help with our favorite
material, nanoporous alumina!

Thank you to everyone past and present in the cell lab for making it
such a fun environment to work in, and for all the helpful advice we’ve
shared. Natalia, thank you so much for answering all my questions I had on
nanoporous alumina, for teaching me the blood isolation procedure, and for
always being supportive and encouraging, even when it came to the endless
LDH experiments! Viviana (V), thank you so much for your company and
help down in the cell lab, for reminding me to take everything one day at a
time, and for being my gym buddy.

Thank you to Jonatan for helping me with any computer problem I
ever had, and thanks to Ingrid R and Sara, for all your help with any admin-
istrative question and calls to Migration board for me!

Thank you to all my collaborators that I have had the honor to work
with these past 4 years. A huge thanks to the Tang lab at the University of
Texas at Arlington for all the help on my in vivo experiments. Dr. Tang, I
am so thankful that I have had the privilege to work with you throughout my
masters and doctorate studies. If it wasn’t for you, I would have never been introduced to Sweden and working in Uppsala. Thank you so much for always having my best interest at heart, for welcoming me back to the lab every winter, for the support and help you have given me, and for always holding me to a higher standard. Dr. Shen and Dr. Hong Weng, thank you so much for helping me with the in vivo studies and for showing me how to run histology! Thank you to Professor Andreas Heilmann and Annika Thormann for fabricating the nanoporous alumina samples. Thank you to Stefano Rubino, for helping me with all the microscopy analysis for the HA particles, and for being patient with me when I was super stressed! Thank you to Viktoria Westlund, for making the HA particles and for being so helpful with all the questions I had when we were trying to make them ourselves. Thank you to Thomas Lind, for helping me with all my PCR experiments and for answering any question I had on bone biology. Thank you to Katarina, Ling, and Beata at the zebrafish platform for introducing me to the world of zebrafish, for being so helpful with all my experiments, and for always being a phone call away to discuss any questions or concerns I had.

Thank you to everyone at MAIIA for making it so easy to transition to work over there. I am really looking forward to working with you all!

A huge thank you to my 2nd family, the Karlssons and Otts! Thank you to Jan, Fari, Mina, Marjam, Sascha, Nora, Milo, and Dante for welcoming me into your house and hearts 6 years ago. I was so scared in the beginning to move so far away from home, but you guys are family to me, so it doesn’t feel like I am living away from home anymore. Jan and Fari, you have been so supportive and encouraging in anything that I have done, be it from brainstorming ideas for my experiments, to helping me find a flat to live in. I am forever grateful to have you in my corner and so excited that the next chapter in my life will involve working for you guys at MAIIA. Sascha, thank you so much for your humor, for always being so easy to talk to, and for answering all my chemistry questions that I have had. Nora, Milo, and Dante, you guys are the best kids ever and I am so happy that we get to do so many fun things together! Your smiles really brighten even the grayest of days. Mina, you were my first friend in Uppsala and I have never forgotten that! Thank you so much for always being there for me, for listening when I needed to talk, for being a step ahead in what good TV show is on, and for having the same taste in clothes that I have, so that we can share and swap!

A big thanks to all my friends here in Uppsala that made living here so much like home for me. Leanne, I am so thankful to have you as a friend and for being there for me every step of the way, be it from Uppsala or Japan! Friendship really knows no distance and I am so grateful for that, miss you loads! Nadja and family, thank you so much for taking care of me, feeding me amazing food, for being so incredibly supportive all the time. Our meet cute will always be my golden standard to compare to! Sharn and Marcus, aka Sharcus you guys are so lovely, thank you so much for all the gym classes, for the amazing dinners and desserts, and for helping me with all my
editing! Aila, it’s been so much fun playing flute duets with you, thank you for helping me channel my stress out constructively through music! Johan, Lucie, and Maël, such a cute family! Thank you for all the times we have spent in and outside of Ångström, from our fika Fridays, to our intolerable obsession with Roger Federer, it’s been a blast to get to know you. Arvid, I feel so lucky that you were seated right next to me at my first gasque at Norrlands nation. Thanks for all the conversations we have had at our version of “the Restaurant” and for the matches we have watched together. Kaspar, thank you for all our fika Fridays, for your honesty and optimism. James, thanks for understanding what a good fry up can do for the soul, and thanks for the fish! ;) Nico and family, you guys always put a smile to my face, thank you for all our talks, dinners, and words of encouragement you have given me. Sujit and Zankruti, thank you for all the masala chais, for the endless laughs we have shared, and for all those late nights in the lab! A special thanks to the Stockholm gang, Jhunumaashi, Mesho, Sumit, Ipsita, and Ashish for the time we have spent together, making fun of each other, for all the food we have gorged ourselves over, and taking care of me, always.

Even with the distance and a 7 hour time difference, my friends and family back in Dallas have always made me feel like I have never left. Mr. Reyna, you are one of the best science teachers I have ever had, and I thank you for inspiring me at such a young age to reach for more. Mrs. Woolf Barbknecht, you have made a lasting impact on my life, always making me feel that anything is possible if you try your best, thank you so much for everything. A huge thanks to the TAMS girls, for all the craziness that we have gotten ourselves into over the last 12 years, for always making time for me when I came home every year, and for all the nights we spent talking the night away! To all my friends at Lakeview, thank you for staying in touch with me throughout the years. Victoria, you have been such a good friend and I am so happy that after 15+ years nothing has changed. Your ability to persevere through anything is a total inspiration, and has impacted how I approach everything, thank you for being that for me. Ashwin and Sapna, thank you for always being only a phone call away, for guiding me through my worst days in the lab, and for coming to meet me whenever I’m home. Rahil, thank you will never be enough for what you have done for me. I am grateful to you, for our time we have had together, for the Target, Ross, Payless shopping sprees, to our annual NBA games we go to. Congratulations to you and Martha, and I wish you all the best for the next chapter in your life together! Ashleigh, aka AshMcNash, I was trying to remember how we first met, and I don’t even think that memory exists, because it feels like I have known you my whole life. Thank you for being my partner in crime, for always being there for me no matter what time what place, for answering my calls at 2 am, for our shared love of RF, and for always knowing exactly what to say at any given time. You are the best!
To my amazing family, you guys are my rock, and without you I wouldn’t know which way to go. *Ma and Papa,* how do I even begin to explain to you what you guys mean to me. You are the best parents and friends I could have ever asked for. Thank you for showing me what the value of hard work, dedication, and discipline can get you in life. You guys have this unshakeable belief in me and that has been the thing that keeps me going. Even though our professions are different, you have always shown interest in every experiment I have done, you deserve this thesis as much as I do.

Thank you for all the sacrifices you have made for me, for being my number 1 fan for 28 years, for setting the best example as people and raising the bar. I hope one day I can repay you for all the things you have done for me, but I would need over 100 lives to even come close. *Dadu and Appa,* you both have inspired me at an early age to never settle for less, always reach your full potential, and for always being curious, thank you for being my guiding light everyday. *Ratki (and Wilbur),* the apple of my eye, the best sister in the world, thank you for being you, for being the yin to my yang, for visiting me, and for all the chocolate covered hot dogs ;) *Michael (2),* I am so happy that you are part of our family now, and so thankful to have you in my life. Thanks so much for visiting me, and keeping an eye on my sister while I am away! Last but certainly not least, a huge thank you to my wonderful husband, *Michael (1).* Not only are we partners in life, but partners at work. Many people asked me if it was hard to work with my husband, but I know I can say that its been the most rewarding, challenging, unforgettable experience of my life! Thank you so much for never letting me give up, for always pushing my limits and making me redraw new boundaries, for fully accepting me for who I am. I have learned and grown so much from you as a person, and I am so thankful to have you by my side. You will always be my proudest accomplishment. I love you more than french fries, *always.*
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A doctoral dissertation from the Faculty of Science and Technology, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science and Technology. (Prior to January, 2005, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Science and Technology”.)