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MACROPHAGE PHAGOCYTOSIS OF POLYETHYLENE PARTICULATE IN VITRO

by

Irina Voronov

**A thesis submitted in conformity with the requirements
for the degree of Master of Applied Science
Graduate Department of Chemical Engineering and Applied Chemistry
University of Toronto**

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Macrophage phagocytosis of polyethylene particulate in vitro

Master of Applied Science, 1997

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ABSTRACT

Polyethylene particles are the major constituents of the material debris formed as a result of orthopaedic implant wear. The purpose of this study was to develop an *in vitro* model which would allow the introduction of polyethylene particulate into macrophages. UHMWPE (18-20 μ m) and HDPE (4-10 μ m) were characterized by XPS and FT-IR and suspended in soluble collagen type I which was subsequently solidified on glass coverslips. Mouse cell line macrophages (IC-21) were established on the collagen-particle substrata and maintained for up to 96 hours. The response of the cells to the particles was examined by light microscopy, TEM, and SEM. Histological analysis of the samples revealed that the macrophages surrounded larger particles (18-20 μ m) and the cells appeared to be attached to the surface of the particles, while the smaller particles (4-10 μ m) had been phagocytosed within two hours. Inflammatory cytokines, lysosomal enzymes, and prostaglandin E₂ were released into the medium, and IL-1 α , IL-1 β , PGE₂, β -galactosidase and hexosaminidase levels were significantly increased over control values. These results validate the model as means of studying the specific *in vitro* interactions of polyethylene with cells.

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1. INTRODUCTION

1.1. CLINICAL PROBLEM

It has been estimated that 300,000 to 400,000 total joint replacements are implanted each year in North America alone.¹ Aseptic loosening of total joint prosthesis has been established as the number one cause of implant failure.¹⁻⁶ It is defined as a loosening of the prosthesis in the absence of an infection.^{1,4-6} Numerous factors, both biological and mechanical in nature, are responsible for prosthesis failure. The patient's general health, predisposition to allergies, patient's lifestyle, infection, and osteolysis represent the biological factors, while surgical problems, prosthesis design, mechanical wear, and stress shielding are the mechanical factors that contribute to implant failure.

Revision surgeries of the failed implants have demonstrated the presence of a membrane at the bone-implant interface.⁶ This membrane forms a thick inflammatory tissue in some patients, and remains thin and non-progressive in others.⁶ Histological evaluations of the membranes retrieved at revision surgeries reveal that thin, non-progressive membranes contain mainly fibroblasts and a few macrophages, with almost no particulate debris, while thicker inflammatory membranes have abundant macrophages, fibroblasts, and foreign body giant cells which were surrounding and engulfing particulate debris.^{2,3} The characterization of these membranes have demonstrated the presence of various types of particulate wear debris consisting of metal, polyethylene and polymethyl methacrylate, with sizes ranging from submicron values up to hundreds of microns.⁶ Immunohistochemical evaluation revealed the presence of a number of inflammatory mediators which are known to take part in inflammation, wound healing, and bone remodeling.⁵⁻⁷

Aseptic loosening of orthopaedic implants is often accompanied by severe osteolysis.^{4,6,8} Histological evaluation of the osteolytic lesion has demonstrated active bone resorption.⁶ It has been hypothesized that the inflammatory mediators, including $\text{TNF-}\alpha$, IL-1, IL-6, PGE_2 , which have been released by activated macrophages are capable of stimulating osteoclasts to resorb bone.^{4,6,8} These mediators are thought to inhibit bone deposition by osteoblasts, therefore,

breaking the balance between osteoclasts and osteoblasts in bone turnover.^{4,6,8,9} Another factor contributing further to bone lysis could be the presence of lysosomal enzymes which are released as a result of cell death or/and frustrated phagocytosis. These enzymes can cause tissue damage as well as lower the pH of the surrounding tissue. Furthermore, it has been shown that activated macrophages themselves are capable of direct bone resorption thus contributing to bone loss at the bone-implant interface.^{7,10,11} Therefore, three biological mechanisms contribute to bone resorption at the bone-implant interface and all of these processes are in part related to particulate activation of macrophages.^{3,6,8,12}

Polyethylene particulate is considered to be the most abundant particle of the wear debris and is believed to be causing a stronger inflammatory response than any other constituent of the wear debris.⁶ It is not fully understood why numerous wear debris are produced in some cases, while there is no accumulation of wear particles at the bone-implant interface in others. It is also not clear how the particles migrate from the high friction areas of the implant to the inflammatory membrane at the bone-implant interface, however, several mechanisms have been proposed. Schmalzried et al.¹³ introduced a concept of the effective joint space in order to explain the migration of the wear debris around the implant. According to Schmalzried's hypothesis, wear debris is dispersed in joint fluid. Depending on contact between the bone and the prosthesis, particulate debris is carried by joint fluid to and along the bone-implant interface, and through the soft tissues and bone.¹³ Thus, the particles are delivered to distant sites along the bone-implant interface and stimulate bone resorption far from the place of wear generation. Therefore, the integrity of the bone-implant interface is crucial to limiting the transport of particles and hence ensuring the survival of the prosthesis.⁶

Since the inflammatory membrane is located at the bone-implant interface, and the inflammatory mediators are believed to have an effect on bone remodeling,^{4,8,9} a continuous cycle of bone resorption/wear formation can ensue in the following manner. The bone resorption ultimately provokes mechanical instability which accelerates implant loosening by increasing the production of more wear debris. This, in turn, increases the flux of inflammatory

cells to the injured site, which is then followed by an increased secretion of inflammatory mediators. This propagates the subsequent bone resorption around the implant.

Even though numerous clinical, *in vivo*, and *in vitro* studies have been performed, the exact mechanism of aseptic loosening is not well understood.⁶ In order to treat (or prevent) aseptic loosening by means other than revision surgery, it is crucial to understand the processes involved. The potential therapies could include blocking the inflammation, blocking bone resorption, or enhancing bone healing and formation around the implants. However, prior to achieving these goals a better understanding of mechanism of aseptic loosening is clearly required. Part of this understanding will come from the study of particulate interactions with cells and specifically macrophages. The purpose of this thesis was to develop a model that would allow for the study of polyethylene particulate phagocytosis. Polyethylene particles are of specific interest since they are the least studied component of particulate wear debris, but yet the most abundant particle type found in the inflammatory membrane at the bone-implant interface. This model may be further used to contribute to a better understanding of the mechanism of aseptic loosening and to investigate the process of bone lysis by cells which have been activated via exposure to polyethylene and other particulate matter.

1.2. PHYSICAL/CHEMICAL FACTORS CONTRIBUTING TO ASEPTIC LOOSENING

1.2.1. General design of total hip prostheses

Total hip replacements were first introduced in England by two independent investigators, McKee and Charnley.¹⁴ Between 1958 and 1961 three hundred total hip prostheses were implanted by Charnley. Currently about 400,000 total joint replacements are performed each year in North America alone.¹ In the last 35 years the general design of joint implants has remained fairly constant. Total hip implants usually consist of a metal stem with a metal ball and a metal socket covered with a polymer (usually UHMWPE).^{14,15} The metallic components of the prostheses are attached to the bone with polymethyl methacrylate cement in older and less active patients, while cementless prostheses are usually recommended for younger

recipients. The association of bone cement particles (PMMA) with the observation of aseptic loosening had originally led scientists to refer to the clinical problem as “cement disease”.⁶ As a result, different methods of fixation were developed and cementless prostheses were introduced. Fixation of uncemented components was enhanced by making the surface of the metallic stem porous, e.g. by coating the surface with beads. This system facilitated implant fixation by allowing bone ingrowth to occur within the porous structure.¹⁵ This close contact between bone and implant is very important because it provides stability to the implant and prevents the development of a conduit for particles and, therefore, the development of osteolysis.

1.2.2. Materials used in total hip prostheses

The choice of materials for total hip prostheses has been narrowed down to three popular polymers (acrylate cement, polyethylene, and Silastic®) and fourteen metal alloys (three iron-based, seven cobalt-based, and four titanium-based).¹⁴ Composite structures, such as the combination of cobalt alloy femoral heads and titanium alloy stems have also been used in order to take advantage of high wear resistance of cobalt alloys and the high fatigue strength of titanium alloys.¹⁶

1.2.3. Factors contributing to the mechanical properties of UHMWPE

UHMWPE has been used as the material of choice for the sockets of total joint implants since 1962.¹⁴ This material has a higher resistance to wear and creep than does Teflon®, and since the 1970s the polyethylene/metal pairing has become the most common choice for joint prosthesis of the hip, knee, ankle, shoulder, elbow, and wrist.¹⁴ UHMWPE has a molecular weight in the order of millions as compared to 10,000 to 500,000 for other polyethylenes.¹⁴ UHMWPE is characterized by high notched bar impact strength, high abrasion resistance, chemical resistance, and a low friction coefficient.¹⁴

1.2.3.1. Synthesis. UHMWPE is synthesized by chain polymerization when ethylene molecules are added to catalyst particles.¹⁴ The catalysts usually employed in the process are made from titanium chloride and organoaluminum compounds such as triethylaluminium or

diethylaluminium chloride. These catalyst particles are usually 5-10 μm in size, while newly synthesized UHMWPE particles are not uniform and vary in size from 63 to 250 μm .¹⁴

1.2.3.2. Contaminants/residues. Different types of residues are encountered in UHMWPE powder. Titanium and aluminum salts, which can be present as hydroxides, chlorides, and hydroxychlorides, as well as chlorine ions have been detected.¹⁴ Small amounts of the calcium salt of an organic acid are added as a corrosion inhibitor and therefore are also present in the powder.¹⁴

1.2.3.3. Processing. After synthesis, the powder of UHMWPE is processed into implant parts using two processing methods: the compression sintering technique and the injection moulding technique.¹⁴ UHMWPE has a very high molecular weight which does not permit the material to be transformed into a free-flowing mass by heating.¹⁴ Using the compression sintering technique, UHMWPE powder is inserted into the moulds and then air is forced out of the moulds by an oil-hydraulic mechanism.¹⁴ During the plasticizing phase, heat is applied to the moulds (between 200°C and 250°C) until the powder is plasticized.¹⁴ Then the heat is slowly and uniformly removed in order to minimize the shrinkage stress from the cooling process and to achieve optimal physical properties.¹⁴ The injection moulding technique requires high injection pressures, processing temperatures between 200°C and 230°C, and mould temperatures between 40°C and 70°C.¹⁴

1.2.3.4. Sterilization. Implants made of UHMWPE are sterilized by γ -irradiation.¹⁴ Even though this method of sterilization has numerous advantages, such as a high microbicidal effect, it changes the characteristics of the material.¹⁴ Formation of radicals, polymerization, depolymerization, and oxidation take place after the polymer has been exposed to γ -rays. It has been shown that these changes in material structure can lead to changes in the polymer's ultimate mechanical properties, which, in turn, may affect the function of the prosthesis and cause the formation of wear debris.¹⁴

1.2.4. Formation of wear particles.

1.2.4.1. Hypothesis of wear formation. It has been proposed by Clarke et al.¹⁷ that in stable, well-fixed implants, the main trigger mechanism for osteolysis and accompanying loosening is the release of UHMWPE and metal particulates from the articulating surfaces. Wear of the joint's bearing surface is an inevitable consequence of activity. The more the patient uses the joint and the more active he or she is, the greater the amount of wear debris that will be released into the tissues and the greater the risk of bone loss.¹⁷ Clarke's studies estimated that 20 million to 40 billion UHMWPE particles shed into the joint space every year. Clarke et al.¹⁷ hypothesized that even in well-fixed implants there is micromotion between (1) the stem of the implant and the bone cement and (2) between the polyethylene socket and the metal ball. This micromotion produces some metal and polyethylene debris. Any debris smaller than 5 μm is phagocytosed by macrophages, which in turn secrete inflammatory mediators as a result of their activation.¹⁷ Therefore as more particulate is produced at these interfaces, more particles are phagocytosed by macrophages, and more inflammatory cytokines are released into surrounding tissues.¹⁷ Hence, extensive bone loss and loosening of the implant are the inevitable results of this process.

However, Clarke's scenario has not been the only hypothesis put forward. Pienkowski et al.¹⁸ postulated a hypothesis based on the study of virgin UHMWPE powder. This group observed that submicron size spheres, which exist within the processed material, retain their morphological identity throughout the processing, sterilization, storage, and wear.¹⁸ Pienkowski et al.¹⁸ hypothesized that submicron UHMWPE wear debris may originate not only from wear processes and friction *in vivo*, but also from release of these submicron particles found in virgin UHMWPE. These particles will then contribute to macrophage activation and bone resorption.

1.2.4.2. Particulate characterization. Since it has been suggested that, in the presence of debris, macrophages can release various cytokines and factors that induce bone loss, it has been hypothesized that the composition, the size, the amount, and possibly the shape of the particles may be important variables that influence macrophage response and subsequent osteolysis.⁶ As a result of these thoughts the characterization of wear debris has stimulated an

Reference	Digesting agent	Particle type	Particle size	Particle shape
12	NaOH	UHMWPE	0.07-6.3 μm 0.57-12.2 μm	rounded elongated ("fibrils" and "shreds")
19	KOH	UHMWPE Ti	0.1-2.0 μm 1.2-10.0 μm 1-200 μm 10-400 μm	spheroids fibrils shards flakes of wire
20	concentrated nitric acid	polyethylene, metal	0.63 μm	no consistent particle morphology
21	Soluene 350	polyethylene metal	2.0-13.0 μm 0.8-1.8 μm	did not evaluate shard-shaped
22	crude papain, NaOH, KOH	HDPE, Ti	did not evaluate	did not evaluate

Table 1.1. Summary of particulate characterization studies.

extensive area of research. A summary of particulate characterization studies is presented in Table 1.1. There are some variations in the methods for particulate isolation. In order to digest retrieved inflammatory membranes Campbell et al.¹² and Shanbhag et al.¹⁹ used strong bases, Margevicius et al.²⁰ employed concentrated nitric acid, while Lee et al.²¹ utilized a commercial tissue solubilizer. Campbell et al.²² found that crude papain offered more complete digestion than either purified papain or sodium hydroxide, but they still noted apparently undigested cellular debris in filtrates. The methods of analysis and the results obtained varied from group to group. Lee et al.,²¹ on the basis of light microscopy, suggested that the mean size of the polyethylene particles was two to four by eight to thirteen micrometers; other authors have recognized that many particles are smaller than the resolution of the light microscope.¹² Margevicius et al.²⁰ measured the particles with an electrical resistance particle-size analyzer (Coulter Multisizer) and studied the particle morphology with scanning electron microscopy. They estimated that the majority of the particles retrieved from the synovial membranes are submicron in size; other investigators^{12,19,21,22} have reported similar results. However, it was

reported by Margevicius et al.²⁰ that this particular Coulter Multisizer particle analyzer has finite detection windows and therefore cannot analyze particles below 0.58 μm and above 20 μm .²⁰ It has been observed that the particles were irregularly shaped, and did not have rough edges. This could be due to the effect of strong bases and acids during the extraction processes.^{12,19,21,22} All of the above studies^{12,19,21,22} have concentrated on the isolation of UHMWPE particles, since it is believed that polyethylene is a key particle and plays an important role in macrophage activation and bone resorption. Nevertheless, metal and PMMA particles were also present in the samples. However, it has been observed that PMMA particles melted and clumped together as a result of tissue digestion used for the particle recovery.²⁰

1.3. BIOLOGICAL FACTORS CONTRIBUTING TO ASEPTIC LOOSENING

1.3.1. Characterization of the inflammatory membrane.

It has been consistently observed that an inflammatory membrane is formed at the bone-implant interface and is associated with particulate debris.⁶ Histological evaluation of this tissue has demonstrated that it consists of fibroblasts, macrophages, foreign body giant cells, and occasional T cells and B cells.^{4-6,8,9,23} Characterization of this tissue has also shown that the inflammatory membrane has all the characteristics of a synovial membrane and, therefore, is also referred to as a pseudosynovial membrane. Just like the synovial membrane, the periprosthetic tissue contains collagen type I and type III as the predominant extracellular matrix proteins.²⁴ Closer analysis of the inflammatory membrane has revealed the presence of particulate wear debris.^{4-6,8,9,23} It was observed that all ($<5 \mu\text{m}$) small particles were associated with macrophages, while large ($>10 \mu\text{m}$) particles were surrounded by foreign body giant cells.¹⁷ It has been reported that the most noticeable cellular reactions, such as inflammation and foreign body granuloma formation, are associated with the response to polymeric (polymethyl methacrylate and/or polyethylene) particulate.⁶

Immunohistochemical evaluation of the inflammatory membranes confirmed that macrophages were the most abundant cells in the tissue and that they were actively involved in phagocytosis of the particulate.^{2,5,6,23} It was also demonstrated that T cells made up about 10% of the inflammatory cells, while B cells were occasional.² The presence of T cells could imply their involvement in the amplification of the inflammatory response, or in a specific delayed hypersensitivity reaction to particulate debris.² Thus, macrophages are the cell type responsible for particle phagocytosis and the development of a model to study the macrophage-particle interactions would be useful in understanding the mechanism of aseptic loosening.

1.3.2. Macrophages and their role in aseptic loosening.

Macrophages (or mononuclear phagocytes) are the scavenger cells of the immune system.²⁵⁻²⁹ They play an important role in host defense, bacteria and foreign body destruction, and in wound healing and tissue repair. Macrophages are mature and fully differentiated cells that are located in different parts of the body and, therefore, have different properties.²⁵ These cells belong to the hemopoietic system in the bone marrow.^{26,29} The progenitor of macrophages is the granulocyte-monocyte colony forming unit (CFU-GM) stem cell; it gives rise to the monoblast, which divides once to form two promonocytes; their division leads to the formation of two monocytes. Monocytes then migrate from the bone marrow into the circulation, where after about 24 hours they migrate into tissues to become macrophages.^{26,29} Depending on the type of tissue, different types of macrophages are formed. These cells do not divide and their number depends on the influx of fresh monocytes into the tissues.²⁶

Macrophages perform numerous functions. The classical function of these cells is phagocytosis, killing, and digestion of microorganisms. Macrophages secrete a great variety of biologically active molecules, such as enzymes, components of the complement system and coagulation cascade, growth factors, cytokines, reactive oxygen and nitrogen intermediates (see Table 1.2).²⁵⁻²⁹

Polypeptide hormones and cytokines:

Interleukin-1 α and β (IL-1 α , β), interleukin-6 (IL-6), tumour necrosis factor α (TNF- α), interferon γ (IFN- γ), platelet-derived growth factor (PDGF), fibroblast growth factor, transforming growth factor β (TGF- β), colony stimulating factor-1 (CSF-1), granulocyte colony stimulating factor (G-CSF), granulocyte-macrophage colony stimulating factor (GM-CSF), monocyte-derived neutrophil chemotactic factor

Bioactive lipids:

6-ketoprostaglandin F₁; 12-hydroxyeicosatetraenoic acid; leukotrienes B₄, C, D, E; platelet activating factor (PAF); prostacycline; prostaglandins E₂, F₂, I₂; thromboxane B₂

Coagulation components:

factors V, VII, IX, X; prothrombin; prothrombinase; plasminogen activator inhibitors; tissue thromboplastin

Complement components:

C1, C2, C3, C4, C5; factors B, D, H, I (C3b inactivator); properdin

Enzymes:

Acid lysosomal hydrolases:

aryl sulfatase; cathepsins B and D; cholesterol esterase; deoxyribonucleases; glycosidases; phosphatases; proteinases; ribonucleases; tryglyceride lipase

Neutral proteinases:

angiotensin convertase; collagenases; elastase; plasminogen activator; stromelysin

Others:

arginase; lipoprotein lipase; lysozyme; phospholipase A₂; transglutaminase

Reactive oxygen and nitrogen metabolites:

O₂⁻, H₂O₂, OH⁻; NO⁻, NO₂⁻

Steroid hormones:

1,25-dihydroxyvitamine D₃

Table 1.2. Secretory products of macrophages. Adapted from Austyn et al.²⁵ and Nathan.²⁷

Macrophages possess a variety of surface receptors that allow these cells to react quickly to different types of stimuli.²⁵ Some receptors enable the cell to recognize the biochemical features of the pathogen, while other receptors distinguish host proteins attached to the surface of the foreign body.³⁰ Major surface receptors of macrophages are summarized in Table 1.3.

Receptor	Abbreviation	CD	Ligand
Fc receptors	Fc γ RI	CD 64	monomeric IgG2a
	Fc γ RII	CD 32	ag-complexed IgG1, IgG2b
	Fc γ RIII	CD 16	IgG2b, IgG2a
	Fc α R		IgA
	Fc ϵ RII	CD 23	IgE
Complement receptors	CR3	CD11b/CD18	complement C3bi, others
	CR1	CD 35	complement C3b
Carbohydrate receptor	MFR		high-mannose polysaccharides
Chemotactic peptide receptor	FMLPR		f-met-leu-phe
Extracellular matrix receptors	Fibronectin R		fibronectin
	VLA proteins		various

Table 1.3. Summary of macrophage surface receptors.^{25,30}

Immunoglobulin Fc receptors (FcR) and complement receptors (CR) enable macrophages to bind to opsonized bacteria and/or foreign bodies. Opsonization of foreign material by antibodies and complement components enhances the process of phagocytosis by macrophages.²⁵ The mannosyl-fucosyl receptor (MFR) is utilized by macrophages to bind glycoproteins that contain fucose-mannose residues, such as zymosan and LPS, and, therefore, is responsible for removal of non-opsonized bacteria.²⁵ Chemotactic peptide receptors trigger

macrophages to move towards the site of inflammation by following the gradient of chemotactic peptide, such as f-Met-leu-phe.²⁵ Extracellular matrix receptors allow macrophages to bind to the proteins of extracellular matrix, which become exposed during tissue injury, and therefore, to remain at the site of inflammation.²⁵

It has been observed that phagocytosis via complement receptors requires a “second signal”.²⁵ Furthermore, it has been demonstrated that the function of complement receptors is somehow linked to extracellular matrix receptors and that, in order for phagocytosis via complement receptors to be triggered, the cell has to be attached to some component of extracellular matrix.²⁵ This phenomenon can be linked to the developmental or activation stages of the cell, since it has been observed that freshly isolated human monocytes and resident mouse macrophages are unable to phagocytose via complement receptors, while activated and inflammatory cells can.²⁵

Since monocytes and macrophages have surface receptors for serum proteins, the engulfment occurs according to the “zipper mechanism” (cellular membrane extends around the particle/microorganism, and ligand-receptor interaction resembles a zipper action).²⁶ The foreign object becomes surrounded by the cell membrane thus forming a phagosome. Later, the phagosome fuses with lysosomes (cytoplasmic granules), containing numerous enzymes (Table 1.2), creating a phagolysosome, where the killing and digestion of microorganism occurs.³¹ These enzymes act together with reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI) for better efficiency of foreign body digestion.^{25,31}

The phagocytosis of particulate wear debris from orthopaedic hip implants is believed to proceed via similar mechanism as those described above, however, the fate of the particle following phagocytosis by a macrophage is not clear. It can be conceived that as a result of phagocytosis, macrophages could try to digest the ingested particles, however, following their inability to digest these foreign bodies, the cells could release the undigested particles as well as products of degradation. As well, macrophages are known to clear the debris (cellular and particular) from the inflammation site by migrating to lymph nodes as well as to other tissues.

Therefore, in aseptic loosening macrophages could be transferring the wear debris to other organs of the body, possibly creating a systemic response in addition to the loosened implant.⁶

1.3.3. Lysosomal enzymes.

As mentioned above, the engulfment and destruction of foreign matter (bacteria and other foreign bodies) is one of the primary functions of macrophages. After the foreign body is phagocytosed, it becomes incorporated into the phagosome, which later fuses with another cellular body, the lysosome, in order to form a phagolysosome where the digestion of the engulfed material occurs.²⁵ Lysosomes (also known as cytoplasmic granules) are packaged with numerous hydrolytic enzymes (see Table 1.2), which act at low pH and are capable of degrading proteins, lipids, polysaccharides, peptidoglycans, and nucleic acids.^{25,31,32} It has been observed that distinct populations of granules with different constituent hydrolases are present within macrophages.³² After being synthesized, the fate of each lysosomal enzyme can be either associated with the degradation of the engulfed foreign body or secretion out of the cell via exocytosis.^{31,32} A knowledge of the levels of lysosomal enzymes secreted out of the cell can provide valuable information about cell activation and about the toxicity of the phagocytosed material.^{25,32}

As well, the release of lysosomal enzymes by macrophages at the bone-implant interface could play an important role in aseptic loosening. For example, the synthesis and release of an enzyme like collagenase could lead to digestion of collagen present in the bone, while the resulting low pH could cause dissolution of the bone mineral, thus enhancing the process of bone lysis.

1.3.4. Inflammatory mediators released in response to wear particles.

Activation of the macrophage by the process of phagocytosis leads to increased production and secretion of various inflammatory mediators (Table 1.2). These mediators have numerous effects and can be chemoattractive to other types of cells, thus causing influx of fresh cells (neutrophils, monocytes, T cells, B cells, fibroblasts) to the site of inflammation. The

presence of inflammatory cytokines in the retrieved inflammatory membranes has been illustrated by numerous investigators.^{9,13,17,21} Specifically, tumour necrosis factor α (TNF- α), interleukin 1 (IL-1), prostaglandin E₂ (PGE₂), and platelet-derived growth factor (PDGF) were shown to be present in the inflammatory membranes as demonstrated by *in situ* hybridization as well as by tissue culture.^{5,6}

1.3.4.1. Cytokines. “Cytokines” is a name used for a group of proteins that are produced and secreted by numerous cells in the response to a stimulus.³³⁻³⁵ This group of proteins is involved in immunity and inflammation where they regulate the amplification and duration of the response.³³ Even though cytokines are a diverse group of proteins, they share a number of common properties. These proteins have low molecular weight (<80 kDa) and are often glycosylated. Cytokines are extremely potent agents and usually act in an autocrine and a paracrine manner at picomolar concentrations.³³⁻³⁵ Since their role is to be a messenger between the cells, cytokines are rarely produced at a constant rate and, instead, are induced or suppressed in response to a stimulus. Their half-life is usually short thus limiting their action. Cytokines are involved in a wide range of processes including control of cell proliferation and differentiation, regulation of the immune response, bone remodeling, wound healing and other processes.³³⁻³⁵

Three cytokines (IL-1, IL-6, and TNF- α) are considered to be the most important inflammatory cytokines since they mediate a number of both local and systemic inflammatory responses. IL-1 and TNF- α are produced primarily by monocytes and macrophages in response to exposure to stimuli such as bacterial endotoxin, and immune complexes.³⁵ IL-6 is secreted by a range of cells in response to stimulation by a number of factors, including other cytokines such as IL-1 and TNF- α .³⁵ The detailed summary of the targets and activities of major inflammatory cytokines is shown in Table 1.4.

Cytokines are believed to play a crucial role in aseptic loosening.^{5,6,8-10} IL-1 and TNF- α are produced primarily by macrophages in response to phagocytosis of cell debris and wear particulate.⁶ These cytokines have the potential to recruit fresh monocytes to the site of inflammation and induce the formation of new blood vessels in the inflammatory membrane. At

Cytokine	Producer cells	Target cells	Activities
IL-1 α and β	monocytes, macrophages, other cells	immune cells, osteoblasts, osteoclasts, fibroblasts, hepatocytes, endothelium	<ul style="list-style-type: none"> -promotes breakdown of cartilage and bone -stimulates osteoclast-like cell formation in vitro -induces cytokine synthesis -cofactor for T-cell activation -cofactor for B-cell activation -promotes hematopoiesis -activates neutrophils -activates vascular endothelial cells -mitogenic for fibroblasts
IL-6	monocytes, macrophages, osteoblasts, T-cells, mast cells, endothelium, fibroblasts, marrow stromal cells	osteoblasts, osteoclasts, B-cells, T-cells, hepatocytes	<ul style="list-style-type: none"> -stimulates osteoclast formation -stimulates growth and differentiation of precursors for osteoclasts -stimulates bone resorption -co-stimulates T-cells and thymocytes -induces B-cell differentiation -competence factor to confer responsiveness to hematopoietic growth factors

Table 1.4. continued on next page

TNF- α	monocytes, macrophages, T-cells, fibroblasts	osteoblasts, osteoclasts, T-cells, B-cells, other cell types	<ul style="list-style-type: none"> -activates granulocytes and macrophages -promotes bone resorption by osteoclasts -stimulates osteoclast-like cell formation in vitro -stimulates both proliferation and differentiation of precursors for osteoclast-like cells to osteoclasts -induces cytokine secretion e.g. IL-2 and IL-6 -cytolytic and cytostatic for transformed and virus infected cells -protects cells from viral infection -co-stimulates T-cell proliferation -activates vascular endothelial cells
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Table 1.4. Targets and activities of major inflammatory cytokines.^{33,35-37}

the same time, they cause an increase in secretion of other cytokines, such as IL-1 and IL-6, therefore, increasing the inflammatory response. Aside from these activities, IL-1, TNF- α , and IL-6 have the potential to interfere with cell differentiation in bone marrow, thus affecting the balance between osteoclasts and osteoblasts towards increased bone resorption.^{6,8,9,11,36}

1.3.4.2. Prostaglandin E₂. Prostaglandins belong to a family of arachidonate metabolites (eicosanoids) and play an important role in inflammation.³⁸⁻⁴⁰ They are synthesized by various enzymes from free arachidonic acid released from membrane phospholipids during the process of inflammation.³⁸⁻⁴⁰ Prostaglandin E₂ is synthesized via the cyclooxygenase pathway by

conversion of arachidonic acid to PGH_2 by the action of an enzyme called PGH synthase.⁴⁰ PGH_2 is a common precursor for many prostanoids (PGD_2 , PGE_2 , $\text{PGF}_{2\alpha}$, PGI_2 , and TxA_2), it is rapidly converted into prostanoids, and its fate is cell specific.^{38,40} PGE synthesis can be induced by IL-1, $\text{TNF-}\alpha$, lipopolysaccharides, and components of the complement cascade.⁴⁰ It has been shown *in vitro* that PGEs affect the secretion of IL-2, IL-4, and $\text{IFN-}\gamma$ by T cells, upregulate the expression of IL-1 receptors on fibroblasts and peripheral blood monocytes, as well as enhance the synthesis of GM-CSF, the cytokine which promotes the production of macrophages from bone marrow.⁴⁰

It has been observed in *in vitro* experiments³⁶ that even though macrophages exposed to wear particles produce low levels of PGE_2 , the osteoblasts, exposed to macrophage-conditioned medium, secrete large amounts of PGE_2 . Horowitz et al.³⁶ have hypothesized that the phagocytosis of PMMA particles by macrophages leads to the production of $\text{TNF-}\alpha$, which acts on osteoblasts to produce other mediators including PGE_2 . This process leads to the recruitment of more macrophages, osteoclasts, and other inflammatory cells, which ultimately results in bone resorption and, therefore, aseptic loosening of the prosthesis. It is believed that Horowitz's hypothesis³⁶ also applies to polyethylene particulate.⁶

1.3.5. In vivo and in vitro studies of wear particulate.

In vivo models have been used to illustrate the foreign-body reaction to wear particulate, study synovial tissue formation, and determine cytokine levels from retrieved tissues. There are a few commonly used animal models. Howie et al.⁴¹ developed a rat model in which a non-weight-bearing sterile plug of acrylic cement was placed in the distal part of the femur, in continuity with the knee joint, after which particles of polyethylene were injected into the knee. In this study Howie et al. was able to demonstrate resorption of bone and the formation of connective tissue at the interface between acrylic cement and bone. This group associated the findings with a macrophage and giant-cell response to particles of polyethylene that had been injected into an adjacent joint. However, 200 μm particles were used in this study and it was noted that the foreign-body response was higher than is usually observed in human samples.

A rabbit model has also been used to study aseptic loosening. Goodman et al.¹⁷ put a bulk pellet of UHMWPE directly into a 6 mm drill hole in the proximal tibia. This research group demonstrated synovial membrane formation, bone resorption in the experimental animals, and elevated levels of PGE₂. This was an interesting finding since PGE₂ was known to play a major role in bone remodeling and therefore it was concluded that PGE₂ could be an important factor in the case of aseptic loosening and bone resorption. However, since there was no direct contact of the particles with joint synoviocytes, this model can not be directly related to the process of aseptic loosening in humans.

A canine model was developed by Spector et al.⁴² In this model total hip replacements were implanted into dogs and then bone cement particulate was placed into the femoral canal just before insertion of the stem. Spector et al.⁴² demonstrated synovial membrane formation identical to the membranes found in humans; biochemical analysis showed elevated levels of PGE₂ in these dogs. Treatment of cells from the synovium-like tissue *in vitro* with naproxen, reduced the levels of PGE₂. Spector and his group used 500 µm particles and assumed that the particles would become smaller as a result of abrasion in the joint; histological evaluation revealed the presence of particles of size >100 µm, which is still considerably larger than the size of particles recovered from retrieved human membranes.^{12,19,21,22} All of the above animal models consistently demonstrated the formation of an inflammatory membrane at the bone-implant interface, a certain degree of bone resorption and elevated levels of inflammatory mediators in response to wear particulate.

While *in vivo* models can illustrate the body's reaction to particulate and other foreign objects, *in vitro* experiments can provide the opportunity to study the response to different stimuli at a cellular level. There are currently no *in vitro* "models" which have described the process of phagocytosis and bone resorption in a similar manner as to the animal models summarized above. Most *in vitro* studies to date have used primary cells (e.g. monocytes,^{11,43-45} fibroblasts^{46,47}) and cell lines (e.g. macrophages,^{9,10} fibroblasts,¹⁰ osteoblasts^{9,36}), and have exposed them to different types of particulate (titanium,^{10,11,44,45} bone cement,^{9,11} polystyrene,^{10,11} polyethylene⁴³⁻⁴⁶) with various sizes (submicron^{10,11,43-45,47} to 100 µm⁹). Some investigators⁹⁻

^{11,36,43-45,47} have studied the direct effect of the particulate on cell function, proliferation, and secretion of inflammatory mediators. Others^{9,10,36} have used the conditioned medium from cells, exposed to the particulate, to analyze its effect on cell proliferation, differentiation, and cytokine secretion. Almost all reported investigations demonstrate elevated levels of IL-1, IL-6, TNF- α , PGE₂, and other inflammatory cytokines. A common shortcoming of these studies is the absence of histological confirmation of particulate phagocytosis. Histological evaluation of the cells could provide information not only about internalization of the particulate, but also could show the effect of phagocytosis on cellular morphology, activation, and viability.

1.3.6. In vitro studies of polyethylene particulate

Despite the fact that UHMWPE is one of the major constituents of wear debris, detected in inflammatory membranes retrieved at revision surgeries,^{2,3,6,8} few studies have examined the phagocytosis of polyethylene particles by macrophages *in vitro*.^{43-46,48} This may be due in part to technical difficulties associated with handling the polyethylene particulates. The material's hydrophobic character render it particularly non-compatible with aqueous media. As well, it has a relatively lower density than water and resides at the surface of the aqueous medium rather than within the medium or at the bottom of the tissue culture wells. This hinders the ability to study cell-polyethylene interactions by methods established for PMMA and polystyrene particles.^{4,9,10} Some models which have been employed for the investigation of polyethylene/macrophage interactions have included work by Harada,⁴³ Chiba,⁴⁶ Shanbhag,^{44,45} and Shirata.⁴⁸ Harada et al.⁴³ used agarose to keep the polyethylene particles (purchased from Polysciences, 1-10 μ m) attached to the surface, but the level of macrophage activation in the presence of agarose alone was sufficiently elevated to make it particularly difficult to assess the specific biochemical responses induced by the particles. Shanbhag et al.^{44,45} used serum to suspend retrieved and fabricated submicron particles of polyethylene. Both groups noted increases in cytokine production (IL-1 β , IL-6, TNF- α , PGE₂), but there was little discrimination in cytokine release compared to control groups. Shirata et al.⁴⁸ utilized an inverted culture system in order to enable cells from a mouse macrophage cell line IC-21 to phagocytose the

fabricated polyethylene particulate. This group demonstrated a statistically significant increase in IL-1 β release. Of all the studies encountered in the current literature, only Chiba et al.⁴⁶ have shown histological evidence (an SEM) of retrieved UHMWPE phagocytosis by human monocytes. Unfortunately, the method of introduction of polyethylene into the cells was not clearly described in the abstract.

1.4. THE ROLE OF EXTRACELLULAR MATRIX PROTEINS IN CELLULAR RESPONSE TO BIOMATERIALS

In response to the implantation of any type of prosthesis, the body reacts with an initial phase of inflammation. The processes involved in this reaction are similar to those in the tissue repair mechanism, exhibited as a normal response to tissue injury.⁴⁹ However, since the implant is also present at the site of the tissue injury, it can influence the outcome of the repair mechanism by augmenting the inflammatory response and, possibly causing a state of chronic inflammation.⁴⁹ It has been established that several material parameters can influence the speed and the result of such tissue repair. These include but are not limited to surface morphological characteristics (rough versus smooth), size, chemical composition, and location of the implant.⁴⁹

As was described in Section 1.2.1 of this thesis, the change in porosity of a prosthesis surface, achieved by coating the surface with beads, can increase bone ingrowth and, therefore, augment the healing process. However, changing the surface morphology of the implant is not the only method available for promoting tissue healing around implants. It is believed that coating the surface of the implant with biologically relevant materials can enhance the fixation of the implant, promote tissue ingrowth, and reduce capsule formation.⁵⁰⁻⁵⁴ Collagen type I and hydroxyapatite are the most common materials used for this purpose.⁵⁰⁻⁵⁴ It has been demonstrated *in vitro*, that a mixture of hydroxyapatite and collagen promotes the production of a calcified collagenous matrix by human osteoblasts.^{50,53} The matrix has been reported to be similar in nature to bone tissue. It has also been shown that a sponge layer of collagen,

mechanically bonded to silicone sheets and implanted in rats, exhibits a reduction in capsular formation in comparison to control silicone sheets without the collagen.⁵¹ In another study, it was demonstrated that the immobilization of collagen onto the surface of porous polyethylene proved to be very effective in forming a firm bond with soft connective tissues and in reducing the incidence of tumour formation.⁵⁴ All these studies demonstrate the importance of extracellular matrix protein at the implant/tissue interface.

1.4.1. Collagen type I

Collagen is the most abundant protein in vertebrates.^{38,39,55,56} Due to its unique tensile strength, collagen is a major protein of the connective tissue.^{38,39,55,56} At least 18 distinct types of collagen have been discovered; these types exist in a variety of tissues forming fibers, filaments, sheet-like structures, and anchoring fibrils.⁵⁶ The main types of collagen found in connective tissue are types I, II, III, V, and XI, while the most common collagen is type I, which is found in skin, lung, bone, and many other tissues (Table 1.5).^{38,39,55,56} The types of collagen found in the inflammatory membrane retrieved at revision surgeries of failed orthopaedic implants are types I and III.²⁴

TYPE OF COLLAGEN	CHAINS	AGGREGATE TYPE	TISSUE DISTRIBUTION
I	$[\alpha 1(I)]_2\alpha 2(I)$	fibrils	most connective tissues, except cartilage and basement membranes (skin, bone, tendon, blood vessels, cornea)
II	$[\alpha 1(II)]_3$	fibrils	cartilage, vitreous humour
III	$[\alpha 1(III)]_3$	fibrils	mostly colocalized with type I collagen
V	$\alpha 1(V)\alpha 2(V)\alpha 3(V)$	fibrils	minor components in many tissues
XI	$\alpha 1(XI)\alpha 2(XI)\alpha 3(XI)$	fibrils	cartilage, vitreous humour

Table 1.5. Main types of collagen. Adapted from Tryggvason⁵⁶ and Voet.³⁸

Collagen molecules form triple-stranded helical structures, in which three polypeptide chains are wrapped around each other.^{38,39} Each chain is composed of a series of repeating sequences with the general formula (Gly-X-Y)_n. While glycine is the most abundant amino acid in collagen, X is often proline and Y is often hydroxyproline. This combination creates very strong hydrogen bonds between the chains thus making a well-packed and rigid structure, characterized by unusual tensile strength. Collagen fibers are insoluble at biological pH due to the crosslinking of the chains via hydrogen bonds. However, it is soluble at acidic pH.^{38,39} This transformation between a collagen solution and an insoluble crosslinked collagen matrix has been used for cell culture studies, since it allows for the generation of a three dimensional matrix for the cells in laboratory conditions.⁵⁷

As one of the major components of the extracellular matrix,⁵⁵ collagen is vital to cell attachment, division and growth.⁵⁵ In cell culture, substrates are commonly coated with extracellular matrix proteins, such as collagens type I and II, fibronectin, and laminin, depending on cell type, to promote attachment of the cells.⁵⁷

1.4.2. The effect of collagen type I on macrophages *in vitro*

Collagen type I is the most commonly used protein in cell culture and is known to enhance growth or differentiation of numerous mammalian cell types.^{55,57} It has been previously described that exposure to extracellular matrix proteins can enhance phagocytosis.⁵⁸ For example, Kaplan et al.⁵⁹ demonstrated that monocytes cultured on collagen rather than glass phenotypically resemble resident tissue macrophages, while Newman et al.⁶⁰ found that the phagocytic ability of monocytes adhered to type I collagen gels increased 2.5-12 times. It has also been shown that coating plastic substrates with collagen type I modifies the cytokine gene expression of such inflammatory cytokines as TNF- α ,⁶¹ IL-1 β and IL-6,⁶² thus demonstrating the importance of extracellular matrix proteins.

It has been demonstrated that collagen is present in the inflammatory membrane at the bone-implant interface.²⁴ The presence of collagen could have numerous effects on macrophage activation and function according to these studies. While it remains difficult to extrapolate the

in vitro experiments discussed above⁵⁸⁻⁶² to the *in vivo* environment of the inflammatory membrane, it would be desirable to consider implementation of collagen into *in vitro* studies in order to render the system more analogous to the *in vivo* environment of the inflammatory membrane, since it could promote cell differentiation, phagocytosis of the particulate, and cytokine expression.

1.5. SCOPE OF THESIS

Aseptic loosening remains the most common reason for implant failure. Polyethylene particulate has been implicated as the most abundant component of the wear debris associated with aseptic loosening. However, polyethylene remains the least studied particle due to its unique physical properties; it has a low density, it is hydrophobic, and it is transparent, therefore, making it difficult to study. At this point, there is little data available⁴⁶ that describes the process of internalization of polyethylene particulate.

1.5.1. Objective

The purpose of this study was (1) to develop a simple and versatile *in vitro* model that would allow macrophages to phagocytose polyethylene particulate; and (2) to investigate the effect of the phagocytosis on cellular activation.

1.5.2. Experimental approach and rationale

In order to introduce polyethylene into macrophages, this study describes the method by which the particles are coated with collagen and then adhered to glass coverslips. In these studies the macrophages are plated on top of the particle/collagen matrix and incubated for different periods of time, ranging from 2 to 96 hours. The use of a coverslip system is intentional, and permits one to perform any type of histological analysis (conventional staining, scanning electron microscopy (SEM), transmission electron microscopy (TEM)) without the

time consuming necessity to cut tissue sections. Post incubation, the cells are analyzed histologically to confirm the internalization of the polyethylene particles. The media are tested for the presence of major inflammatory mediators (IL-1, IL-6, TNF- α , PGE₂), as well as nonspecific lysosomal enzymes (β -GAL and HEX). The selection of the above mediators was made in order to confirm the activation of macrophages by polyethylene particulate phagocytosis.

Macrophages are of particular interest to this study because they are the primary cells associated with phagocytosis of wear debris in aseptic loosening of the orthopaedic implants. These cells are believed to play an important role in internalization of the wear debris and release of inflammatory mediators that are capable of not only augmenting the inflammatory response, but also of breaking the balance in bone remodeling, therefore, causing bone resorption, more loosening of the implant, and production of more wear debris, initiating the vicious circle leading to implant failure.

The use of collagen in the model has been rationalized by the following. Type I collagen is an extracellular matrix protein that is present in inflammatory membranes retrieved from the bone/implant interface of failed prostheses. Aside from the fact that collagen is the most abundant protein in the body, it has some unique properties that are vital to cell function, both *in vivo* and *in vitro*. Collagen provides an extracellular matrix for the cells, promotes cell differentiation, augments phagocytosis by macrophages, and modulates cytokine expression. At the same time, collagen has the ability to crosslink and form an insoluble matrix that can be used for attachment of the particles to the bottom of tissue culture well.

In summary, the development and characterization of a model that allows polyethylene phagocytosis by macrophages can help towards gaining a better understanding of the mechanisms involved in aseptic loosening. It could have an impact on the development of alternatives to the revision surgery of a loose implant, such as blocking of the inflammatory cytokines; or stimulate a production of new types of prostheses, either particle-free or made from a type of material that does not cause an inflammatory response by the macrophages.

2. MATERIALS AND METHODS

2.1. PARTICLES.

Two types of polyethylene particles (18-20 μm ultra high molecular weight polyethylene (UHMWPE) and 4-10 μm high density polyethylene (HDPE) particles, obtained in-kind from Shamrock Technologies, Newark, NJ) were used in the experiments. It was originally desired to have submicron particles of UHMWPE since this would most appropriately reflect the type of particle generated from orthopaedic implant devices. However, no commercial source of UHMWPE in submicron size was found. As a substitute, 4-10 μm HDPE was used. While the chemistry of the polymer chain does not differ from that of UHMWPE, the two materials differ in their molecular weight. Physical properties for both types of polyethylene were provided by Shamrock in Technical Data Sheets and are summarized in Table 2.1. The particles were sterilized by γ -irradiation in air (2.5 MR) (Chemical Engineering Department, University of Toronto) and stored at 4°C.

Properties	UHMWPE	HDPE
Size (microns)	18-20	4-10
Melt point, °C	166	118
Specific gravity	0.9	0.95

Table 2.1. Physical properties of UHMWPE and HDPE

2.2. PARTICLE SURFACE CHEMICAL CHARACTERIZATION BY X-RAY PHOTOELECTRON SPECTROSCOPY (XPS).

X-ray photoelectron spectroscopy is a technique commonly used for surface analysis of polymers in the biomaterials field, providing a total elemental analysis of the top 10-200 Å of the surface.⁵⁸ XPS spectra of the polyethylene particles were obtained by Dr. Rana Sodhi using a Leybold MAX 200 XPS system, located at the Centre for Biomaterials, University of Toronto.

An unmonochromatized $\text{MgK}\alpha$ x-ray source operating at 12 kV and 25 mA was used. The take-off angle for the analysis was 90° .

The energy range was calibrated against Cu 2p_{3/2} and Ag 3d_{5/2} at 932.7 eV and 368.3 eV, respectively, and scaled to place the main C peak at 285.0 eV.⁶³ As no observable differential charging of the surface was produced with this source, external charge compensation was not employed. Features in the resultant spectra due to excitation from the x-ray satellite components were subtracted using an algorithm supplied with the instrument and based upon a program by van Attekum and Trooster.⁶⁴ Atomic ratios of C and O were derived from spectra run in a low energy mode (pass energy=192 eV) which had been normalized to correct for the transmission function of the spectrometer. Both the transmission function and the sensitivity factors for oxygen and carbon (O=0.78, C=0.34) were empirically derived by the manufacturer of the XPS system. Further information on the nature of the substrate surfaces were obtained from the C 1s region run in a high resolution mode (pass energy=48 eV). Binding energies and peak areas were obtained using the curve-fitting routines provided with the spectrometer.

Non-sterile UHMWPE and HDPE particles and sterile (γ -irradiated) UHMWPE and HDPE particles were analyzed (n=3), and differences in particle surface chemistry after γ -irradiation were assessed. In order to analyze the powders, the samples were attached to the sample holder by using double sided copper tape.

2.3. PARTICLE BULK CHEMICAL CHARACTERIZATION BY FT-IR.

Infrared spectroscopy is a simple and rapid method for identifying a structure of an organic compound.⁶⁵ Non-sterile and sterile UHMWPE and HDPE particles were analyzed (n=3) by infrared spectroscopy with nitrogen purge (IFS85, Bruker, Bruker Spectrospin (Canada) Ltd., Milton, ON). For the analysis, 1% (by weight) of polyethylene particles in dehydrated KBr pellets were prepared by pressing ($0.2 \times 10^{-3} \text{ N/m}^2$) and measuring the absorbance spectra (100 scans/spectrum). The differences in bulk material chemistry between both types of the particles, and the effect of γ -irradiation on bulk properties were then assessed.

2.4. ENDOTOXIN TEST.

HDPE particles were tested for the presence of endotoxin (lipopolysaccharides from the walls of gram-negative bacteria) using a standard E-TOXATE detection kit (Sigma, St. Louis, MO). This test is based on the ability of *Limulus* amoebocyte lysate (LAL) to change viscosity and opacity when exposed to minute quantities of endotoxin.⁶⁶

In order to obtain a sample, the polyethylene particles were mixed with sterile ddH₂O overnight on a shaker, and the water was later collected for analysis. The pH was tested and was approximately 7.0. Samples, along with positive and negative controls provided with the kit, were exposed to E-TOXATE working solution, mixed gently, and incubated for 1 hour at 37°C. After incubation, the tubes were examined for evidence of gelation.

UHMWPE particles were not analyzed for the presence of lipopolysaccharides since they were found to be too large to be phagocytosed by the cells and were not used in characterization of the collagen model.

2.5. PARTICLE SUSPENSION PREPARATION FOR THE TRITON X-100 MODEL.

Approximately 1 gram of UHMWPE particles was mixed thoroughly with 0.5 mL of 2%(vol.) Triton x-100 (Sigma, St. Louis, MO) in PBS (Mount Sinai Hospital, Media Preparation Department) and the particle mixture was then resuspended in PBS. The number of the particles was counted using a Coulter Counter (model ZM, Coulter Electronics, Hialeah, FL) and the final concentration of the Triton x-100 was then determined, based on the amount of the particle suspension required to provide 10⁵ particles. The concentration of Triton x-100 used in the following experiments (see Section 2.6) was 0.0083%(vol.). Triton X-100 was selected for these experiments since this surfactant is routinely used in cell culture.⁶⁷

2.6. EXPERIMENTS USING MOUSE PERITONEAL MACROPHAGES.

Mouse peritoneal macrophages were obtained from 6- to 8-week-old specific pathogen-free female Swiss Webster mice as described by Nathens et al.⁶⁸ in the facility located at the Toronto Hospital, General Division, Toronto. Briefly, mice were injected with 2 mL of

thioglycollate broth (Difco Laboratories, Detroit, MI) into the peritoneal cavity and peritoneal exudate macrophages were collected by peritoneal lavage with ice-cold Hanks' balanced salt solution (Gibco BRL, Burlington, ON), five days after the injection. The cells were centrifuged at 200g (1500 rpm) for 10 minutes, washed twice with HBSS, resuspended in fully supplemented media (RPMI-1640 (Sigma, St. Louis, MO) supplemented with 10% FCS (Gibco BRL, Burlington, ON)), and then counted using a hemocytometer. The cells were diluted to a final concentration of 1×10^6 cells/mL in fully supplemented media and plated in 24-well plates (Falcon, Fisher Scientific, Whitby, ON) at 1mL/well. At this point an aliquot of Triton x-100-particle solution and Triton x-100-control was added and the cells were incubated for 24 and 48 hours. The media was then collected and analyzed for TNF- α , β -GAL, and HEX release (see sections 2.15.3 and 2.15.4 for details on the biochemical analysis methods).

2.7. PARTICLE SUSPENSION PREPARATION FOR COLLAGEN MODEL.

Approximately 1 gram of particles was mixed thoroughly with 0.5 mL of dimethyl sulfoxide (DMSO, BDH Laboratories) using a sterile quartz pestle and mortar. The particle mixture was then resuspended in 14.5 mL of 0.01% solution of collagen type I monomer, derived from calf skin (C-8919, Sigma, St. Louis, MO) at 4°C. The number of suspended particles was then counted using a Coulter Counter (model ZM, Coulter Electronics, Hialeah, FL) and the final concentration of particles adjusted to 10^7 particles/mL.

2.8. COVERSIP PREPARATION.

Microscope coverslips 22×22 mm (Fisher Scientific, Whitby, ON) were sterilized by dry heat at 200°C for 2 hours in the oven. Aliquots of cold collagen solution with suspended particles (10^5 particles/10 μ L) were spread evenly on each coverslip and the collagen was then allowed to polymerize at room temperature and the solution was then evaporated. A typical distribution of particles in the collagen matrix is shown in Figure 3.3a. Uncoated coverslips, coverslips coated with DMSO; collagen type I; and a mixture of DMSO with collagen type I, were also prepared and used as the controls for these experiments. All samples were then placed

in 6-well tissue culture plates (Falcon, Fisher Scientific, Whitby, ON) and exposed to UV light in a sterile tissue culture hood for overnight and for 1 hour immediately before use. Following the initial overnight UV sterilization, the plates were wrapped in clean non-sterile polyethylene bags, covered with aluminum foil, and stored at 5°C until required.

2.9. EXPERIMENTS USING THE MOUSE MACROPHAGE CELL LINE.

A mouse macrophage cell line was used to characterize the collagen model because of their well characterized biological function as well as their commercial availability. The mouse macrophages are fully differentiated cells, capable of secreting various cytokines and lysosomal enzymes, and do not require special isolation and purification procedures that are necessary with primary cultures.

IC-21 mouse macrophage cell line obtained from ATCC (TIB-186, Rockville, MD) was maintained in tissue culture flasks (Falcon, Fisher Scientific, Whitby, ON) in RPMI-1640 medium (Sigma, St. Louis, MO) supplemented with 10% heat inactivated FCS (Gibco BRL, Burlington, ON), 1% penicillin-streptomycin (Gibco BRL, Burlington, ON), and 2 mM L-glutamine (Gibco BRL, Burlington, ON) at 37°C in 5% CO₂ atmosphere with 100% humidity. Immediately prior to the experiment, cells were collected by incubation with Ca²⁺/Mg²⁺ free PBS (Mount Sinai Hospital, Media Preparation Department), centrifuged for 10 minutes at 170 g (1100 rpm), resuspended in fully supplemented medium as described above, plated on the previously prepared coverslips at 1×10^6 cells/2 mL/well and then incubated for up to 96 hours. At the end of each incubation period, the cultures were washed twice with PBS (1 mL/well) and then fixed for histology (LM, SEM, or TEM) according to the protocols described further in this chapter (sections 2.11, 2.13, and 2.14). The media were collected for cytokine and lysosomal enzyme analysis (methods described in section 2.15). The cell viability was checked by a standard trypan blue exclusion method (see section 2.10).⁶⁹

2.10. VIABILITY TEST.

Cell viability was determined by trypan blue exclusion test.⁶⁹ The cells were plated on glass coverslips, glass coverslips coated with collagen-DMSO mixture, and glass coverslips coated with particle mixture and incubated for 24 hours. At the end of the incubation period, the media was removed and the coverslips were washed twice with PBS (1mL/well). Then 200 μ L of 20% (vol.) trypan blue in PBS was added per well and the coverslips analyzed under the microscope. The number of the cells that uptake the dye was then determined.

2.11. HEMATOXYLIN AND EOSIN STAINING.

The cells were fixed using a mixture consisting of 100% methanol and 10% formaldehyde (2:1 per volume) for 2 hours (2 mL/well), then stained with hematoxylin and eosin using the standard staining procedure⁷⁰ at Mount Sinai Hospital, Pathology Department. Briefly, the fixed coverslips were rinsed with water, stained with hematoxylin for 5 minutes, rinsed with water, counterstained with eosin for 2 minutes, and rinsed with water again. The coverslips were then dehydrated by graded alcohols (70% to 100%) and then by xylene (100%). The coverslips were then mounted on microscope-grade glass, dried, and photographed both under regular and polarized light, using a VANOX AHB3 Olympus (Carsen Group Inc., Markham, ON) microscope.

2.12. CELL/AREA COUNT.

Hematoxylin and eosin stained slides were analyzed for cell number and area percent count using a monochrome image analyzer equipped with Q500MC software by Leica Cambridge Ltd. located at Mount Sinai Hospital, Pathology Department, Toronto. The assessment was performed on an "Orthoplan" Leitz microscope using the 4 $\times$ objective (N.A. 0.1) coupled with JVC TK-128OU colour video camera. Twenty fields (560 $\times$ 512 pixels, 1 pixel = 3.22 μ m, as determined by the microscope calibration built into the program before each use) per slide were analyzed. Gray levels were detected and cell number, area of the gray and area percent were calculated. In this software, the cell count was defined as the number of isolated

features ending within the measured frame, and the area percent was defined as the proportion of detected pixels in the image, expressed as a percentage from 0 to 100.

2.13. SEM.

Scanning electron microscopy is a histological technique that allows one to investigate the overall morphological features of a specimen.⁷⁰ The cells incubated for 2, 6, and 24 hours (Experiment 2 in Table 1 of the Appendix) were fixed with 2.5 % glutaraldehyde in phosphate buffer, dehydrated in graded alcohols, critical-point dried from CO₂ (Ladd Research Industries Inc., Burlington, VT), sputter-coated with platinum (~3 nm) (Polaron Instrument Inc., Doylestown, PA) and examined by Mr. Robert Chernecky using an Hitachi (model 2500) scanning electron microscope, located in the Faculty of Dentistry, University of Toronto.

2.14. TEM.

Transmission electron microscopy is a histological technique that allows for the investigation of the internal structure of the specimen.⁷⁰ Following incubation for 20 hours, cells were scraped off the coverslips into 1 mL of PBS, pelleted in a microcentrifuge at 530g (2500 rpm), and then fixed with 2.0% glutaraldehyde in 0.1M cacodylate buffer, postfixed with 1% osmium tetroxide in the same buffer, dehydrated in ethanol, and embedded in Epon, and sectioned. The analysis was performed by Ms. Julia Hwang at the Histology Department, Hospital for Sick Children.

2.15. MEDIA ANALYSIS.

After each incubation period the media were collected and stored at -80°C in 200 µL aliquots. Mouse interleukin-1α (IL-1α), interleukin-1β (IL-1β) and interleukin-6 (IL-6) ELISA kits (Endogen, Cambridge, MA) were used to analyze for cytokine release at 24 hours. Tumour necrosis factor α (TNF-α) release was also analyzed by ELISA as described by Nathens et al.⁶⁸ Prostaglandin E₂ (PGE₂) release was determined by radioimmuno assay using PGE₂ RIA kit (Dupont, Boston, MA). β-galactosidase (β-GAL) and hexosaminidase (HEX) activity were

measured as described by Hubbes et al.⁷¹ All cytokine and lysosomal enzyme data were normalized by area % counts.

2.15.1. IL-1 α , IL-1 β assay

The samples and kit supplied standards (50 μ L) were incubated at room temperature with biotinylated antibody reagent (50 μ L) in anti-mouse IL-1 α /IL-1 β precoated well plate for 2 hours. Recombinant *E.coli*-derived mouse IL-1 α /IL-1 β was used as a standard. All samples and standards were analyzed in duplicates. After the incubation, the wells were washed three times with wash buffer and then 100 μ L of streptavidin-HRP (horse radish peroxidase) was added to each well and the plate was incubated for another 30 minutes at room temperature. Then the plate was washed three times with wash buffer and 100 μ L of TMB substrate solution was added to each well. The plate was then incubated in the dark at room temperature for 30 minutes. The reaction was stopped with 100 μ L of stop solution (0.18 M sulfuric acid) and the absorbance of the plate was determined on an ELISA plate reader set at 450 nm. The results were calculated using Microsoft Excel software.

2.15.2. IL-6 assay

The samples and kit supplied standards (50 μ L) were incubated at 37°C in a humidified incubator with plate reagent (50 μ L) in anti-mouse IL-6 precoated well plate for 2 hours. Recombinant *E.coli*-derived mouse IL-6 was used as a standard. All samples and standards were analyzed in duplicates. After the incubation, the wells were washed five times with wash buffer and then 100 μ L of anti-mouse IL-6 conjugate reagent was added to each well and the plate was incubated for another hour at 37°C in a humidified incubator. Then the plate was washed five times with wash buffer and 100 μ L of TMB substrate solution was added to each well. The plate was then incubated in the dark at room temperature for 30 minutes. The reaction was stopped with 100 μ L of stop solution (0.18 M sulfuric acid) and the absorbance of the plate was determined on an ELISA plate reader set at 450 nm. The results were calculated using Microsoft Excel software.

2.15.3. TNF- α assay

The cytokine was measured with a double sandwich enzyme-linked immunosorbent assay technique. The plates were coated with monoclonal hamster anti-murine TNF- α antibody (Genzyme, Cambridge, MA) (100 μ L/well, overnight, at 4°C). The samples/standards (100 μ L) were added to coated wells, incubated for 3 hours at room temperature on a rotating shaker, washed, and then incubated overnight at 4°C with a polyclonal rabbit anti-murine TNF- α antibody (Genzyme, Cambridge, MA) (100 μ L). Then 100 μ L of alkaline phosphatase-conjugated goat anti-rabbit IgG was added to each well and the plates were incubated for 1 hour on a rotating shaker at room temperature. The fluorogenic substrate 5-fluoro-salicyl phosphate (100 μ L) and developing reagent (terbium-ethylenediamine tetraacetic acid) (100 μ L) were then added. Fluorescence was measured with a time-resolved fluorimeter located in the Department of Biochemistry, Mount Sinai Hospital, Toronto. The concentration of TNF- α in the samples was calculated by an automatic immunoanalyzer (Cyber Fluor 615; Cyber Fluor, Toronto, ON). Recombinant *E.coli*-derived murine TNF- α (Genzyme, Cambridge, MA) was used as a standard. All samples and standards were analyzed in duplicates.

2.15.4. β -GAL and HEX assays

β -GAL activity was measured by using 4-methylumbelliferyl- β -D-galactopyranoside (4MU-gal) (Koch-Light laboratories). Briefly, 60 μ L of sample/standard were mixed with 190 μ L of substrate solution (0.56 mM 4MU-gal in 0.1M citrate buffer, pH 4.3) and incubated at 37°C for 30 minutes. All samples and standards were analyzed in duplicates. The reaction was terminated with 2 mL of 0.1 M 2-amino-2-methyl-1-propanol buffer, pH 10 (MAP) and the fluorescence was determined at an excitation wavelength of 365 nm and an emission wavelength of 450 nm on the spectrofluorimeter (Perkin-Elmer 650-40 Fluorescence Spectrophotometer). 4-Methylumbelliferone solution was used as a standard.

Hexosaminidase activity was determined in a similar manner using 4-methylumbelliferyl- β -N-acetyl-glucosaminide-6-sulfate (4-MUGS) as a substrate. The analysis has been carried out in Dr. J. W. Callahan's laboratory at the Hospital for Sick Children.

2.15.5. PGE₂ assay

The principle of this radioimmunoassay is based on competitive binding of radioactive and non-radioactive antigen to a fixed number of antibody binding sites, i.e. as the amount of unlabeled antigen is increased in samples/standards, the amount of the tracer bound to the antibody is decreased. Briefly, samples/standards (100 μ L) were incubated with 100 μ L of tracer (PGE₂[¹²⁵I]) and 100 μ L of antiserum (rabbit anti-PGE₂ antibody) overnight at 4°C. 100ng/mL solution of PGE₂ in acetonitrile was used as a standard. At the end of the incubation period, 1 mL of cold precipitating agent (16% polyethylene glycol PEG6000 and 0.05% sodium azide in 50 mM phosphate buffer, pH 6.8) was added to the tubes, the tubes were vortexed thoroughly, and then incubated for 30 minutes at 4°C. The tubes were then centrifuged in a microcentrifuge at 13800 g (13000 rpm) for 30 minutes, the supernatants were decanted, and the tubes were then counted in a gamma counter for 1 minute. The results were calculated as described in the protocol supplied with the kit using Microsoft Excel software. All samples/standards were analyzed in duplicates.

2.16. STATISTICAL ANALYSIS

The means, standard errors, and standard deviations were calculated utilizing the statistical program built into SigmaPlot 1.02 scientific graph system (Jandel Scientific) and GraphPad InStat software package. The comparison between the groups to determine the significance of the differences was performed using student paired T-test utilizing the GraphPad InStat software package.

3. RESULTS

In this section a summary of the results is presented. The data were organized in a logical sequence corresponding to the development of this research project starting with characterization of the particles, and followed by development of an *in vitro* model for polyethylene particle phagocytosis and detailed characterization of the model.

3.1 PARTICLE CHARACTERIZATION

XPS analysis (see Table 3.1) of the γ -irradiated particles and non- γ -irradiated particles showed that there was no difference in oxygen content on the particle surface following sterilization for UHMWPE, however, there was a statistically significant increase in oxygen content ($p < 0.05$) for HDPE, suggesting oxidation of HDPE due to γ -irradiation. The levels of silicon were low, thus demonstrating that the samples were not contaminated with silicon. Typical XPS spectra for UHMWPE and HDPE are presented in Figure 3.1a,b.

TYPE OF POLYETHYLENE	ATOMIC % O 1s	ATOMIC % C 1s	ATOMIC % Si 2p
UHMWPE	3.60 ± 1.06	95.97 ± 1.29	0.40 ± 0.17
UHMWPE, γ -irradiated	3.73 ± 0.91	95.93 ± 0.95	0.30 ± 0.00
HDPE	0.83 ± 0.12	99.03 ± 0.15	0.13 ± 0.06
HDPE, γ -irradiated	1.53 ± 0.40	98.33 ± 0.46	0.13 ± 0.06

Table 3.1. XPS analysis of UHMWPE and HDPE

FT-IR analysis demonstrated that there was no difference in bulk chemistry due to γ -irradiation for both types of particles, however, there was a distinction between UHMWPE and HDPE spectra (Figures 3.2a-d). This may result from differences in the degree of crystallinity which has been reported for the two types of polyethylenes.⁷²

Figure 3.1. XPS spectra of polyethylene particles

Peak	Peak strength	Range	Group
2949 cm^{-1}	very strong	2990-2850 cm^{-1}	-CH ₃ and -CH ₂ - in aliphatic compounds
2845 cm^{-1}	strong	2850-2650 cm^{-1}	-CH ₃ attached to O or N
1474 cm^{-1}	strong	1475-1450 cm^{-1}	-CH ₂ - in aliphatic compounds
1464 cm^{-1}	strong	1475-1450 cm^{-1}	-CH ₂ - in aliphatic compounds
731 cm^{-1}	medium	740-720 cm^{-1}	-(CH ₂) _n - hydrocarbons

Table 3.2. Summary of the absorbance peaks in FT-IR spectra (Lambert et al.⁶⁵)

As can be seen from the spectra (Figure 3.2) and from the peak assignment (Table 3.2) all major peaks correspond to the hydrocarbon structure which is typical for polyethylene. At the same time, the presence of absorbance peaks at approximately 3330 and 1640 cm^{-1} suggests the presence of trace contaminants in the samples of HDPE (Figure 3.2 a,b). These may have been introduced during polymer synthesis. Similar peaks were not found in the UHMWPE samples (Figure 3.2 c,d). Despite the fact that the XPS data indicated the presence of oxygen, the 1720 cm^{-1} absorbance peak that represents carbonyl (C=O) groups in ketones and is commonly associated with polyethylene oxidation⁷³ was not observed in any of the samples. This suggests the absence of oxidation in the bulk properties of both polyethylenes.

The two particle characterization methods have demonstrated that while the bulk chemistry of both polyethylenes was not seemingly affected by γ -irradiation, the surface of HDPE particles was altered as a result of the same treatment.

SEM was performed on HDPE particles in order to investigate the morphology of the phagocytosable particles and the distribution of the particles within the collagen matrix on the coverslips (Figure 3.3a-b). Figure 3.3a represents a typical particle distribution on the coverslip, while Figure 3.3b demonstrates the surface morphology of the particles.

Endotoxin test, performed on HDPE particles, demonstrated that the particles were free of endotoxin.

Figure 3.2. FT-IR spectra of polyethylene particles

(a) HDPE particles, not γ -irradiated; (b) HDPE particles, γ -irradiated

Figure 3.2. FT-IR spectra of polyethylene particles

(c) UHMWPE particles, not γ -irradiated; (d) UHMWPE particles, γ -irradiated

Figure 3.3 Scanning electron micrograph of HDPE particle distribution in collagen matrix on a coverslip. (a) $\times 100$ original magnification; (b) $\times 5000$ original magnification

3.2 MODEL DEVELOPMENT

The purpose of this study was to develop a model that would allow the investigation of interactions between polyethylene particles and macrophages. The reason for the absence of such a model in the current scientific literature could be explained by the inherent physical properties of polyethylene particulate. These particles are hydrophobic (i.e. incompatible with an aqueous environment) and have a low density (see Table 2.1) and hence they float on the surface of aqueous solutions which have a higher density. Therefore, one of the major difficulties encountered in this work was to prepare a particle suspension that would enable the interaction of individual particles with the cells in aqueous media and to achieve this with processing aids that would be non-toxic to the cells.

Several reagents (such as ethanol, carageenan, serum) were considered to be used for preparation of the particle suspension in the process of the model development. Triton x-100 was one of the surfactants which was extensively studied. A limitation in the use of this surfactant was the fact that at high concentrations of the reagent the cells would disintegrate. It was found that the cut-off concentration of triton x-100 which would be tolerated by the cells was approximately 0.018% (vol.). However, even after achieving a suspension of the particles in triton-x at such a low concentration, the particles did not remain evenly dispersed in the media for extended periods of time and would eventually rise to the surface in a matter of a few minutes. This was undesirable since the cells were attached to the bottom of the tissue culture plate. Another problem observed with utilizing triton-x was the fact that even at concentrations of 0.0083%(vol.), the surfactant rendered cellular membranes more permeable and caused leaching of the cellular contents, such as cytokines and lysosomal enzymes, into the surrounding environment (see control samples in Figure 3.4). As can be observed in Figure 3.4, the levels of TNF- α , β -GAL, and HEX release are significantly higher in the groups with triton-x than in groups with particles and triton-x. As a result of these elevated background values it would be impossible to study the effect of particles on the release of lysosomal enzymes and cytokines from macrophages. Furthermore, the use of triton x-100 as a surfactant would require

Figure 3.4. Media analysis of mouse peritoneal macrophages exposed to UHMWPE particles, suspended in Triton x-100, for 24 and 48 hours.

(a) β -GAL release; (b) HEX release; (c) TNF- α release

employment of a non-adherent cell line since the particles would reside in suspension and not remain at the bottom of the tissue culture plate. Such a system would not be relevant to the in vivo environment of the inflammatory membrane. In addition, a system with a shaker would be required to keep the cells and particles agitated and this would not make the model practical or easy to use.

An alternate approach was taken in attempt to embed the particles in a matrix which could simulate the biological environment. Based on communications with Dr. Alex Hinek (Research Institute, Hospital for Sick Children), the use of collagen was suggested. It is a biological agent which is routinely used in cell culture to promote cell adhesion to different surfaces.⁶⁹ At the same time the inherent feature of collagen to crosslink³⁸ could be utilized to trap the polyethylene particles in a stable matrix. Because collagen is not a surfactant and does not produce a single particle suspension by itself, DMSO was required in order to wet the particles before they were resuspended in collagen solution. Following subsequent coating on glass coverslips, the particles were trapped in the crosslinked collagen. In this form they remained attached to the surface for a prolonged period of time (the longest time period tested was 96 hours in culture). As a result of the latter analysis the collagen model was then selected for further study. The model's characterization is presented in the following sections.

3.3 MODEL CHARACTERIZATION

3.3.1 Histology

Histological characterization of the cells with hematoxylin and eosin staining permits a fast but valuable evaluation of the general condition of the cells, allows for distinguishing dead and live cells, and provides a preliminary evaluation of their association with the polyethylene particles. While SEM analysis provides a three dimensional image of the cell surface, TEM illustrates an accurate image of the intracellular events and provides evidence for the process of particle internalization by the cells. Histological evaluation of polyethylene particles by light microscopy also permits easy identification of the particles. Since polyethylene is transparent, it

is not easily distinguished in cell culture, however, it becomes readily visible under polarized light.

Histological analysis of the cells exposed for 20 hours to the larger UHMWPE particles (18-20 μm) revealed that all large particles were surrounded but not phagocytosed by the macrophages (Figures 3.5a,b). In the experiment the cells were evenly distributed at the outset, but by 20 hours most of the macrophages were associated with particles, leaving large cell free areas suggesting that the cells were attracted to the particles.

Since it was desired to develop the model to specifically look at polyethylene particle phagocytosis, further work on the model characterization was performed only with the smaller HDPE (4-10 μm) particles. It was found that after 24 hours in the wells containing HDPE all cultured cells had phagocytosed the particles with most cells having engulfed several particles (Figures 3.6a,b). When the mouse macrophages were exposed to smaller particles, phagocytosis was progressive over time and engulfment of particles was observed after 2 hours (see Figure 3.7).

Upon SEM analysis at 2 hours, even though several particles had been phagocytosed, the cells appeared to be very motile (Figures 3.7a,c), and cell pseudopodia could be seen in contact with particles (Figure 3.7c). Beyond 6 hours and up to 24 hours in culture no unphagocytosed particles were found. At these later time points the cells were less motile, with numerous pseudopodia attached to the substratum (Figure 3.7e,f). The cells did not stop dividing at any time point indicating that the particles did not have an apparent toxic effect on the cells. Upon division the phagocytosed particles appeared to be equally distributed between the two daughter cells (Figure 3.7d). The cells in the control cultures (glass, DMSO, and DMSO with collagen) were similar to those shown in Figures 3.7b,f for all three categories.

TEM analysis (Figure 3.8a,b) was performed at 20 hours of incubation and confirmed the internalization of the PE particles. An examination of Figure 3.8a showed eight particles located within a cross-section of a single cell. The particles were surrounded by double layer membranes and numerous vacuoles were distributed along the interface (Figure 3.8b).

Figure 3.5. H&E staining of the IC-21 cells exposed to UHMWPE particles for 20 hours.

(a) normal light; (b) polarized light; original magnification $\times 2000$

The transparent particles become visible under polarized light.

(a)

(b)

Figure 3.6. H&E staining of the IC-21 cells exposed to HDPE particles for 24 hours.

(a) normal light; (b) polarized light and Nomarski optics;

original magnification $\times 1000$

The transparent particles become visible under polarized light.

Figure 3.7. Scanning electron micrograph of IC-21 cells exposed to HDPE particles for up to 24 hours. (a) cells with particles, 2 hours of incubation; (b) control, no particles, 2 hours of incubation.

Figure 3.7. Scanning electron micrograph of IC-21 cells exposed to HDPE particles for up to 24 hours. (c), (d) cells with particles, 2 hours of incubation.

Figure 3.7. Scanning electron micrograph of IC-21 cells exposed to HDPE particles for up to 24 hours. (e) cells with particles, 24 hours of incubation; (f) control, no particles, 24 hours of incubation.

Figure 3.8. Transmission electron micrograph of IC-21 cells exposed to HDPE particles for 20 hours; (a) original magnification $\times 11920$; (b) original magnification $\times 48600$.
* HDPE particle; ➤ lysosome.

3.3.2. Cell counts

The analysis of the histological slides for the cells exposed to 4-10 μm particles was carried out using an image analyzer and demonstrated differences in cell/area counts between the control and particle containing groups (see Table 2 in Appendix for the complete listing of cell count data). A representative sample of the cell count data is shown in Figure 3.9a-b. The data shows that after 24 hours of incubation the cell counts for collagen with DMSO groups had statistically higher values over groups where the cells were exposed to particles. This observation of cell number differences was repeated in four out of six experiments, whereas the area measurement data were significant in three out of six experiments (see Table 2 in the Appendix). At 48 hours of incubation, cell counts as well as area counts were significantly higher in collagen with DMSO groups than in particle groups in three out of four experiments, thus suggesting inhibition of cell proliferation. In one experiment, where the cells were exposed to the particles for longer periods of time (72 and 96 hours) the differences were more significant ($p < 0.001$). Therefore, these data demonstrate that exposure to particles appears to have affected cellular proliferation and this effect was progressive with time (see Figure 3.9). The cells exposed to the particles for shorter periods of time (2 and 6 hours of incubation) did not demonstrate significant differences between the groups for cell number as well as for area % (Figure 3.10a,b), probably, due to shortness of the incubation periods. However, the cell incubation experiments for the 2 and 6 hours exposure periods was only carried out once.

Since the image analysis work was a computerized process, it was believed that area % counts provided a more sensitive measure of the cell number than did the cell counts. Because of the nature of the experiments, the cells would group around the particles making it impossible for the computer or operator to distinguish how many cells were in the clump. Even though the computer was set up to pick up the dark staining of the nucleus, it could mistakenly count a few cells located in one clump as one single cell, while area counts would remain the same independently of how many cells were in the clump. Based on this assessment, all cytokine and lysosomal enzyme data were normalized by area % counts.

Figure 3.9. Changes in cell number and cell area % with time
(see Table 2 in the Appendix for n values)

Figure 3.10. Changes in cell number and cell area % with time
(see Table 2 in the Appendix for n values)

3.3.3. Cytokine and lysosomal enzyme analysis

The secretion of the cytokines and lysosomal enzymes is an important parameter that can be used to estimate the inflammatory effect of the particles when engulfed by the cells. In this study seven experiments were carried out to analyze the cytokine and lysosomal enzyme release by the cells at different time points (see Table 3.3.).

Experiment #	Particle type	Incubation periods	Analyses performed	# of cell/well plated
0 *	UHMWPE	20	H&E, TNF- α , β -GAL, HEX	1×10^6
1	HDPE	20	H&E, TNF- α , β -GAL, HEX	1×10^6
2	HDPE	2, 6, 24	H&E, SEM, TEM, TNF- α , β -GAL, HEX, IL-1 β , PGE ₂	1×10^6
3	HDPE	24, 48, 72, 96	H&E, TNF- α , β -GAL, HEX, IL-1 α , IL-1 β , IL-6, PGE ₂	0.5×10^6
4	HDPE	24, 48	H&E, TNF- α , β -GAL, HEX, IL-1 β	0.5×10^6
5	HDPE	24, 48	H&E, TNF- α , β -GAL, HEX, IL-1 β	0.5×10^6
6	HDPE	24, 48	H&E, TNF- α , β -GAL, HEX, IL-1 β	0.5×10^6

Table 3.3. Summary of the performed experiments (* image analysis was not performed on Experiment 0)

The cells exposed to large UHMWPE particles (18-20 μ m) for 20 hours released TNF- α , β -GAL, and HEX (Figure 3.11 a-c), however, this response was not statistically significant when compared to collagen controls (this study corresponds to Experiment 0 in Tables 3, 5, and 6 of the Appendix). It was also observed that while the TNF- α data for the glass control were

dramatically lower than for collagen groups, the same samples had significantly higher values of lysosomal enzymes than for the collagen groups.

The cells exposed to smaller HDPE particles (4-10 μm) also released cytokines and lysosomal enzymes and typical profiles (based on the results from Experiment 3, recorded in Tables 2-9 of Appendix) for cytokine and lysosomal enzyme release data at the 24 hour incubation time point are shown in Figure 3.12(a-d) and Figure 3.13(a-c). The levels of IL-1 α (Figure 3.12c) and IL-1 β (Figure 3.12d) in the media were significantly higher ($p < 0.05$) as compared to non-particulate controls at the 24 hour incubation period. Since biological activities of IL-1 α are similar to those of IL-1 β ,²⁵ the supernatants were analyzed for the presence of IL-1 α only once, while IL-1 β release was measured in five experiments and was statistically significant over all control groups in all experiments (Figure 3.14). Even though the levels of IL-1 β production by the cells in response to the particles are different for the four experiments shown in Figure 3.14, the pattern of the cytokine release remained the same. The differences in cytokine release could be attributed to the fact that different kits from different lots as well as two different ELISA plate readers were employed during the course of the analyses.

At 24 hours, TNF- α (Figure 3.12a) and IL-6 (Figure 3.12b) release were not statistically significant over collagen with DMSO values ($p > 0.1$). As well, it can be seen from Table 2 in the Appendix that TNF- α release was consistently not significant ($p > 0.05$) in all experiments, including the experiments with 48, 72, and 96 hour time points (see Figure 3.15a). At these later time points, a decrease in TNF- α production was observed.

The release of both β -GAL and HEX (Figures 3.13a,b) was statistically significant at the 24 hour incubation period ($p < 0.05$) for particle groups over collagen with DMSO values. This was noted in three out of six experiments (see data in Tables 5 and 6 of the Appendix). At 48 hours of incubation, particle phagocytosis resulted in significant differences for β -GAL release in three out of four experiments (see data in Table 5 of the Appendix), and for HEX release in two out of four experiments ($p < 0.05$) (see data in Table 6 of the Appendix). An increase in lysosomal enzyme production was observed from 72 to 96 hours of incubation (Figure 3.16b).

Figure 3.11. Media analysis of IC-21 cells exposed to 18-20 μ m UHMWPE particles; 20 hours incubation period; (a) β -GAL; (b) HEX; (c) TNF- α (*) samples were compared using paired student's t-test (see Tables 3, 5, 6 in the Appendix for n values)

Figure 3.12. Cytokine release by IC-21 cells exposed to HDPE particles; 24 hour time point (a) TNF- α ; (b) IL-6; (c) IL-1 α ; (d) IL-1 β ; (*) samples were compared using paired student's t-test (see Tables 3, 4, 8, 9 in the Appendix for n values)

Figure 3.13. Lysosomal enzyme and PGE₂ release by IC-21 cells exposed to HDPE particles 24 hour time point; (a) β-GAL; (b) HEX; (c) PGE₂ (*) samples were compared using paired student's t-test (see Tables 5, 6, 7 in the Appendix for n values)

Figure 3.14. Summary of IL-1 β results based on experiments 3-6;

S1 - experiment 5; S2 - experiment 6; S3 - experiment 4; S4 - experiment 3;

1 - glass, 24 hrs inc; 2 - coll+DMSO, 24 hrs inc; 3 - HDPE, 24 hrs inc;

4 - glass, 48 hrs inc; 5 - coll+DMSO, 48 hrs inc; 6 - HDPE, 48 hrs inc

Figure 3.15. Summary of TNF- α results based on experiments 3-6;

S1 - experiment 6; S2 - experiment 5; S3 - experiment 4; S4 - experiment 3;

1 - glass, 24 hrs inc; 2 - coll+DMSO, 24 hrs inc; 3 - HDPE, 24 hrs inc;

4 - glass, 48 hrs inc; 5 - coll+DMSO, 48 hrs inc; 6 - HDPE, 48 hrs inc

Figure 3.16. IC-21 cells exposed to HDPE particles for 24, 48, 72, and 96 hours; (a) TNF- α ; (b) β -GAL; (c) PGE₂ (see Tables 3, 5, 7 in the Appendix for n values)

Interestingly, the lysosomal enzyme release data for glass and DMSO alone were similar to those for all groups that contained collagen (see Figures 3.13 and 3.16, and Tables 5 and 6 of the Appendix). This suggests that collagen did not have an effect on cellular activation.

PGE₂ (Figure 3.13c) release was not significant at 24 hour incubation period ($p>0.05$), nor at 48 and 96 hour time points (Figure 3.16). The only difference between the collagen with DMSO and the particle groups appeared to be at the 72 hour time point ($p<0.05$ within samples of a specific experiment). However, 48, 72, and 96 hours of incubation experimental time points were only investigated once. A decrease in PGE₂ production relative to 24 hours was observed at 48, 72, and 96 hours of incubation (Figure 3.16).

3.3.4. Viability test

Cell viability was assessed by using trypan blue stain and was always above 95% for all test groups. Histological evaluation of the samples also confirmed that the cells appeared viable and exhibited features that are usually associated with normal cell morphology.⁶⁹

4. DISCUSSION

The purpose of this study was to develop a model that would allow phagocytosis of polyethylene particulate *in vitro*. A need for such an *in vitro* system exists since polyethylene is the major contributor to prosthetic wear and, at the same time, is the least studied component of the wear debris that has been isolated from patients with orthopaedic implant failure. Of particular concern at this time is the fact that despite the *in vivo* findings showing abundance of polyethylene particulate,⁶ there is little^{43,46,48} *in vitro* data available that has demonstrated phagocytosis of polyethylene particulate, and no histological findings from such studies that confirm the engulfment of the particles.

The absence of histological evidence of particulate phagocytosis in *in vitro* studies (not only polyethylene, but also PMMA and metal) could be explained by the fact that aseptic loosening has been investigated by two distinct approaches: (1) to determine the role of material in the process of aseptic loosening by measuring the levels of inflammatory mediators in response to the material *in vitro*, and; (2) to establish the role of these mediators in the aseptic loosening by utilizing *in vivo* models and studying retrieved inflammatory membranes. The first approach has employed biochemical methods of analysis, such as ELISA, producing large quantities of data without histological confirmation of either phagocytosis or cell viability. The second approach has utilized histological methods as well as immunohistochemistry to analyze retrieved specimens for cellular responses to particulate debris, however, these *in vivo* models and retrieved membranes do not provide the detailed information about individual cellular responses to particles, at the mechanistic level. Thus, a vacuum in the basic research of cell/particle interactions exists, producing a necessity for a well characterized *in vitro* model studying macrophage-particle interactions.

The new *in vitro* model presented in this work enables us to consistently introduce micron size polyethylene particles into macrophages. The internalization of particles has been repeatedly demonstrated by SEM (Figure 3.7) and TEM (Figure 3.8). The *in vitro* system described herein is simple and versatile, and, furthermore, allows for the media analysis of

cytokines and lysosomal enzymes. Thus, it incorporates the traditional methods of histological analysis and permits biochemical characterization of the model.

4.1. HISTOLOGICAL CHARACTERIZATION

As mentioned before, there are some difficulties associated with handling polyethylene particulate: it is hydrophobic and has a low density, and, therefore, is incompatible with aqueous media. This problem was overcome in the following manner. Type I collagen was selected for use in this system because polymerization of the collagen was considered to be a practical tool for trapping the particles in a solidified matrix formed in the culture system. In part, this mimics the *in vivo* environment, since collagen type I is the most abundant protein in the bone, and it is present in the inflammatory membrane.²⁴ At the same time, type I collagen is commonly applied in tissue culture to promote cell attachment onto different surfaces.⁶⁹

The use of collagen type I for incorporation of the polyethylene particles into the cells has biochemical significance. It has been previously described (see Section 1.4) that proteins of the extracellular matrix have numerous effects on cell differentiation and behavior. Apparently, the exposure to collagen can also increase the phagocytic activity of macrophages. Newman et al.⁶⁰ demonstrated that monocytes adhered to type I collagen gels phagocytosed 2.5-12-fold more opsonized bacteria than plastic adherent monocytes. This group discovered that the adherence to collagen gels activated complement receptors, as well as Fc receptors for phagocytosis, thus causing the increase in phagocytic ability of the cells. Newman et al.⁶⁰ hypothesized that the adherence of monocytes to the extracellular matrix during inflammation could activate the cells for enhanced phagocytic bactericidal activity. There are a few observations made in this study that could be extrapolated to the current research project. Polyethylene particles, as well as other constituents of wear debris, become opsonized by serum proteins *in vivo* and *in vitro* (since the culture media contains 10% fetal serum)(Boynton, Sandhu, unpublished data⁷⁴). The presence of collagen in the inflammatory membranes as well as in the *in vitro* model could activate the complement receptors,²⁵ thus enhancing the phagocytosis of the particles. That

phenomenon renders the *in vitro* model even more relevant to the *in vivo* situation since it not only mimics the surrounding environment of the inflammatory membrane, but also imitates the processes involved in phagocytosis of the wear particulate.

This *in vitro* model takes advantage of another useful property of collagen. As mentioned in the Introduction (section 1.4), collagen promotes differentiation of human blood monocytes into macrophages. Kaplan et al.⁵⁹ demonstrated that monocytes matured on collagen matrices appear to be tissue macrophage-like cells with enhanced Fc- and C₁-mediated phagocytosis. Even though this property of the model may not be relevant to the present study with fully differentiated macrophages, it has been useful for the development of experiments with human peripheral blood monocytes in our laboratory.⁷⁵

The histological evaluation of the cells exposed to the polyethylene particles demonstrated the ability to perform any type of histological analysis. Conventional hematoxylin and eosin staining, SEM, and TEM are easily performed with this model and the histological findings in this study are consistent with that reported in scientific literature.⁴⁶ Two different types of cellular reactions, associated with particle phagocytosis, are observed *in vivo*: (1) foreign body giant cell formation in response to large particles and; (2) the phagocytosis of small (< 5 µm) particles. The *in vivo* processes involved in response to particulate wear debris are mimicked by the model presented in this thesis. Histological evaluation of particle phagocytosis was demonstrated by hematoxylin and eosin staining (Figures 3.5, 3.6) and by SEM (Figure 3.7) analysis of the cultures. The majority of cells have engulfed particulate polyethylene. TEM analysis further confirmed that small particles were internalized by the macrophages, where they were incorporated into vacuoles (Figure 3.8) and not just surrounded by the cells. The cells appeared to be bigger in groups that contained particles as compared to control groups. As well, the former cells contained numerous lysosomes, which is a feature that is consistent with macrophage activation.⁷⁶ It should be noted that lysosome formation may not only be due to particle digestion but also result from collagen phagocytosis and digestion.

Despite the fact that there was no apparent cytotoxicity in response to phagocytosis of polyethylene particulate, based on histologically observed specimens, the particles appeared to

have had a significant effect on cellular function. In order to assess the effect of the particles on macrophage proliferation, the cell number and area percent counts were determined (complete data are given in Table 2 of the Appendix). When evaluating cell counts (Figures 3.9 and 3.10), a consistent inhibition of cell proliferation by polyethylene particles ($p < 0.1$) was demonstrated at 24 hours of incubation in four out of six experiments. At 48 hours of incubation three out of four experiments illustrated significant ($p < 0.05$) differences between collagen/DMSO controls and the particle groups. Only one experiment was designed to look at later time points and the differences between the groups were even more dramatic for 72 and 96 hours of incubation ($p = 0.0007$ at 72 hours and $p < 0.0001$ at 96 hours, see Figure 3.9). The evaluation of cell numbers using area % counts demonstrated a similar picture. It can be suggested from these data that the inhibition of cell proliferation was consistent between experiments and progressive with time. Nevertheless, the cell viability, as verified by trypan blue, was 98% for all test groups, and, therefore, was not affected by particulate phagocytosis. Thus, polyethylene phagocytosis was not toxic to the cells but affected the cell proliferation.

The observed decrease in the rate of macrophage proliferation during particle phagocytosis was consistent with the data available in literature.^{10,77} Shanbhag et al.¹⁰ demonstrated that P388D1 mouse macrophage cells exposed to polystyrene and titania particles generally incorporated less thymidine than controls, thus illustrating the decrease in DNA synthesis. At the same time, human fibroblasts exposed to PMMA particles demonstrated an increase in fibroblast proliferation,⁴⁷ while the experiments with bovine synovial fibroblasts illustrated a correlation between particulate material, particle size, particle concentration and cell proliferation.⁷⁷ An increase in particle concentration usually corresponded with a decrease in proliferation rate.⁷⁷ It should be noted that even though the results obtained with image analysis correlate to those performed by other methods of analysis, the whole concept of inhibiting cell proliferation may not be relevant to the *in vivo* situation at all, since macrophages *in vivo* are fully differentiated cells and they do not divide at the bone-implant interface. Nevertheless, the fact that cell proliferation was affected in a cell line may suggest that either the cells 'slow down' while being occupied with digesting the engulfed material, or the cells secrete

an inhibitor of proliferation as a response to particle phagocytosis. The later hypothesis can be supported by studies performed with human fibroblasts, where the cells were exposed to conditioned media from macrophage cultures that had been exposed to particulate material.¹⁰ In the latter study it was observed that exposure of macrophages to titania particles inhibited fibroblast proliferation, while the exposure to polystyrene particles had an inhibitory effect at low concentrations and a stimulating effect at high concentrations.¹⁰ These results demonstrate that the cells secrete different "sets" of cytokines in response to different types of particles. Therefore, it appears that in response to polyethylene phagocytosis, macrophages could potentially secrete an inhibitor of cell proliferation.

Even though image analysis was utilized in this study to assess cell number and to normalize the cytokine and lysosomal enzyme data, this method was found not to be very sensitive for determining slight differences in cell number, between the groups. The conventional methods of determining the rates of cell proliferation, such as tritiated thymidine incorporation to measure DNA synthesis^{47,67}, and protein measurements,⁴⁷ are more sensitive assays and could be used in future experiments. Nevertheless, image analysis is a simple method that does not require any additional procedures to be performed on existing histological slides and can provide a quick estimate of cell number or cell area when conventional methods are not available.

4.2. BIOCHEMICAL CHARACTERIZATION

It was noted in the Introduction, that differences exist between the resting and activated macrophage. The main features exhibited by activated macrophages include increase in size, ruffling of the plasma membrane, increase in the number of pinocytic vesicles, increase or decrease of different surface receptors and the release of inflammatory mediators.⁷⁶ Of all of these events the latter has been believed to be of greater importance.⁷⁶ Cytokines, prostaglandins, and lysosomal enzymes are considered to be markers of macrophage activation.^{32,76} Cytokines and PGE₂ have numerous activities, including the ability to enhance

phagocytosis, to activate other cells and to inhibit or promote cellular proliferation.²⁵⁻²⁹ On the other hand, the lysosomal enzymes carry out a completely different set of activities. It has been described that the release of lysosomal enzymes by macrophages can occur by undamaged cells by exocytosis. This is particularly noted with older macrophages and in locations near toxic or irritating substances.³² Salthouse et al.³² has also observed that geometry, surface, and other characteristics of the implant can affect the levels of lysosomal enzyme activity. Therefore, in order to confirm increased cell activation during phagocytosis of the particles, the media from cell cultures were collected and cytokine and lysosomal enzyme profiles determined.

4.2.1. Cytokines and prostaglandin E₂

As shown in Figures 3.12, 3.14, a significant increase of IL-1 α and IL-1 β for the cells exposed to the particles, at 24 hours of incubation, was demonstrated. The release of IL-6, TNF- α , and PGE₂ was not significant at 24 hours of incubation (Figures 3.12, 3.13, 3.15, 3.16). These findings correlate in part with the analyses of the human retrieval specimens^{5,7}, as well as with cell culture results obtained from the study of different particle types.^{6,43-45} Al Saffar et al.⁷ utilized immunohistochemistry on tissue sections and demonstrated the presence of IL-1 β containing cells (80% of these cells were macrophages) in the retrieved tissues. Jiranek et al.⁵ employed hybridization of the tissue sections and observed that IL-1 β mRNA was predominantly associated with macrophages in response to polyethylene, PMMA, and metal debris present in all specimens.

The results presented in this thesis differ somewhat from Shanbhag et al.^{44,45} who demonstrated a significant increase in IL-1 β , IL-6, and PGE₂ production in response to the exposure of fabricated submicron polyethylene particles to human peripheral blood monocytes and an increase in PGE₂ release in response to retrieved polyethylene particles. The mean size of the retrieved polyethylene particles was $0.47 \pm 0.2 \mu\text{m}$, and the mean size of the fabricated polyethylene particles was $0.66 \pm 0.6 \mu\text{m}$. However, when their data were compared to controls the only statistically significant increase in cytokine production was observed at a particle/cell surface area ratio of 10. This corresponded to approximately 1130 particles per cell for

fabricated particles and 3520 particles per cell for retrieved particles. Not surprisingly, the cell viability at these concentrations for both particle types was only 50%. It is hard to imagine the relevance of such concentrations to the *in vivo* situation. Moreover, Shangbag's study showed no histological evidence to confirm internalization of the polyethylene particulate. Hence, the cytokine release may have well been due to cell death and not to particle phagocytosis. To the contrary, the data presented in this thesis show that an increase in cytokine production (IL-1 α , IL-1 β) was observed at a ratio of 1 particle per 10 cells, with a cell viability of 98%.

It must also be considered that the variance in cytokine release data between the thesis study and Shanbhag's work,⁴⁵ could be explained by the differences in cell types (monocytes⁴⁵ versus fully differentiated mouse macrophages) and in particle types (retrieved or fabricated submicron UHMWPE particulate⁴⁵ versus 4-10 μ m HDPE particles). As well, the retrieved particles in Shanbhag's work could have residual protein adsorbed to the surface of the particles. The presence of denaturated proteins on the surface can cause unpredictable responses from the cells.⁷⁸ Despite the variance in cytokine response, the development of this model will allow detailed investigation of these differences.

The presence of collagen in the system may also have played an important role in the macrophage response to the particles. At the same time, the data presented in this thesis are confirmed by the results obtained from Shirata et al.⁴⁸ This group used IC-21 cells and an inverted tissue culture system that enabled them to introduce HDPE particles into the macrophages. They demonstrated a statistically significant increase in IL-1 β release, however, the latter was the only cytokine measured in their study. Even though collagen was not employed in Shirata's model, their results correlate to the results presented in this thesis. Since both studies were carried by different protocols and different research groups, it is difficult to determine if collagen's role in the particle phagocytosis was specific. Future studies could simultaneously examine both models in order to assess if the presence of collagen increases the rate and levels of particle phagocytosis as well as the production and release of the cytokines.

The rationale for why the levels of TNF- α , one of the major pro-inflammatory mediators, were not statistically significant for particles versus no particles and why this finding differs

from that of other *in vitro* studies^{43,46} could possibly be explained by the fact that a mouse cell line was employed in this work. There may be specific differences between mouse and human macrophages in relation to interactions with polyethylene particulate. For this reason, future studies with the model will be performed with human derived macrophages. However, cell type may not be the only factor contributing to the differences in the cell response. As demonstrated by XPS (Table 3.1) and FTIR (Figure 3.2) data, there can be distinctions in surface chemistry and bulk properties between UHMWPE and HDPE. The combination of particle type and surface chemistry (i.e., metal versus PMMA versus PE), differences from the specific wear particles found *in vivo*, along with mouse cells could explain the differences in the TNF- α response. It has been reported in the recent literature^{6,45}, that particle size, shape, and surface chemistry are critical factors determining cell response. The particles retrieved from human specimens range from submicron values up to 100 μm and contain not only polyethylene, but other significant materials, such as metal and polymethyl methacrylate. The particle surface can be altered by repeated exposure to high concentrations of lysosomal enzymes and other oxidative agents produced by the inflammatory cells. Metal ions embedded into polyethylene particles⁷² could act as catalysts, increasing the rates of oxidation and changing particle surface chemistry. One could hypothesize, that oxidized particles may have the ability to cause enhanced cellular activation in comparison to non-oxidized particles. The significance of the material chemistry and structure emphasizes the importance of having a well characterized *in vitro* model available to study the different variables affecting cellular activation. Particle size is another parameter to be considered.^{12,19-22} It has been reported in literature that the majority of the retrieved polyethylene particles are submicron in size.^{12,19-22} Since the particles employed in this study were 4-10 μm in size instead of submicron, this could in part explain some of the differences in cytokine response. The cellular reaction could vary with the particle size simply due to the increase in reactive surface area. Nevertheless, the goal of introducing polyethylene particles into macrophages was achieved and an increase in cytokine production demonstrated cellular activation.

It should be considered that there may be another explanation as to why TNF- α levels were consistently not significant when comparing particle groups to collagen controls. It has been observed, that collagen has numerous effects on monocyte/macrophage responses, including enhancement of phagocytosis,⁶⁰ increase in surface receptors expression,^{25,60} and modulation of cytokine expression.⁶⁰⁻⁶² Eierman et al.⁶¹ observed that monocytes adhered to collagen produced significantly higher levels of TNF- α than monocytes cultured on tissue culture plastic. This phenomenon was repeated in the experiments presented in this thesis: the cells cultured on collagen secreted more cytokine than the cells in control groups (glass, DMSO)(see Figures 3.12 and 3.14). The collagen effect on the cells can bring about new considerations into the investigation of the mechanism of aseptic loosening. It is generally believed, that in aseptic loosening, the pro-inflammatory cytokine TNF- α is secreted by macrophages in response to wear debris phagocytosis.^{4,6,36,43,46} However, the only significant increases in TNF- α production that have been reported were in experiments with PMMA particles of a non-specified size,^{4,36} retrieved polyethylene particles,⁴⁶ and polyethylene particles embedded in agarose.⁴³ In experiments with PMMA,^{4,36} two groups of particles (1) < 300 μm and (2) 300-1000 μm were utilized, and the significant increase in TNF- α release was demonstrated. However, this phenomenon can be due to cell death rather than to particle phagocytosis. In the case of retrieved polyethylene particles,⁴⁶ the cells could be stimulated by proteins still attached to the particles, while Harada et al.⁴³ observed that agarose caused monocyte activation even in the absence of particles. That may bring one to hypothesize that TNF- α could be secreted not in response to the particulate phagocytosis, but in response to exposure to extracellular matrix proteins, especially collagen, which is abundant in the inflammatory membrane.²⁴ Nevertheless, the role of this cytokine still remains the same: augmentation of the inflammatory response, enhancement of cytokine production, and promotion of bone damage, however, the mechanism of the cytokine induction could be different. This hypothesis is confirmed by the model described in this thesis, since all collagen groups (collagen, collagen with DMSO, and collagen with DMSO and particles) secreted similar levels of TNF- α and polyethylene particulate phagocytosis did not have any measurable effect on

the production of this cytokine. Thus, the model mimics the *in vivo* environment of the inflammatory membrane by providing extracellular matrix to the cells and could be used in determining the roles and the mechanisms of induction of various cytokines in response to different types of stimuli.

4.2.2. Lysosomal enzymes

As shown in Figures 3.13, 3.16 a significant increase of β -GAL and HEX levels in cells exposed to the particles for 24 hours was demonstrated. While there were some experiments (see Tables 5, 6 in Appendix) which did not show the levels of lysosomal enzymes to be significant, it is important to emphasize that only the levels of released enzymes were measured in this study. Since, the primary function of lysosomal enzymes is to digest foreign material inside of the lysosome/phagolysosome^{27,31} and not to serve as an inflammatory mediator, the levels of the released enzymes may not have the same significant effect as the levels of released cytokines. At the same time, a knowledge of the amount and types of enzymes released by the cells can provide valuable information about the condition of the cells. For example, one could presume that in the case of frustrated phagocytosis the levels of released enzymes would be dramatically increased due to the processes involved, whereas a non-damaged cell would maintain relatively low levels of the released enzymes. As well, the levels of the lysosomal enzymes can also provide information about cell numbers. For example, Landegren⁶⁷ demonstrated that the levels of total hexosaminidase increased with an increase in cell number and the results corresponded to the data obtained by the thymidine incorporation assay. However, since the levels of intracellular HEX were not measured in the current study it was not possible to apply this technique to confirm the cell count values. Nevertheless, the presence of non-specific lysosomal enzymes, such as β -GAL and HEX, was demonstrated and a significant increase in enzyme release in particle groups compared to controls was observed.

4.3. SUMMARY

The preliminary characterization of this novel *in vitro* model has opened the possibility of studying the response of cells to wear debris in culture. The model has now been expanded and human macrophages are used in the system.⁷⁵ Histological evaluation of the cell response may allow one to detect minute differences in cell morphology in reaction to different types of particles, including polyethylene, PMMA, and metal, with biochemical confirmation of these differences. The versatility of the model allows one to utilize co-culture techniques and, therefore, provides a tool to study the mechanism of aseptic loosening and bone lysis due to macrophage activation by polyethylene particulate.

5. CONCLUSIONS

1. A novel *in vitro* model that allows one to consistently introduce polyethylene particulate into cells has been developed. It differs from other attempts to model polyethylene phagocytosis in that it utilizes the extracellular matrix protein - collagen - to provide the entrapment for the particles as well as a matrix for the cells. This model is simple and versatile making it an easy task to perform histological as well as biochemical analyses with the cells.
2. The model had been characterized both histologically and biochemically.
3. Histological evaluation of the model confirmed the internalization of the polyethylene particles and provided unique information about morphological changes in the cells in response to polyethylene particulate phagocytosis.
4. Biochemical evaluation of the model demonstrated the cytokine and lysosomal enzyme release in response to particulate phagocytosis, verifying cellular activation observed histologically, and established a peak cytokine response at 24 hours of incubation.

5. The presence of type I collagen played an important role by providing extracellular matrix for the cells, entrapment for the particles, and may have facilitated macrophage phagocytosis of the particles.
6. Image analysis was employed to assess the cell number in order to normalize the cytokine and lysosomal enzyme release data. Even though it is a quick and easy method of analysis, it may not be sensitive enough to detect minute changes in cell proliferation in response to particle phagocytosis.
7. While only the levels of the released enzymes have been measured in this study, the data show significant levels of enzymes for particles versus controls in several experiments. However, it was concluded that the levels of intracellular enzymes could provide more information about cellular response to particle phagocytosis.

6. RECOMMENDATIONS

1. The model could be used to investigate the response of macrophages to different particle types (metal, PMMA, polyethylene), different particle concentrations, and different particle sizes by utilizing histological as well as biochemical techniques.
2. The model could be employed to study the response to particulate phagocytosis by different cell types (mouse vs. rat vs. human, etc.) in order to provide information about differences between the species.
3. The model could be utilized to determine the effect of particulate phagocytosis on bone resorption using co-culture systems or macrophage conditioned media.
4. The model could allow for the investigation and identification of proteins/lysosomal enzymes adhered to the surface of the particles inside of the phagolysosome by immunohistochemistry. If particles with a chemically altered surface are used, the differences in protein adsorption to the particle surface could be determined and further assessed for specific cellular responses.

5. The model could be used in conjunction with *in vivo* experiments in order to determine the similarities and differences in one-cell type system versus multiple-cell type system.
6. The employment of immunohistochemical techniques could be used to study the effect of particulate internalization on cell structure and morphology.
7. It is highly recommended to utilize conventional methods of determining the rates of cell proliferation, such as protein and DNA analysis, for normalization of cytokine and lysosomal enzyme release data.
8. A further study on the potential role that collagen plays with respect to the uptake of particles must be investigated. A comparison study between collagen model versus the inverted system would probably be valuable in providing this information.
9. The model could be utilized to study bone resorption by particle-activated macrophages, since it is believed that macrophages are capable of direct bone resorption when stimulated by particle phagocytosis and, therefore, contribute to bone lysis in aseptic loosening.

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8. APPENDIX

Table 1. Summary of the performed experiments

Experiment #	Particle type	Incubation periods	Analyses performed	# of cell/well plated
0 **	UHMWPE	20	H&E, TNF- α , β -GAL, HEX	1×10^6
1	HDPE	20	H&E, TNF- α , β -GAL, HEX	1×10^6
2	HDPE	2, 6, 24	H&E, SEM, TEM, TNF- α , β -GAL, HEX, IL-1 β , PGE ₂	1×10^6
3	HDPE	24, 48, 72, 96	H&E, TNF- α , β -GAL, HEX, IL-1 α , IL-1 β , IL-6, PGE ₂	0.5×10^6
4	HDPE	24, 48	H&E, TNF- α , β -GAL, HEX, IL-1 β	0.5×10^6
5	HDPE	24, 48	H&E, TNF- α , β -GAL, HEX, IL-1 β	0.5×10^6
6	HDPE	24, 48	H&E, TNF- α , β -GAL, HEX, IL-1 β	0.5×10^6

** image analysis was not performed on Experiment 0

Table 2. Summary of cell and area % counts

Group type	Incubation time (h)	Cell count $\pm$ std. error	p, cell count	Area % count $\pm$ std. error	p, area count
Experiment 1					
GLASS	20	2005 $\pm$ 42.5		6.422 $\pm$ 0.142	
COLL+DMSO	20	2164 $\pm$ 54.3	*	7.853 $\pm$ 0.227	*
HDPE	20	1957 $\pm$ 39.3	*p=0.0038	6.566 $\pm$ 0.160	*p=0.0008
Experiment 2					
GLASS	2	1522 $\pm$ 75.7		5.008 $\pm$ 0.258	
COLL+DMSO	2	1832 $\pm$ 67.4	*	5.381 $\pm$ 0.206	*
HDPE	2	1817 $\pm$ 54.5	*p=0.8657	4.944 $\pm$ 0.141	*p=0.0881
GLASS	6	2022 $\pm$ 28.1		5.299 $\pm$ 0.103	
DMSO	6	1876 $\pm$ 28.3		6.139 $\pm$ 0.100	
COLL+DMSO	6	1834 $\pm$ 57.9	*	6.643 $\pm$ 0.251	*
HDPE	6	1865 $\pm$ 61.3	*p=0.7119	6.114 $\pm$ 0.258	*p=0.1499
GLASS	24	1944 $\pm$ 61.0		8.112 $\pm$ 0.282	
DMSO	24	1995 $\pm$ 39.1		8.722 $\pm$ 0.138	
COLL+DMSO	24	2288 $\pm$ 43.2	*	8.873 $\pm$ 0.218	*
HDPE	24	2219 $\pm$ 74.0	*p=0.4183	8.585 $\pm$ 0.368	*p=0.5048
Experiment 3					
GLASS	24	1244 $\pm$ 35.7		5.382 $\pm$ 0.152	
DMSO	24	1395 $\pm$ 41.6		5.417 $\pm$ 0.162	
COLLAGEN	24	1632 $\pm$ 40.2		6.953 $\pm$ 0.239	
COLL+DMSO	24	1628 $\pm$ 25.1	*	6.525 $\pm$ 0.149	*
HDPE	24	1438 $\pm$ 45.9	*p=0.0008	5.653 $\pm$ 0.185	*p=0.0007
GLASS	48	2275 $\pm$ 44.7		11.917 $\pm$ 0.242	
DMSO	48	2060 $\pm$ 59.3		10.347 $\pm$ 0.327	

COLLAGEN	48	2338 ± 59.1		11.756 ± 0.414	
COLL+DMSO	48	2410 ± 32.8	*	12.341 ± 0.284	*
HDPE	48	1839 ± 121	*p<0.0001	8.949 ± 0.698	*p<0.0001
GLASS	72	2744 ± 86.6		11.383 ± 0.367	
DMSO	72	2905 ± 84.9		13.865 ± 0.442	
COLLAGEN	72	3715 ± 65.8		17.005 ± 0.460	
COLL+DMSO	72	3813 ± 81.9	*	18.213 ± 0.483	*
HDPE	72	3336 ± 101	*p=0.0007	15.629 ± 0.423	*p=0.0003
GLASS	96	4972 ± 53.9		18.290 ± 0.328	
DMSO	96	5354 ± 42.5		19.071 ± 0.427	
COLLAGEN	96	5973 ± 55.6		24.324 ± 0.545	
COLL+DMSO	96	5152 ± 81.0	*	20.174 ± 0.364	*
HDPE	96	3672 ± 289	*p<0.0001	14.713 ± 1.211	*p=0.0001
Experiment 4					
GLASS	24	1277 ± 31.6		5.206 ± 0.149	
COLL+DMSO	24	1169 ± 22.2	*	4.196 ± 0.126	*
HDPE	24	1089 ± 36.1	*p=0.0675	3.933 ± 0.147	*p=0.1825
GLASS	48	2265 ± 60.7		9.187 ± 0.272	
COLL+DMSO	48	2184 ± 52.9	*	8.103 ± 0.245	*
HDPE	48	2026 ± 91.4	*p=0.1426	8.178 ± 0.375	*p=0.8680
Experiment 5					
GLASS	24	2066 ± 84.6		7.478 ± 0.411	
COLL+DMSO	24	2086 ± 82.4	*	7.800 ± 0.346	*
HDPE	24	1859 ± 81.8	*p=0.0582	6.469 ± 0.378	*p=0.0133
GLASS	48	2858 ± 75.4		10.360 ± 0.229	
COLL+DMSO	48	3161 ± 84.4	*	12.519 ± 0.286	*
HDPE	48	2671 ± 86.4	*p=0.0002	10.656 ± 0.325	*p=0.0001

Experiment 6					
GLASS	24	1972 ± 177		7.043 ± 0.639	
COLL+DMSO	24	2125 ± 200	*	7.851 ± 0.952	*
HDPE	24	1849 ± 154	*p=0.2808	6.295 ± 0.649	*p=0.1848
GLASS	48	2753 ± 194		12.066 ± 1.121	
COLL+DMSO	48	3103 ± 187	*	13.991 ± 0.928	*
HDPE	48	2001 ± 262	*p=0.0015	8.985 ± 1.316	*p=0.0036

* the samples were compared using student's paired t-test

Table 3. Summary of TNF- α data

Group type	Incubation time (h)	TNF- α , ng/mL mean $\pm$ std. Error	TNF- α / area %, pg/ml/area% mean $\pm$ std. error	p
Experiment 0	n=6			
GLASS	20	0.492 $\pm$ 0.022		
COLLAGEN	20	4.058 $\pm$ 0.125		*
UHMWPE	20	4.154 $\pm$ 0.192		*p=0.6759
Experiment 1	n=3			
GLASS	20	0.095 $\pm$ 0.016	17.79 $\pm$ 2.51	
COLL+DMSO	20	2.750 $\pm$ 0.081	362.65 $\pm$ 15.23	*
HDPE	20	2.902 $\pm$ 0.389	441.97 $\pm$ 60.22	*p=0.2707
Experiment 2	n=3			
GLASS	2	0.137 $\pm$ 0.007	27.36 $\pm$ 1.98	
DMSO	2	0.137 $\pm$ 0.019		
COLL+DMSO	2	1.133 $\pm$ 0.070	210.56 $\pm$ 15.30	*
HDPE	2	1.143 $\pm$ 0.166	231.19 $\pm$ 34.22	*p=0.6113
GLASS	6	0.175 $\pm$ 0.013	33.03 $\pm$ 2.54	
DMSO	6	0.181 $\pm$ 0.001	29.48 $\pm$ 0.51	
COLL+DMSO	6	2.366 $\pm$ 0.321	356.16 $\pm$ 50.16	*
HDPE	6	2.245 $\pm$ 0.046	367.19 $\pm$ 17.22	*p=0.8455
GLASS	24	0.179 $\pm$ 0.011	22.07 $\pm$ 1.56	
DMSO	24	0.222 $\pm$ 0.006	25.45 $\pm$ 0.80	
COLL+DMSO	24	2.399 $\pm$ 0.382	270.37 $\pm$ 43.56	*
HDPE	24	2.163 $\pm$ 0.087	251.95 $\pm$ 14.81	*p=0.7094
Experiment 3	n=8 at 24	n=6 at 48	n=4 at 72	n=2 at 96
GLASS	24	1.356 $\pm$ 0.080	251.95 $\pm$ 16.48	

DMSO	24	1.337 ± 0.144	246.82 ± 27.59	
COLLAGEN	24	4.402 ± 0.341	633.11 ± 53.66	
COLL+DMSO	24	4.716 ± 0.674	722.76 ± 104.61	*
HDPE	24	4.437 ± 0.518	784.89 ± 95.16	*p=0.6671
GLASS	48	0.588 ± 0.332	49.34 ± 27.88	
DMSO	48	0.340 ± 0.092	32.86 ± 8.95	
COLLAGEN	48	0.585 ± 0.120	49.76 ± 10.36	
COLL+DMSO	48	0.665 ± 0.085	53.89 ± 7.00	*
HDPE	48	1.480 ± 0.602	165.38 ± 68.50	*p=0.1364
GLASS	72	0.081 ± 0.056	7.12 ± 4.92	
DMSO	72	0.198 ± 0.067	14.28 ± 4.85	
COLLAGEN	72	0.388 ± 0.021	22.82 ± 1.38	
COLL+DMSO	72	0.262 ± 0.161	14.39 ± 8.85	*
HDPE	72	0.633 ± 0.041	40.50 ± 2.84	*p=0.0308
GLASS	96	0.000 ± 0.000	0.00 ± 0.00	
DMSO	96	0.150 ± 0.096	7.87 ± 5.04	
COLLAGEN	96	0.498 ± 0.032	20.47 ± 1.39	
COLL+DMSO	96	0.049 ± 0.049	2.43 ± 2.43	*
HDPE	96	0.177 ± 0.177	12.03 ± 12.07	*p=0.5172
Experiment 4	n=4			
GLASS	24	0.190 ± 0.155	36.40 ± 29.79	
COLL+DMSO	24	3.110 ± 0.682	741.06 ± 163.93	*
HDPE	24	2.481 ± 0.654	630.69 ± 167.82	*p=0.6546
GLASS	48	0.005 ± 0.005	0.544 ± 0.544	
COLL+DMSO	48	0.000 ± 0.000	0.00 ± 0.00	*
HDPE	48	0.396 ± 0.330	48.36 ± 40.46	*p=0.2771
Experiment 5	n=4			

GLASS	24	0.099 ± 0.052	13.17 ± 6.99	
COLL+DMSO	24	2.590 ± 0.531	332.05 ± 69.59	*
HDPE	24	3.623 ± 0.562	559.98 ± 92.83	*p=0.0971
GLASS	48	0.094 ± 0.086	9.03 ± 8.33	
COLL+DMSO	48	0.315 ± 0.063	25.19 ± 5.03	*
HDPE	48	0.940 ± 0.365	88.21 ± 34.33	*p=0.1192
Experiment 6	n=4			
GLASS	24	0.400 ± 0.163	56.79 ± 23.65	
COLL+DMSO	24	2.206 ± 0.539	280.92 ± 76.58	*
HDPE	24	1.592 ± 0.215	252.82 ± 42.94	*p=0.7598
GLASS	48	0.384 ± 0.024	31.78 ± 3.56	
COLL+DMSO	48	0.575 ± 0.031	41.10 ± 3.53	*
HDPE	48	1.155 ± 0.389	128.49 ± 47.21	*p=0.1144

* the samples were compared using student's paired t-test

Table 4. Summary of IL-1 β data

Group type	Incubation time (h)	IL-1 β , pg/mL mean $\pm$ std. error	IL-1 β / area %, pg/ml/area% mean $\pm$ std. Error	p
Experiment 2	n=3			
GLASS	24	0.288 $\pm$ 0.138	0.036 $\pm$ 0.017	
DMSO	24	0.978 $\pm$ 0.828	0.112 $\pm$ 0.095	
COLL+DMSO	24	1.530 $\pm$ 0.421	0.172 $\pm$ 0.048	*
HDPE	24	19.462 $\pm$ 1.244	2.267 $\pm$ 0.174	*p<0.0001
Experiment 3	n=4			
GLASS	24	2.020 $\pm$ 0.720	0.375 $\pm$ 0.134	
DMSO	24	4.550 $\pm$ 2.990	0.840 $\pm$ 0.553	
COLLAGEN	24	3.290 $\pm$ 0.460	0.473 $\pm$ 0.068	
COLL+DMSO	24	3.960 $\pm$ 0.610	0.607 $\pm$ 0.095	*
HDPE	24	48.810 $\pm$ 4.440	8.634 $\pm$ 0.835	*p<0.0001
GLASS	48	2.110 $\pm$ 0.320	0.177 $\pm$ 0.027	
DMSO	48	2.190 $\pm$ 0.710	0.212 $\pm$ 0.069	
COLLAGEN	48	4.640 $\pm$ 0.680	0.395 $\pm$ 0.059	
COLL+DMSO	48	2.440 $\pm$ 0.420	0.198 $\pm$ 0.034	*
HDPE	48	4.210 $\pm$ 0.580	0.470 $\pm$ 0.074	*p=0.0159
Experiment 4	n=4			
GLASS	24	1.185 $\pm$ 0.306	0.228 $\pm$ 0.059	
COLL+DMSO	24	1.116 $\pm$ 0.523	0.266 $\pm$ 0.125	*
HDPE	24	9.944 $\pm$ 1.071	2.528 $\pm$ 0.288	*p=0.0004
GLASS	48	0.243 $\pm$ 0.092	0.026 $\pm$ 0.010	
COLL+DMSO	48	1.438 $\pm$ 0.092	0.177 $\pm$ 0.013	*
HDPE	48	1.622 $\pm$ 0.460	0.198 $\pm$ 0.057	*p=0.7327

Experiment 5	n=4			
GLASS	24	1.668 ± 0.365	0.223 ± 0.050	
COLL+DMSO	24	2.909 ± 0.338	0.373 ± 0.046	*
HDPE	24	5.024 ± 0.512	0.777 ± 0.091	*p=0.0076
GLASS	48	0.270 ± 0.173	0.026 ± 0.017	
COLL+DMSO	48	0.332 ± 0.232	0.027 ± 0.019	*
HDPE	48	0.842 ± 0.451	0.079 ± 0.042	*p=0.3199
Experiment 6	n=4			
GLASS	24	0 ± 0	0 ± 0	
COLL+DMSO	24	0.155 ± 0.146	0.020 ± 0.019	*
HDPE	24	7.439 ± 0.560	1.182 ± 0.151	*p=0.0003
GLASS	48	0.270 ± 0.135	0.022 ± 0.011	
COLL+DMSO	48	0.467 ± 0.063	0.033 ± 0.005	0*
HDPE	48	0.717 ± 0.226	0.080 ± 0.028	*p=0.1741

• the samples were compared using student's paired t-test

Table 5. Summary of extracellular β -GAL data

Group type	Incubation time (h)	β -GAL, nm/h/mL mean $\pm$ std. error	β -GAL/ area %, nm/h/mL/area % mean $\pm$ std. error	p
Experiment 0	n=6			
GLASS	20	26.167 $\pm$ 2.120		
COLLAGEN	20	8.667 $\pm$ 0.211		*
UHMWPE	20	9.500 $\pm$ 0.342		*p=0.0925
Experiment 1	n=3			
GLASS	20	29.333 $\pm$ 2.333	4.568 $\pm$ 0.377	
COLL+DMSO	20	21.333 $\pm$ 0.667	2.813 $\pm$ 0.122	*
HDPE	20	31.333 $\pm$ 2.333	4.772 $\pm$ 0.374	*p=0.0076
Experiment 2	n=3			
GLASS	2	10.090 $\pm$ 0.500	2.015 $\pm$ 0.144	
DMSO	2	9.650 $\pm$ 0.600		
COLL+DMSO	2	10.130 $\pm$ 0.120	1.883 $\pm$ 0.075	*
HDPE	2	10.330 $\pm$ 0.120	2.089 $\pm$ 0.064	*p=0.1053
GLASS	6	14.610 $\pm$ 1.150	2.757 $\pm$ 0.224	
DMSO	6	13.310 $\pm$ 0.080	2.168 $\pm$ 0.038	
COLL+DMSO	6	12.280 $\pm$ 1.460	1.849 $\pm$ 0.231	*
HDPE	6	13.070 $\pm$ 0.300	2.138 $\pm$ 0.103	*p=0.3159
GLASS	24	35.090 $\pm$ 0.510	4.326 $\pm$ 0.163	
DMSO	24	35.560 $\pm$ 0.270	4.077 $\pm$ 0.072	
COLL+DMSO	24	31.420 $\pm$ 1.810	3.541 $\pm$ 0.222	*
HDPE	24	34.250 $\pm$ 0.080	3.990 $\pm$ 0.171	*p=0.1848
Experiment 3	n=8 at 24	n=6 at 48	n=4 at 72	n=2 at 96
GLASS	24	17.730 $\pm$ 0.250	2.761 $\pm$ 0.072	

DMSO	24	17.980 ± 0.150	2.371 ± 0.074	
COLLAGEN	24	17.130 ± 0.210	2.609 ± 0.100	
COLL+DMSO	24	17.090 ± 0.260	2.619 ± 0.072	*
HDPE	24	17.370 ± 0.270	3.072 ± 0.111	*p=0.0041
GLASS	48	25.350 ± 1.520	2.127 ± 0.135	
DMSO	48	27.770 ± 2.360	2.684 ± 0.243	
COLLAGEN	48	25.170 ± 2.240	2.141 ± 0.205	
COLL+DMSO	48	23.880 ± 1.440	1.935 ± 0.125	*
HDPE	48	23.970 ± 1.630	2.679 ± 0.277	*p=0.0345
GLASS	72	42.890 ± 1.180	3.768 ± 0.160	
DMSO	72	44.200 ± 1.040	3.188 ± 0.126	
COLLAGEN	72	43.540 ± 0.850	2.560 ± 0.085	
COLL+DMSO	72	44.030 ± 0.510	2.418 ± 0.070	*
HDPE	72	42.020 ± 0.980	2.689 ± 0.096	*p=0.0627
GLASS	96	56.220 ± 2.560	3.074 ± 0.150	
DMSO	96	57.110 ± 1.840	2.995 ± 0.117	
COLLAGEN	96	60.440 ± 4.550	2.485 ± 0.195	
COLL+DMSO	96	59.180 ± 0.480	2.933 ± 0.058	*
HDPE	96	59.070 ± 2.740	4.015 ± 0.379	*p=0.1062
Experiment 4	n=4			
GLASS	24	14.500 ± 0.289	2.785 ± 0.097	
COLL+DMSO	24	13.500 ± 0.289	3.217 ± 0.119	*
HDPE	24	15.250 ± 0.947	3.877 ± 0.281	*p=0.0736
GLASS	48	22.000 ± 0.577	2.395 ± 0.095	
COLL+DMSO	48	20.250 ± 1.436	2.499 ± 0.193	*
HDPE	48	23.500 ± 0.289	2.874 ± 0.136	*p=0.1638
Experiment 5	n=4			

GLASS	24	16.750 ± 1.436	2.240 ± 0.228	
COLL+DMSO	24	14.250 ± 0.250	1.827 ± 0.087	*
HDPE	24	16.250 ± 0.947	2.512 ± 0.207	*p=0.0226
GLASS	48	26.500 ± 0.646	2.558 ± 0.084	
COLL+DMSO	48	23.500 ± 0.289	1.877 ± 0.049	*
HDPE	48	27.750 ± 0.479	2.604 ± 0.091	*p=0.0004
Experiment 6	n=4			
GLASS	24	14.750 ± 0.750	2.094 ± 0.218	
COLL+DMSO	24	15.000 ± 0.707	1.911 ± 0.249	*
HDPE	24	16.250 ± 0.947	2.581 ± 0.306	*p=0.1395
GLASS	48	28.000 ± 0.707	2.321 ± 0.223	
COLL+DMSO	48	24.250 ± 0.947	1.733 ± 0.133	*
HDPE	48	27.500 ± 1.323	3.061 ± 0.472	*p=0.0352

- the samples were compared using student's paired t-test

Table 6. Summary of extracellular HEX data

Group type	Incubation time (h)	HEX, nm/h/mL mean $\pm$ std. error	HEX/ area %, nm/h/mL/area% mean $\pm$ std. error	p
Experiment 0	n=6			
GLASS	20	1295 $\pm$ 51		
COLLAGEN	20	739 $\pm$ 17		*
UHMWPE	20	779 $\pm$ 11		*p=0.1102
Experiment 1	n=3			
GLASS	20	891 $\pm$ 15	139 $\pm$ 3.81	
COLL+DMSO	20	719 $\pm$ 15	95 $\pm$ 3.48	*
HDPE	20	781 $\pm$ 18	119 $\pm$ 3.97	*p=0.0102
Experiment 2	n=3			
GLASS	2	496 $\pm$ 1	99 $\pm$ 5.11	
DMSO	2	494 $\pm$ 3		
COLL+DMSO	2	490 $\pm$ 5	91 $\pm$ 3.62	*
HDPE	2	508 $\pm$ 3	103 $\pm$ 3.03	*p=0.0695
GLASS	6	601 $\pm$ 9	113 $\pm$ 2.78	
DMSO	6	613 $\pm$ 12	100 $\pm$ 2.54	
COLL+DMSO	6	542 $\pm$ 54	82 $\pm$ 8.68	*
HDPE	6	622 $\pm$ 6	102 $\pm$ 4.40	*p=0.1071
GLASS	24	1356 $\pm$ 8	167 $\pm$ 5.88	
DMSO	24	1465 $\pm$ 25	168 $\pm$ 3.87	
COLL+DMSO	24	1465 $\pm$ 106	165 $\pm$ 12.60	*
HDPE	24	1421 $\pm$ 58	165 $\pm$ 9.79	*p=0.9838
Experiment 3	n=8 at 24	n=6 at 48	n=4 at 72	n=2 at 96
GLASS	24	1045 $\pm$ 27	163 $\pm$ 5.53	

DMSO	24	1065 ± 30	140 ± 5.77	
COLLAGEN	24	1001 ± 37	152 ± 7.91	
COLL+DMSO	24	980 ± 21	150 ± 4.70	*
HDPE	24	975 ± 26	172 ± 7.28	*p=0.0222
GLASS	48	2567 ± 65	215 ± 6.99	
DMSO	48	2656 ± 63	257 ± 10.14	
COLLAGEN	48	2126 ± 112	181 ± 11.46	
COLL+DMSO	48	1782 ± 36	144 ± 4.42	*
HDPE	48	1957 ± 74	219 ± 18.96	*p=0.0034
GLASS	72	2706 ± 122	238 ± 13.18	
DMSO	72	2889 ± 125	208 ± 11.20	
COLLAGEN	72	2693 ± 179	158 ± 11.36	
COLL+DMSO	72	2646 ± 21	145 ± 4.02	*
HDPE	72	2689 ± 90	172 ± 7.41	*p=0.0192
GLASS	96	2862 ± 172	156 ± 9.81	
DMSO	96	2615 ± 474	137 ± 25.04	
COLLAGEN	96	2991 ± 90	123 ± 4.61	
COLL+DMSO	96	2913 ± 51	144 ± 3.63	*
HDPE	96	2924 ± 40	199 ± 16.58	*p=0.0853
Experiment 4	n=4			
GLASS	24	960 ± 23	184 ± 6.88	
COLL+DMSO	24	851 ± 12	203 ± 6.68	*
HDPE	24	878 ± 18	223 ± 9.53	*p=0.1280
GLASS	48	1740 ± 44	189 ± 7.40	
COLL+DMSO	48	1578 ± 31	195 ± 7.03	*
HDPE	48	1586 ± 29	194 ± 9.59	*p=0.9441
Experiment 5	n=4			

GLASS	24	1337 ± 27	179 ± 10.49	
COLL+DMSO	24	1187 ± 34	152 ± 8.00	•
HDPE	24	1250 ± 24	193 ± 11.88	*p=0.0286
GLASS	48	2009 ± 41	194 ± 5.80	
COLL+DMSO	48	1724 ± 43	138 ± 4.63	•
HDPE	48	1732 ± 33	162 ± 5.86	*p=0.0161
Experiment 6	n=4			
GLASS	24	965 ± 22	137 ± 12.80	
COLL+DMSO	24	911 ± 23	116 ± 14.37	•
HDPE	24	990 ± 24	157 ± 16.64	*p=0.1102
GLASS	48	1692 ± 44	140 ± 13.53	
COLL+DMSO	48	1528 ± 29	109 ± 7.54	•
HDPE	48	1512 ± 6	168 ± 24.65	*p=0.0621

• the samples were compared using student's paired t-test

Table 7. Summary of PGE₂ data

Group type	Incubation time (h)	PGE ₂ , pg/0.1 mL mean $\pm$ std. error	PGE ₂ /area%, pg/0.1 mL/area% mean $\pm$ std. error	p
Experiment 2	n=3			
GLASS	6	19.3 $\pm$ 2.5	3.642 $\pm$ 0.477	
DMSO	6	21.2 $\pm$ 0.4	3.453 $\pm$ 0.086	
COLL+DMSO	6	20.2 $\pm$ 0.2	3.045 $\pm$ 0.117	*
HDPE	6	21.4 $\pm$ 0.4	3.500 $\pm$ 0.162	*p=0.0849
GLASS	24	10.5 $\pm$ 4.3	1.294 $\pm$ 0.532	
DMSO	24	12.6 $\pm$ 0.6	1.445 $\pm$ 0.072	
COLL+DMSO	24	12.8 $\pm$ 0.4	1.443 $\pm$ 0.057	*
HDPE	24	14.07 $\pm$ 0.87	1.639 $\pm$ 0.123	*p=0.2223
Experiment 3	n=8 at 24	n=6 at 48	n=4 at 72	n=2 at 96
GLASS	24	8.53 $\pm$ 1.19	1.584 $\pm$ 0.226	
DMSO	24	8.58 $\pm$ 1.14	1.584 $\pm$ 0.216	
COLLAGEN	24	9.50 $\pm$ 1.52	1.366 $\pm$ 0.224	
COLL+DMSO	24	9.71 $\pm$ 1.20	1.488 $\pm$ 0.187	*
HDPE	24	11.75 $\pm$ 1.52	2.079 $\pm$ 0.277	*p=0.0994
GLASS	48	5.05 $\pm$ 0.48	0.424 $\pm$ 0.041	
DMSO	48	4.60 $\pm$ 0.51	0.445 $\pm$ 0.051	
COLLAGEN	48	4.26 $\pm$ 0.60	0.362 $\pm$ 0.053	
COLL+DMSO	48	4.12 $\pm$ 0.63	0.334 $\pm$ 0.052	*
HDPE	48	4.49 $\pm$ 0.76	0.502 $\pm$ 0.094	*p=0.1471
GLASS	72	8.73 $\pm$ 2.03	0.767 $\pm$ 0.180	
DMSO	72	5.25 $\pm$ 0.51	0.379 $\pm$ 0.039	
COLLAGEN	72	3.55 $\pm$ 0.26	0.209 $\pm$ 0.016	

COLL+DMSO	72	4.2 ± 0.53	0.231 ± 0.030	•
HDPE	72	5.43 ± 0.1	0.347 ± 0.011	*p=0.0105
GLASS	96	17.6 ± 1.6	0.962 ± 0.089	
DMSO	96	19.6 ± 0.4	1.028 ± 0.031	
COLLAGEN	96	16.4 ± 0.4	0.674 ± 0.022	
COLL+DMSO	96	15.3 ± 0.7	0.758 ± 0.037	•
HDPE	96	16.4 ± 0.4	1.115 ± 0.096	*p=0.0740

* the samples were compared using student's paired t-test

Table 8. Summary of IL-1 α data

Group type	Incubation time (h)	IL-1 α , pg/mL mean $\pm$ std. error	IL-1 α / area %, pg/ml/area% mean $\pm$ std. error	p
Experiment 3	n=4			
GLASS	24	9.12 $\pm$ 0.88	1.695 $\pm$ 0.170	
DMSO	24	7.67 $\pm$ 0.47	1.420 $\pm$ 0.097	
COLLAGEN	24	14.40 $\pm$ 0.18	2.071 $\pm$ 0.076	
COLL+DMSO	24	19.22 $\pm$ 0.63	2.946 $\pm$ 0.118	*
HDPE	24	189.83 $\pm$ 8.58	33.580 $\pm$ 1.874	*p<0.0001
GLASS	48	11.10 $\pm$ 2.33	0.931 $\pm$ 0.196	
DMSO	48	10.92 $\pm$ 2.86	1.055 $\pm$ 0.278	
COLLAGEN	48	27.71 $\pm$ 3.91	2.357 $\pm$ 0.343	
COLL+DMSO	48	25.21 $\pm$ 3.91	2.043 $\pm$ 0.320	*
HDPE	48	59.77 $\pm$ 4.64	6.679 $\pm$ 0.735	*p=0.0012

* the samples were compared using student's paired t-test

Table 9. Summary of IL-6 data

Group type	Incubation time (h)	IL-6, pg/mL mean $\pm$ std. error	IL-6/ area %, pg/ml/area% mean $\pm$ std. error	p
Experiment 3	n=4			
GLASS	24	47.63 $\pm$ 5.81	8.850 $\pm$ 1.108	
DMSO	24	32.20 $\pm$ 11.84	5.944 $\pm$ 2.193	
COLLAGEN	24	1583 $\pm$ 41	228 $\pm$ 9.84	
COLL+DMSO	24	1628 $\pm$ 30	249 $\pm$ 7.33	*
HDPE	24	1589 $\pm$ 51	281 $\pm$ 12.90	*p=0.0774
GLASS	48	0.00 $\pm$ 0.00	0 $\pm$ 0	
DMSO	48	0.00 $\pm$ 0.00	0 $\pm$ 0	
COLLAGEN	48	280 $\pm$ 19	23.81 $\pm$ 1.834	
COLL+DMSO	48	113 $\pm$ 20	9.15 $\pm$ 1.625	*
HDPE	48	61.3 $\pm$ 10.2	6.84 $\pm$ 1.261	*p=0.3043

* the samples were compared using student's paired t-test