ANTIOXIDANT MICROSPHERES AS DRUG DELIVERY VEHICLES FOR THE PREVENTION OF POST-TRAUMATIC OSTEOARTHRITIS

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CHAPTER I

INTRODUCTION

Text adapted from:

Kavanaugh, T. E.; Werfel, T. A.; Cho, H.; Hasty, K. A.; Duvall, C. L. Particle-based technologies for osteoarthritis detection and therapy. *Drug Deliv Transl Res* **2015**.

Overview of Osteoarthritis

Osteoarthritis (OA) is a debilitating disease that affects joints and their surrounding tissues leading to pain and loss of mobility. While there are many factors involved in the initiation of OA, the full context is not thoroughly understood; it is considered to be a complex disease of the whole joint, rather than a specific cellular or matrix component.[8] Many potential risk factors for OA such as genetic predisposition,[9] aging,[10] obesity,[11] and joint trauma or misalignment[12,13] have been investigated. While the mechanism of action and pathogenesis of the disease remains incompletely elucidated, it is clear that a combination of both mechanical and biological factors are involved.[14, 8,15] Figure 1 illustrates the multifaceted, negative impacts that arthritis can have on joints.

Because OA is high prevalence and causes significant morbidity, an improved understanding of the pathogenesis of OA and development of improved OA therapies are significant medical needs. As of 2012, over 25% of the United States population over the age of 45 was afflicted with OA.[16] This number is expected to increase to almost 30% by the year 2032.[16] Not only is OA physically incapacitating, it causes a significant financial burden to patients and

the healthcare system. Healthcare costs related to OA totaled over $60 billion in 2007, and the aggregate cost of OA is expected to increase to $185.5 billion per year based on data from 2007.[17]

Current treatments of OA are primarily focused on pain alleviation and the improvement of joint function and mobility.[18] These treatments can be classified into three main categories: non-pharmacological, pharmacological, and surgical. Non-pharmacological treatments include reduction of weight on the affected joint or braces that mechanically stabilize the joint. These treatments can be effective, but many patients find them difficult to implement for extended periods of time. Joint replacement surgeries are common in patients with severe symptoms and are generally very effective.[19] However, surgery is often only utilized as a last resort after pharmacological treatments have failed and the patient has experienced debilitating pain for many years.[20]

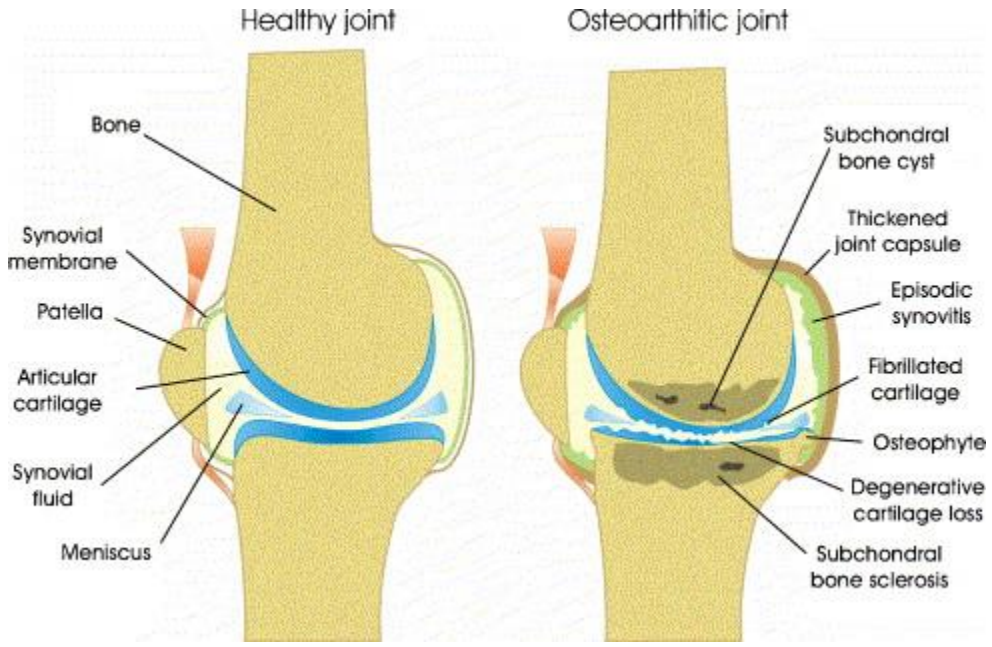

Figure 1: Schematic of a healthy (left) and osteoarthritic (right) knee joint. This schematic shows the detrimental effects caused by the presence of arthritis in a joint. Reproduced with permission from Elsevier. [6]

Unfortunately, no current treatments address the underlying molecular causes of the disease[14,20] or are curative in nature. Pharmacological treatments include the administration of analgesics such as non-steroidal anti-inflammatory drugs (NSAID) and in some cases analgesics such as opioids or narcotics.[21] Intra-articular injections and systemic administration of long-acting glucocorticoids can also be effective during flares of inflammation, but these only work temporarily and can have negative consequences for long term use.[22,23] Other current pharmaceuticals such as hyaluronic acid-based products rely on longitudinal intra-articular injections that each supply only 1-3 months of symptom relief. Injection directly into the joint enables control over dosing the target tissue, but most drugs are quickly cleared from the joint cavity, reducing any potential for long term benefits of treatment.[24] Systemic administration of drugs for OA has been associated with severe side effects with little therapeutic benefit. NSAIDs are known to cause gastrointestinal complications in a significant population of patients and selective cyclooxygenase-2 (COX-2) inhibitors, another class of drug found to be moderately effective at treating symptoms of early-mid stage OA, have been associated with cardiovascular risks.[6] The shortcomings of these conventional therapies motivate not only discovery of new therapeutics but also new delivery systems for targeted and prolonged pharmacological delivery that could lead to better compliance and improved outcomes for patients with OA. Recent studies suggest that inflammatory mediators, proteases, and signaling molecules such as NFκB, ERK1/2, interleukin-1 receptor antagonist, and TLRs may be promising molecular targets for treatment of OA. Without delivery systems, these inflammatory mediators have minimal therapeutic potential because of their lack of persistence at the target site and act to broadly to be effective via oral administration.[14]

The most significant challenge that persists with drug delivery for OA is a lack of vasculature within synovial joints; this is a significant barrier to biodistribution of systemically delivered therapies to the target site. Another challenge is rapid clearance of locally delivered therapeutics due to synovial fluid exchange. The presence of synovial fluid makes the delivery of hydrophobic drugs difficult, as they lack the ability to disperse within the joint. However, hydrophilic therapeutics such as proteins are cleared from the joint via pressure gradients that cause flow of the synovial fluid. Fluid movement within joints is created by ultrafiltration of fluid from the capillaries into the joint cavity and drainage of fluid from the cavity, through the synovial membrane into the lymphatics.[25] Repeated administration of locally-injected therapies on a regular basis is not desirable/feasible; thus this delivery route is not warranted if the benefit is relatively short-lived.[26] The following text with summarize the utilization of polymeric drug delivery vehicles in the context of OA, including nano- and micro-scale materials. Many nano-scale polymeric materials have been investigated for intravenous delivery and preferential targeting/retention at pathological sites; for example, many of the platforms developed for anticancer agents,[27] including polymeric micelles, liposomes, and dendrimers, may also be useful technologies for OA therapy.[28] Micron-scale (microgels, microparticles, etc.) biomaterials made from synthetic and/or natural polymers are also useful for solubilizing and controlling the release of therapeutics for local delivery,[29] and their larger size can be beneficial for reducing the diffusivity and rate of clearance from the joint. These technologies enable specific biochemical interactions and/or physicochemical tuning (charge, size, etc) to be utilized to potentially yield longer-acting and more effective treatments for OA. Prior to reviewing specific OA applications of particulate systems, the general properties and formulation characteristics will be outlined for micelles, liposomes, and solid polymeric particles.

Micelles

Figure 2: Schematic illustrating the multifunctional components that can be utilized in micelle formations [5].

Micelles are nanoscale materials comprising amphiphilic polymers that self-assemble in aqueous solvents (Figure 2).[30] Micelle formation is driven by the entropic hydrophobic effect, wherein energetically unfavorable water-cages are excluded from the hydrophobic polymer blocks, followed by self-aggregation of these segments into hydrophobic compartments stabilized by a hydrophilic corona.[31] In order for micelles to form, a critical concentration of the molecule must be present, known as the critical micelle concentration (CMC). For many amphiphilic diblock copolymers, the CMC is low enough to enable micelle stability under dilute conditions (~10-100 μg/mL)[26]. However, a primary challenge in micelle research is reducing susceptibility to premature disassembly *in vivo* under dilute conditions and/or due to competing interactions with endogenously present serum proteins and cholesterol.[32] Micelle core and shell crosslinking are especially promising for stabilization of these structures.[33,34]

Importantly, the core of the micelles can be utilized to load and solubilize hydrophobic drugs or imaging agents.[35] A primary shortcoming of micelles, on the other hand, is the inability to use hydrophilic drugs unless they are covalently tethered to the micelle. Solvent evaporation

methods, emulsion polymerizations, and nano-precipitation have been successfully utilized to encapsulate hydrophobic drugs within the micelle core and enhance colloidal stability of the poorly soluble drugs. Sizes of micelles are dependent on many factors including but not limited to: the polymer chemistry, degree of polymerization, ratio of hydrophobic to hydrophilic polymer block lengths, packing factor, and drug cargo and amount. The size of micelles ranges broadly, but it is generally accepted that sizes from 20-200nm in diameter are ideal for avoiding rapid renal clearance and passively targeting pathological tissues that have leaky vasculature.[36] A smaller diameter facilitates entry into the lymphatic vessels and transport to lymph nodes and improves diffusivity from the vasculature and throughout target tissues.

In addition to core-loading of hydrophobic cargo, micelles can be designed with multiple functionalities. Micelles can also be targeted for preferential uptake by specific cell type through conjugation of peptides, proteins, antibodies, or other targeting ligands onto the hydrophilic corona of the polymer, which is often polyethylene glycol (PEG).[37,5] PEG is most often employed because it provides micelle shielding or "stealth" from the mononuclear phagocyte (MPS) system, reduces systemic toxicity, and prolongs blood circulation times.[38-41] Even with a PEG corona, micelles, like all nanoparticles, remain susceptible to rapid clearance from the blood by the phagocytic cells of the liver and spleen; this limits the ability to achieve preferential accumulation at pathological sites. Micelles can also be endowed with environmentally-responsive functionalities tuned to trigger drug release or particle uptake in specific pathological environments[27,37,42-48] or to provide endosomal escape functions.[49-54,35]

Liposomes

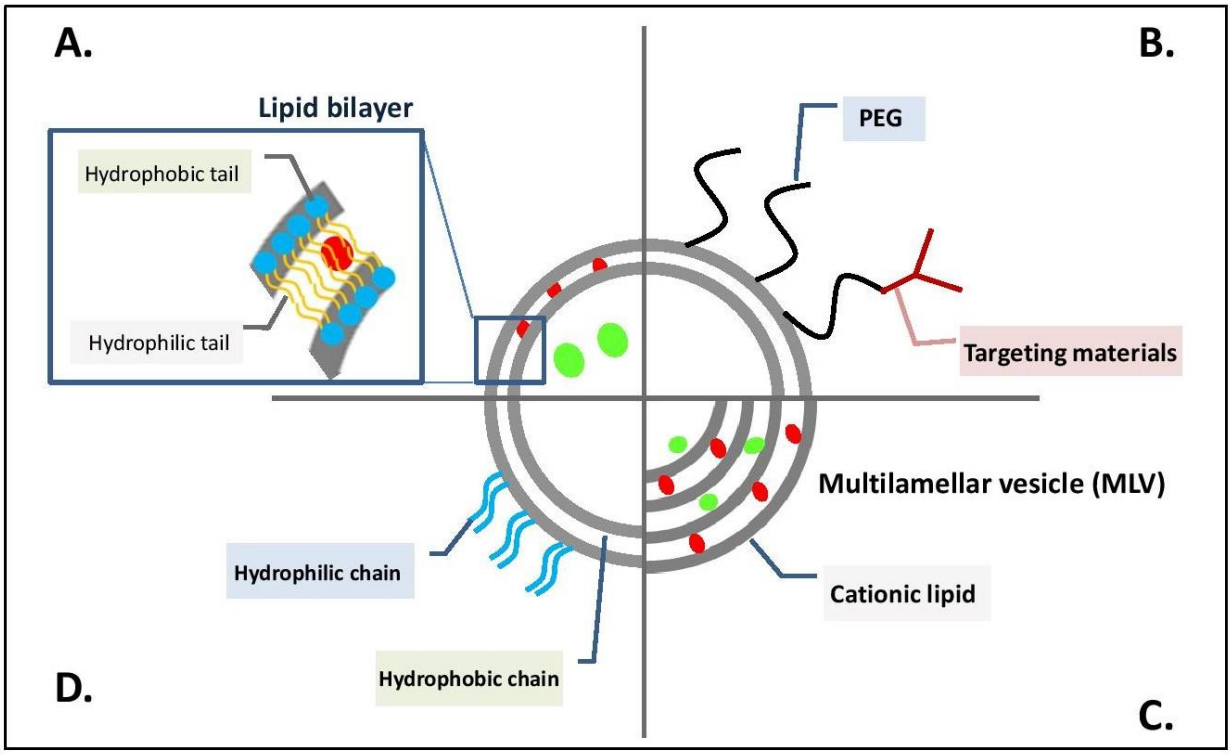

Figure 3: Schematic diagram of various liposomal structures. (a) Illustrates the ability for liposomes to delivery both hydrophilic (green sphere) and hydrophobic (red sphere) drugs either solubilized in the core or embedded within the lipid bilayer. (b)-(d) Represent variations of the traditional liposomes to add targeting ligands or 'stealth' using poly(ethylene glycol). Concept of this figure is adapted from Nature Publishing Group.

Liposomes are aqueous-core vesicles surrounded by a lipid bilayer; Figure 3 schematically illustrates the multifunctional components that can comprise liposomes. Unlike micelles, liposomes contain an aqueous core than can carry hydrophilic drug cargo. Similar to micelles, liposomes suffer from removal by the MPS following IV delivery, and this can be at least partially overcome by PEGylation, which can be used to increase stealth and/or provide chemical handles for functionalization with targeting ligands.[55,56] Liposomes can be formulated using several

different techniques resulting in a wide range of sizes from ~ 50 nm up to 5000 nm depending on the buffer, lipid composition, filtration strategy, and number of lipid bilayers that are present. [57] Formulation techniques for drug loaded liposomes can be broken down into passive and active loading techniques.[58] Passive loading, which occurs during liposome formation, can be further divided into mechanical dispersion, solvent dispersion, and detergent removal methods. Mechanical dispersion can be performed via sonication, extrusion, freeze-thaw cycles, lipid film hydration, micro-emulsification, and membrane extrusion. Active loading of liposomes is performed after liposome formation. A common form of active loading utilizes pH gradients to drive water soluble drugs with protonatable amine functionalities into liposomes after formulation. The drug is precipitated out due to raised pH inside of the liposome.[59] This approach enables very high loading efficiency in liposomal products for delivery of drugs such as doxorubicin (Doxil).[60]

Liposomes have been the focus of several successful clinical trials, mostly for cancer applications. PEGylated liposomal doxorubicin (Doxil/Caelyx), nonPEGylated liposomal doxorubicin (Myocet), liposomal daunorubicin (DaunoXome), and liposomal cytarabine (Depocyte) are all FDA approved drugs, and there are numerous other anticancer liposomal drugs in advanced clinical development.[61] Liposomes have also commonly been used for imaging applications, for example to encapsulate contrast agents such as In or Gd for MRI.[62,57] Liposomes for rheumatoid arthritis (RA) have been investigated by Storm et al. concluding that liposomes can functionally improve the therapeutic performance of anti-inflammatory agents for RA through formation of a depot (local administration) or by attaining site specific drug targeting (IV administration). However, at the time of this thesis, no liposomal therapies for RA have been developed for clinically due to the lack of marketability that is perceived by industry. The benefits of using nanoscale drug delivery systems has not been estimated to outweigh the costs of

integrating these new therapies into the clinic for what is estimated to be of marginal benefit for delivery of the same anti-inflammatory compounds currently used. Liposomes would require IV or IA administration while current drugs are routinely delivered orally. In contrast, liposomal delivery of doxorubicin has proven effective for cancer therapies clinically due to the already invasive nature of previous cancer treatments. Although liposomes can supply a prolonged release of the drugs for the treatment of RA, there were not significant changes in the clinical outcome of particle based therapies compared to free drug therapies.[63]

Dendrimers

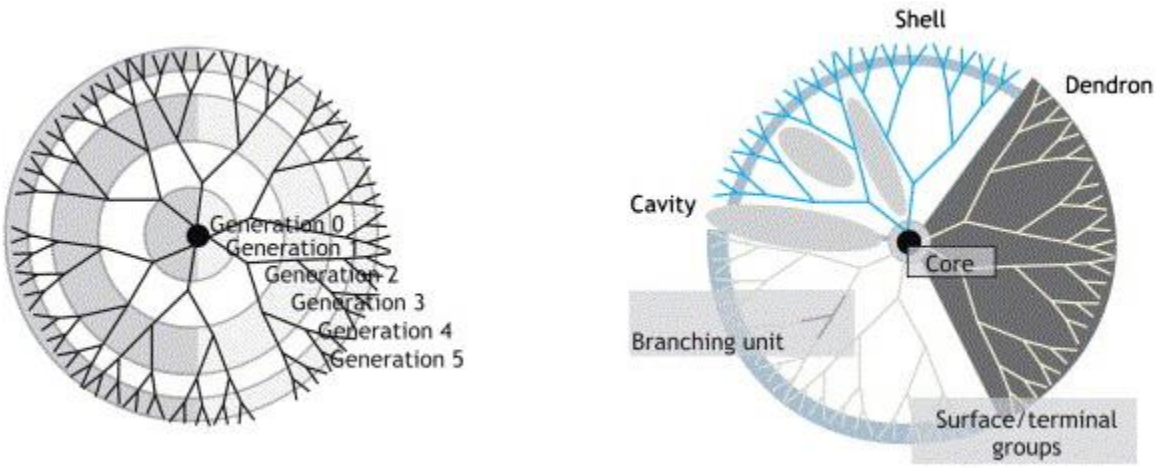

Figure 4: Dendrimer schematic illustrating the well-defined structure that is defined by the number of generations. [4] Figure reproduced with permission from Elsevier.

Dendrimers are repetitively branched molecules that consist of three components: the initiator core, branched interior, and shell, that latter of which provides surface groups that can be utilized for covalent attachment of cargo or targeting ligands (Figure 4). Dendrimers are formed by synthesis of multiple generations that radially branch in 3D from the initiator core. This can be achieved either from a convergent or divergent synthesis approach.[64] The highly branched,

generational architecture makes dendrimers flexible platforms for incorporating different types of cargo. They can be readily designed to incorporate small molecule drugs, imaging agents, therapeutic proteins, peptides, or nucleic acids, and targeting agents.[4,65-67] Moreover, combinations of multiple functionalities can be easily integrated into a single dendrimer design due to the uniqueness of each compartment (initiator, interior, shell) of the molecule.[68] As a results of the sequential nature of dendrimer synthesis, the final product is highly monodispersed compared to many of the other nano- and micro-fabrication techniques used.[69] The surface chemistry of dendrimers is also easily altered and can be optimized to provide ideal properties for a specific application. In a good example of the diversity achievable with dendrimer designs, Tyssen et al. recently performed a high-throughput screen of dendrimers with varying cores, branches, generation numbers, and surface chemistries. Through the screen, they were able to identify a subset of optimally balanced hydrophobic and anionic surface chemistries for the binding and neutralization of HIV-1. Their most promising dendrimer, VivaGel®, is currently in advanced clinical trials. [70] Thus, dendrimers are potentially unique in their ability to be used both as deliver systems and as therapeutics themselves for some select applications (i.e., VivaGel®).

Polymeric materials are appealing for OA therapies for their ability to supply prolonged drug release. However, a disadvantage of classic dendrimer preparations is the burst release of the encapsulated drug. With dendrimers, upwards of 70% of the encapsulated drugs are often released within a few hours of being reconstituted into saline.[71] This can be overcome by covalently bonding the drug to the dendrimer, or formation of a dendrimeric prodrug.[26] Drug encapsulation within dendrimers has been successfully carried out for anticancer, anti-inflammatory, and anti-microbial drugs by both physical encapsulation and chemical coupling.[72]

The surface chemistry and/or charge of many dendrimers enable incorporation of mechanisms for cellular internalization. Although the mechanism is not entirely elucidated, it is generally thought that cationic nanoparticles bind negatively charged glycosaminoglycans (GAGs) of the cell surface in order to drive internalization.[73] Further, dendrimers can be actively targeted for cell uptake by incorporating a ligand that binds the dendrimer to internalizing cell surface receptors. The ability to penetrate chondrocytes and the extracellular matrix would prove crucial for dendrimers for OA applications.[4,74,75]

Polymeric Nano/Microspheres

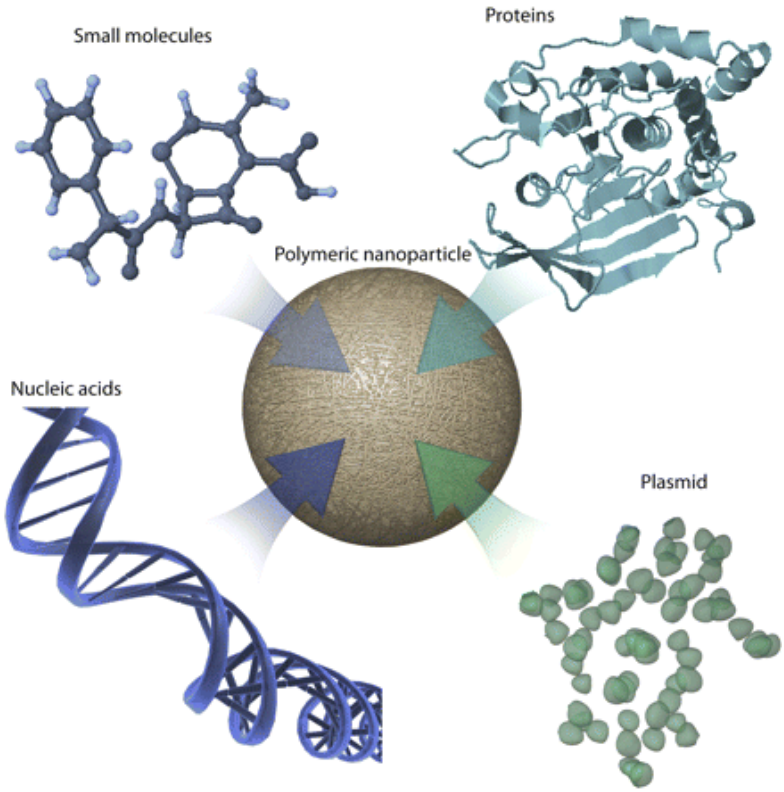

Figure 5: Graphical representation of polymeric nano- and microparticles, which can deliver both hydrophobic and hydrophilic compounds. [1] Figure reproduced with permission from ASPET.

Polymeric particles are a more generalized platform that can be formulated in both nano- and micro-scale size ranges and to encapsulate both hydrophilic and hydrophobic drugs, nucleic acids, and proteins. Sizing of these spheres can be tuned by the type of fabrication technique utilized. There are two main types of polymer spheres. The first is a capsule, or polymersome, which consists of a hydrophilic drug reservoir and a polymeric shell; these are analogous to liposomes but are polymer rather than lipid based. The second is solid spheres comprising a homogenous polymeric matrix loaded with dispersed/entrapped drug. Release kinetics of both have been thoroughly studied and are also tunable depending of formulation technique and the chemical composition and molecular weight of the polymer and the drug.

Varying sizes of particles can be obtained depending on the formulation techniques used. Many nano-sized particles are accomplished through spontaneous assembly based on chemistry.[76] Other techniques for size control of these polymeric particles involve solvent and solute concentrations and volumes, emulsifying time, and solvent evaporation methods. A common method of making polymeric particles is the oil-in-water (O/W) emulsion method that utilizes an oil phase with a hydrophobic drug and polymer that is emulsified into a water phase; a surfactant is typically utilized to stabilize the emulsion. The oil phase solvent is then evaporated, trapping the drug inside of polymeric particles.[77] A similar method can also be employed to encapsulate hydrophilic drugs. This method is known as a water-in-oil-in-water (W/O/W) emulsion. In the W/O/W method, a water phase containing the hydrophilic drug or protein is first emulsified into an oil phase containing the polymer. Then, that water and oil emulsification is dropped into a final water phase containing a surfactant and emulsified. The oil solvent is subsequently removed, leaving the hydrophilic therapeutic trapped within the polymeric sphere.[78] Nano-precipitation is another common method for nanoparticle formulation. Nano-precipitation is accomplished

through rapid mixing under defined flow parameters. Polymer molecules will nucleate into nanoparticles until colloidal stability is reached during the controlled mixing of solvent and a non-solvent. This transition is made with the use of different solvents dependent upon the polymer solubility.[79,80] Nano-precipitation can be more elegantly accomplished under fluid control such as micromixers or the use of microfluidic devices. These approaches to nano-precipitation produce less polydispersed particles under more controlled batch conditions.[81-85] Nano-sized particles can be used for systemic delivery via intravenous injection while micro-sized particles are typically only utilized for local delivery via injections directly into the target site. For nano-particles to maintain a longer circulation time, they are often surface-modified with functionalities such as PEG to increase hydrophilicity and provide stealth shielding.[86] The vast variety of parameters that can be tuned to control the properties of polymeric micro- and nanospheres makes them a good approach for OA applications.

Osteoarthritis Targeting and Drug Delivery

The particle classes summarized above share common characteristics that make them advantageous for OA applications. Polymeric and liposomal particles commonly increase the circulation time and improve the pharmacokinetics of free drugs which can suffer from rapid clearance when delivered systemically. Increased circulation time and systemic persistence effectively increases the probability of targeted or nonspecifically-accumulating drug formulations to accumulate in the inflamed/damaged joints. Generally, polymeric and liposomal particles can be endowed with increased circulation time through surface modification and the addition of PEG.[45,51,52,72,86-88] For targeting the cartilage matrix, micelles, polymeric nanoparticles, and dendrimers can infiltrate the pores within cartilage due to their nanoscale size; these classes of

delivery systems can also be synthesized to have positive surface charge to bind to the inherently negatively charged cartilage matrix. The ability to functionalize the surface of particulate systems with targeting ligands allows for collagen II-binding peptide sequences to be tethered to particles and increase their targeting to cartilage matrix.[89] Polymeric particles also have the ability to target hydroxyapatite that is present in advanced cases of OA when subchondral bone is exposed; this is achieved through the attachment of targeting peptides and bisphosphonates.[26,90] Polymeric systems can also be optimized for targeting the cartilage surface rather than the underlying matrix by conjugating peptides that bind to epitopes exposed following cartilage degradation such as VDIPEN and NITEGE.[26,91] Beyond this more generalizable characteristics, the different classes of delivery systems also have unique characteristics that make them especially promising for overcoming different aspects of the delivery barriers present in OA; these more specific applications of each delivery technology are summarized below.

Micelles

Several applications of micelles have been explored for arthritis (both OA and RA) treatment.[7,92-95] In these studies, several different hydrophobic, small molecule drugs (indomethacin, dexamethasone, camptothecin, and cyclosporin A) have been formulated into micelles and administered either locally[92,93] or systemically.[7] Zhang et al. formulated indomethacin into amphiphilic poly(N-isopropylacrylamide)-polyphosphazene micelles containing ethyl 4-aminobenzoate side groups for enhanced loading efficiency of indomethacin.[92] The micellar formulation of indomethacin had enhanced pharmacokinetics (e.g. longer circulation time in blood plasma[96]). Moreover, a single subcutaneous injection of the indomethacin-loaded micelles provided therapeutic efficacy in carrageenan-induced paw edema. In a second model, these micelles significantly reduced swelling of ankle arthritis induced with complete Freund's adjuvant

(CFA) out to 15 days. In a similar approach, Yue Koo et al. developed targeted, sterically-stabilized micelles consisting of a mixture of PEGylated lipids conjugated to the vasoactive intestinal peptide (VIP) targeting moiety.[93] The VIP targets key effector cells, activated T cells, macrophages, and over-proliferating synoviocytes by their overexpression of VIP receptors, most predominately the VPAC2 receptor. The micelles were used to solubilize camptothecin, a drug that serves as a topoisomerase I inhibitor and is thought to be effective at treating arthritis by triggering apoptosis and cell proliferation of key effector cells in the arthritic joint. Camptothecin micelles abrogated collagen induced arthritis (CIA) in mice following a single subcutaneous administration at as little as 0.3 mg/kg dose, which is significantly lower than the usual anti-cancer dose of free camptothecin. Notably, this dose of campothecin in the sterically stabilized micelles completely reversed paw thickening and decreased arthritis scores by half. A recent follow-up study has shown therapeutic efficacy of the sterically stabilized micelles delivering the VIP peptide alone as well.[97] VIP acts therapeutically in the context of RA due to its anti-inflammatory action on T cells and macrophages. VIP causes a shift of the immune reaction toward an anti-inflammatory Th2 type T cell response and the downregulation of pro-inflammatory Th17 subset of the immune response. In the study, VIP-loaded micelles administered intravenously had 13-fold higher uptake in arthritic limbs than free VIP. VIP-loaded micelles (5 nmol/animal) abrogated negative side effects of VIP such as myelosuppression, hematological toxicity, hemorrhagic renal cystitis, and elevations in liver function tests. It also caused functional improvements in terms of decreasing paw thickness and arthritis score by 50% and 75%, respectively, and at the molecular level, this treatment also decreased inflammatory markers in the arthritic joints (TNF-α, IL-1, MMP-2, and MMP-9).

One promising approach to improving performance of micelles is to covalently crosslink them post-assembly in order to improve their stability. In a recent study by Storm et al, core-crosslinked micelles were developed for delivery of the steroidal anti-inflammatory agent dexamethasone (DEX). The authors validated their design in two animal models of arthritis.[7] Figure 6 highlights this micelle design and the results from this study. In this clever approach, thioether ester-containing DEX derivatives were developed in order to allow for core crosslinking within the micelles, which resulted in varying degrees of hydrolytic release based upon oxidation state of the DEX derivative (sulfide, sulfoxide, and sulfone). The sulfone-containing DEX derivative was chosen for evaluation *in vivo* since it had the fastest release profile; this lead formulation was chosen based on the acute inflammation response characteristic of the animal models tested. Importantly, mice treated with a single intravenous injection of 1, 5, and 10 mg/kg of the DEX-micelles showed significant, dose-dependent reductions in arthritis score and final disease score out to 25 days when compared to the saline control. Free DEX only showed significant reduction in disease score out to day 10 when compared to the saline treated control.

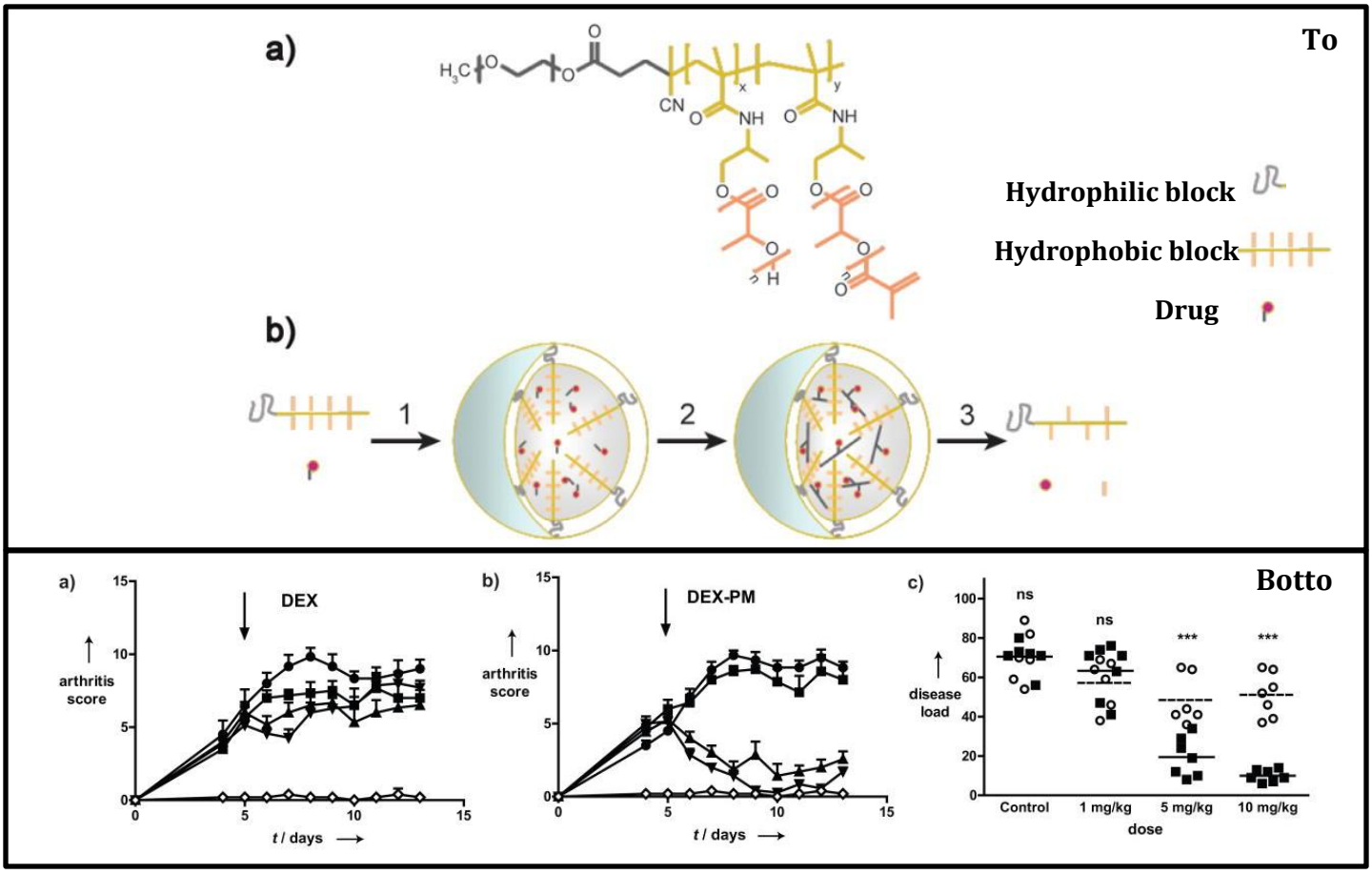

Figure 6: Results and micelle formulation from the study performed by Storm et Al. The top panel is a schematic representation of dexamethasone-loaded core-crosslinked polymeric micelles (DEX-PM). (a) Chemical structure of poly(ethylene glycol)-*b*-poly(*N*-(2hydroxypropyl)-methacrylamide-lactate) (mPEG-*b*-pHPMAmLac$_n$) block copolymers. (b) Illustrates the preparation, degradation, and drug release of DEX-PM. The bottom panels (a-b) highlight the results of their study. (a) and (b) show arthritis score after treatment. The mice received an i.v. injection of DEX (a) or DEX-PM (b) dosed at 1 mg/kg (■), 5 mg/kg (▲), or 10 mg/kg (▼). Control mice (●) received PBS or unloaded micelles. The disease load of each individual mouse upon treatment with free DEX (○) or DEX-PM (■). The disease load was defined as the area under the arthritis score curve from treatment (day 5) until the end of the

study (day 13). The DEX-PM micelles provided a significant therapeutic effect and reduced both arthritis score and disease load compared to free DEX. Figure reproduced with permission from Wiley. [7]

Liposomes

Several reviews have discussed liposomal drug formulations in the context of OA and/or RA.[6,25,98-101] A focus of these articles is the enhanced pharmacokinetics that liposomes can provide.[25,98] However, at the time of these reviews, these liposomal drug formulations were first being developed solely as an encapsulation strategy and to increase the retention time of the drugs in the joint after IA injections. Gerwin at el. discusses many different polymer drug formulations for IA delivery specifically for osteoarthritis and concludes that sustained release of OA drugs is essential for seeing therapeutic outcomes. They argue that polymeric systems can provide this sustained release profile both with new OA drugs and current treatments standards.[6] Hoven et al. focuses on liposomal drugs for the treatment of RA, which differs from OA, but can benefit from similar drug delivery technologies and inform the application of techniques toward OA treatment. Unlike some of the early reviews on this topic, Hoven discusses the benefits of targeting, both passive and active targeting, for liposomal delivery of therapeutics. Unfortunately, Hoven draws the conclusion that clinical development of these formulations may be limited due to the high barrier of entry of these types of drug formulations into the current market.[101] This said, several advances in targeting and environmentally-responsive polymers have been made since the publication of these mentioned reviews. These advances in targeting could provide the benefits that are needed to move some of the liposomal technologies from pre-clinical testing to clinical

trials. The current text focuses on newer technologies which represent these advancements in targeting and environmental-responsiveness.

Liposomes are particularly well suited for OA drug delivery to the cartilage surface, synovial membrane, and intra articular space. The size of liposomes can be tuned to be optimal for targeting these specific components of a joint. Liposomes can also be formulated from lipids that give them a positive surface charge and make them good candidates for targeting the anionic cartilage surface.[26] Liposomes have also been preclinically tested and shown promise in OA applications. For example, in a rat model of OA induced through intrapatellar ligament injection of monosodium iodoacetate, liposomes containing dexamethasone significantly reduced knee joint inflammation.[102] The multilamellar liposomes used in this study were composed of soybean phosphatidylcholine and dipalmitoyl phosphatidylethanolamine at a molar ratio of 95:5. Local injections (dose: 1mg/kg of drug) of liposomal formulations for diclofenac and dexamethasone were administered to these rats. In the same study, the liposomes were given bioadhesive properties by surface functionalization with either hyaluronan or collagen. In all cases, the liposomal drug formulations decreased inflammation of the rat knee joints down to at least 20% of the initial inflammation volume. The best performing formulation was the bioadhesive hyaluronan-surface functionalized liposomes containing both diclofenac and dexamethasone. These liposomes delivered via local injections reduced injury-induced inflammation volume by 12.9% at 17 days after a single injection of liposomes. Un-targeted liposome formulations were not investigated in this study.[102]

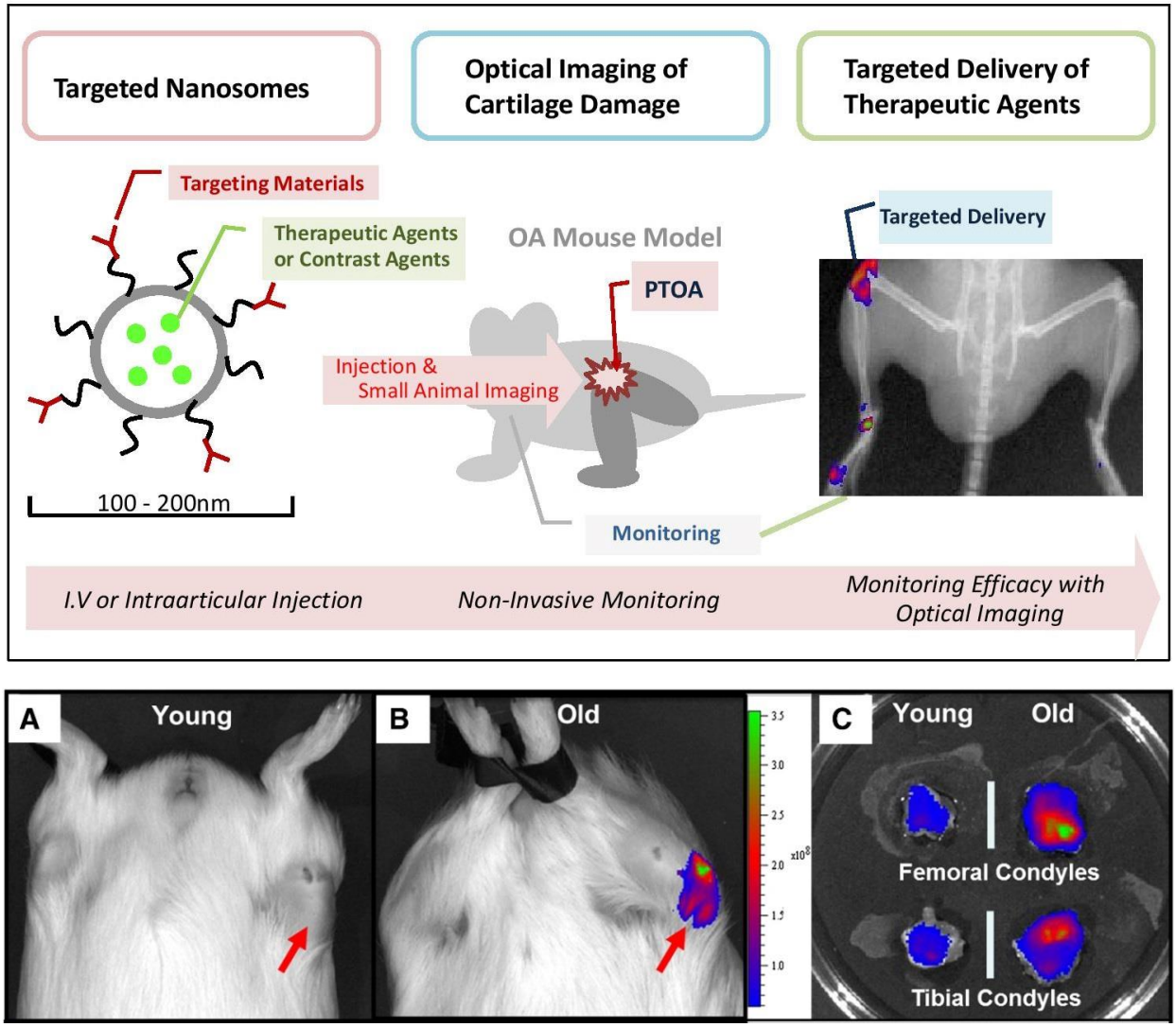

Figure 7: (Top) Schematic diagram of theranostic approach for osteoarthritis-type II collagen antibodies-targeted fluorescent nanosomes. These nanosomes bind on damaged cartilage in an OA animal model, providing a traceable fluorescent signal and delivering therapeutic agents.[2] **(Bottom)** In a spontaneous OA model in guinea pigs, older animal showed higher accumulation of immunoliposomes. Reproduced with permission from Elsevier. [3]

In a very recent study, targeted immunoliposomes (150-250nm in diameter) were developed for early clinical detection of OA. This diagnostic reagent was loaded with a near infrared fluorophore and was surface functionalized with a monoclonal antibody that selectively

binds to collagen II (CII) after it is exposed in damaged cartilage.[3] The immunoliposomes bound specifically to damaged cartilage *ex vivo* and, as detected by fluorescence imaging. Importantly, after intravenous administration, these immunoliposomes also preferentially accumulated *in vivo* in a spontaneous OA model in guinea pigs at sites of arthritic cartilage. These liposomes, highlighted in Figure 7, show binding to the medial condyles of the older animals where OA initiates. Theranostic liposomes have potential to aid evaluation of therapeutic drug development for OA with small animal models allowing longitudinal studies of individual animals. They have also been used in a mechanical overload in the mouse knee which is a useful model for study of post-traumatic osteoarthritis.[2] The top panel in Figure 7 shows the general use of nanosomes for optical imaging of cartilage damage. Based on strong diagnostic performance in multiple, clinically-relevant OA models, these targeted liposomes show tremendous promise for extension into targeted drug delivery applications.

Liposomes have also been investigated by other groups for applications in RA. Although RA has different pathogenesis from osteoarthritis, symptomatic treatment and delivery mechanism of drugs and therapeutics can be similar.[101,103] Liposomes have shown very promising results for treatment of inflammation caused by OA through the delivery of drugs such as celecoxib[104], methotrexate, and dexamethasone.[101] Targeting liposomes to RA-damaged cartilage has also proven successful through use of targeting ligands such as, prednisolone phosphate[103]; this approach has promising for limiting drug off target side effects. Hofkens at el. investigates liposomal targeting of synovial lining macrophages using prednisolone phosphate. Liposomes were formulated using dipalmitoyl phosphatidylcholine (DPPC), PEG, and cholesterol. The lipid film was hydrated in prednisolone disodium phosphate (PLP), which served as the targeting agent. Free PLP was removed from the liposome solution before treatments were given. Mice were given

arthritis using an antigen-induction (AIA) model. The mice were treated 3 days post-induction via IV injection with liposomal PLP or free PLP (both 10mg/kg) or saline. A second mouse model used immune-complex arthritis (ICA) where mice were treated only with the liposomal PLP (10 mg/kg) or saline. Knee joint swelling was measured 1 and 5 days post treatment for the AIA mouse model. Swelling with liposomal PLP decreased by 74% when compared to the saline control and decreased by 64% when compared to the free PLP treatment. By day 5, liposomal PLP almost completely suppressed knee joint swelling. To test the targeting ability of PLP to macrophages in the synovial intima layer, colloidal gold liposomes were injected IV. It was seen via histology that these liposomes were readily taken up by macrophages as they leave the bloodstream and were not taken up by type B synovial fibroblasts. This study then goes into further investigation about macrophage phenotypes in the presences of liposomal PLP. They show that IA and IV injections of liposomal PLP is able to alter suppress M1 synovial macrophage without altering M2 phenotype within the inflamed synovium for both the AIA and ICA mouse models. [103] Thus, this targeting approach may be especially useful for targeting of immunomodulatory compounds.

In addition to targeting macrophages, Vanniisinghe at el. developed a system for synovium-specific targeting that has utility for OA applications. This targeted liposomal drug delivery system was developed to deliver drug cargo to inflamed joints.[105] In this study PEGylated liposomes composed of the lipid DPPC and cholesterol were functionalized with RGD or HAP-1 (SFHQFARATLAS), which targets fibroblast-derived B synoviocytes. The drug cargo that was delivered in this study was a short immunosuppressive peptide (core peptide, CP). CP is a nine amino acid peptide (GLRILLLKV) that is an effective immunosuppressant. Arthritis was induced in rats then the rats were treated with untargeted, RGD, and HAP-1 liposome formulations. Rats were given two IV injections of liposomes two consecutive days (0.5mg drug/0.5 ml/250 g

rat/day). The targeted HAP-1 liposomes produced a 10-fold increase in accumulation in the arthritic rat joints compared to its contralateral unaffected joint. The untargeted liposome and the scrambled HAP-1 control only showed a 5-fold increase in fluorescence in the arthritic joint. The RGD targeted liposomes showed a higher fold increase (7-fold higher) than the non-targeted liposomes but this was lower than the HAP-1 targeted liposomes. The delivery of CP via the targeted HAP-1 liposomes significantly reduced paw swelling at day 7 compared to the control paw (>10% reduction in paw swelling) while the delivery in the RGD liposomes and non-targeted liposomes did not significantly decrease paw swelling.[105] This study highlights the significance targeting ligand choice can have on drug delivery via liposomes and highlights the potential for HAP-1.

Dendrimers

Dendrimers, similar to micelles and liposomes, have the ability to target the major components of healthy and arthritic joints. Sizes of dendrimers span a broad range from tens of nanometers to hundreds of nanometers in size when complexed with one another or with drugs. The smaller diameter dendrimers can target the cartilage matrix and subchondral bone. The end group functionality of dendrimers can be harnessed to tether a targeting ligand or peptide which allows for long retention and greater accumulation of the dendrimers in the joint, leading to prolonged and local drug release. Larger generation dendrimers can also be used for targeting cartilage surface, synovial membranes, and intra articular space, similar to liposomes.[26]

Hayder et al. showed the efficacy of using dendrimers for RA treatment after IV injections. They showed that azabisphosphonate (ABP)-capped dendrimers selectively target monocytes and modulate them toward a more anti-inflammatory phenotype. Intravenous injection (10mg/kg) of

these dendrimers inhibited the development of inflammatory arthritis in two mouse models indicated by a reduction in inflammatory cytokines and absence of cartilage destruction. The first mouse model was an IL-1ra knockout mouse that spontaneously develops arthritis. Treatment with ABP dendrimers reduced the arthritic score by 80% compared to untreated mice. The second mouse model was a K/BxN serum transfer model, which involves transferring serum from an autoimmune K/BxN mouse to a BALB/c A mouse, resulting in an inflammatory immune response and arthritis. With the treatment of ABP, there was a significant decrease in paw swelling and arthritic score compared to the control. Paw swelling decreased by about 30% and arthritic score decrease by almost 70%. It was also shown that ABP dendrimers do not cause off target effects; this was determined though IV injections of ABP once per week for 8 weeks. Histology was performed on the spleen, kidney, lung, liver, and aorta showing no differences between dendrimer treated mice and non-treated mice. The dendrimer treatments also did not cause changes in body weight of treated mice.[90] In this case, the dendrimer itself has inherent therapeutic/anti-inflammatory and targeting capabilities without requiring loading of any additional drug; this and other studies have shown that screening of dendrimer chemistries can uncover entities with desirable function, yielding simpler, more translational systems.[74,90,106]

Polymer Nano/Microspheres

Polymeric particles are generally thought to be better suited for targeting the cartilage surface, synovial membrane, and intra articular space.[26] The ability to synthesize polymeric particles with larger sizes gives them an advantage over smaller particles, as they are not as easily cleared from the intra-articular space since their diffusion is more limited. A study by Singh et al. demonstrated that larger particles of approximately 900nm were retained locally for a significantly

longer time than comparable smaller particles.[107] In this study, poly(2-hydroxyethyl methacrylate)-pyridine (pHEMA-pyridine) was used to create polymeric microspheres at 500nm and 900nm containing fluorescent bovine serum albumin (BSA). In healthy rats, these particles were injected IA, and particle retention over time was measure via fluorescence. The half-life of the 500nm (1.9d) and 900nm particles (2.5d) was significantly higher than the free protein (0.63d). The plateau, measured out to 14 days for the 900nm (30% at 14d) particles was significantly higher than both the retention by the 500nm particles (~5% at 14d) and the free protein (<5% at 14d).

Both natural and synthetic polymers have been used in the creation of spherical polymeric particles. Natural polymers are more disposed towards being immunogenic, which is especially undesirable for OA which is exacerbated by local inflammation. It is also more difficult to achieve reproducibility in production of particles made from natural polymers.[108] Chitosan and gelatin have shown the most promise for drug delivery in OA models and have produced desirable outcomes.[109] For example, chitosan has been used to incorporate Flurbiprofen and extend its time in the joint, leading to extended release locally for more than 24 hours.[110] Gelatin has also been used to deliver many nonsteroidal anti-inflammatory drugs and proteins such as anti-TNF to reduce inflammation.[111,112]

Synthetic polymeric particles are one of the most commonly utilized delivery technologies due to their lack of immunogenicity, tunability, and ability to synthesize them reproducibly. The most widely used synthetic polymer is poly(lactic-co-glycolic acid) (PLGA). PLGA has been used to fabricate particles for delivery of many types of drugs. PLGA has a history of use in FDA-approved systems because it degrades into naturally existing metabolites (lactic and glycolic acid) and is fully resorbable, although lactic acid can be toxic at high levels. One such technology is Lupron®, which is used currently used to deliver drugs such as testosterone, clarithromycin,

lovastatin, and progesterone long term, up to 6 months, to treat prostate cancer, endometriosis, fibroids, and central precocious puberty. Flexion Therapeutics is another company that specialized in the use of PLGA microspheres for OA therapy. They have developed three formulations of PLGA microspheres for OA treatment and therapy, FX006, FX007, and FX005. All formulations are intended for IA injection or local delivery. FX006 is a sustained release steroid injectable in phase 3 development for patients with severe OA pain.[113] FX007 is a PLGA formulation for local administration of TrkA receptor antagonist intended for post-operative pain. FX005, like FX006 is intended for late or end stage OA patients. This formulation is an IA, sustained release particle that delivers p38 MAP kinase inhibitor. This company has seen very promising pre-clinical and clinical results from these formulations for extended pain relief. FX006 (0.28mg) was able to almost completely eliminate painful gait in a rat arthritis model out to day 32. The free drug (Kenalog-40®) reduced painful gait, but not to the extent of the PLGA formulation and at day 32, the gait analysis score for the free drug was ~3x higher than FX006.

Almost all PLGA particles experience an initial burst release of the drug, which is an important dosing consideration for sustained release formulations in order to be sure local drug toxicity does not occur. Other synthetic polymers have also been extensively tested for sustained and targeted drug release. By tailoring the polymer composition, microspheres have been developed that are responsive to a variety of stimuli. For example, polypropylene sulfide (PPS) is responsive to reactive oxygen species (ROS) (undergoes a phase change from hydrophobic to hydrophilic) and was recently utilized for the first time to form microparticles that enabled "on demand" release of antioxidants.[77] OA is a disease associated with inflammation. Reactive oxygen species are major mediators of the inflammatory cycle. By introducing stimuli responsive particles, such as PPS polymeric particles, "environmentally responsive" drug release can be achieved. PPS

has a unique ability to become hydrophilic when exposed to ROS such as hydrogen peroxide, triggering the release of hydrophobic encapsulated drugs. Since ROS triggers the release of the drug, these particles provide local, sustained therapy which is activated "on-demand" during cycles of oxidative stress.

Polymer microparticles have been utilized to deliver a variety of therapeutic cargo relevant to reduction of inflammation in the setting of OA. Interluekin-1 (IL-1) is a positive regulator of inflammation and can be inhibited to stop or slow the progression of OA symptoms. Interleukin-1 receptor antagonist (IL-1Ra) is a natural protein inhibitor of IL-1 and has been one of the most thoroughly investigated biologic drugs for treatment of OA. One model of arthritis, discussed above, involves the spontaneous onset of arthritic symptoms in mice lacking the IL-1Ra gene. Because this gene is an important regulator of RA and OA, IL-1Ra is a widely studied therapeutic for arthritis. Whitmire et al. used self-assembling nanoparticles to deliver IL-1Ra to rat knee joints and showed prolonged retention over free IL-1Ra. The nanoparticles are formed from a block co-polymer consisting of a hydrophobic block (cyclohexyl methacrylate, CHM) and a hydrophilic block (tetraethylene glycol methacrylate, TEGM) with a tethering moiety, paranitrophenol (pNP) used to attached the IL-1Ra protein. The nanoparticle delivery system retained 20% of the delivered IL-1Ra at day 10 which is significantly higher than the free IL-1Ra that remained in the joint at day 10. The half-life of the IL-1Ra nanoparticles was significantly higher at 3.01 +/- 0.09 days compared to the soluble IL-1Ra at only 0.96 +/- 0.08 days. [114] This study highlights the ability of polymeric particles to supply a local depot of drug that is less readily cleared from arthritic joints.

While larger particles have been used to enhance pharmacokinetics through physical size, more convention nanoparticle sizes (i.e., 100-200 nm) have also shown promise, especially when

used in conjunction with targeting ligands that improve binding and retention within OA joints. In a study conducted by Rothenfluh at el., bio-functional polymeric nanoparticles were used to target arthritis and increase retention in cartilage. [89] In this study, PPS nanoparticles are synthesized using PEG-PPS-PEG block copolymers. These nanoparticles were surface functionalized with a peptide (WYRGRL) that targets collagen 1. In healthy mice, fluorescent nanoparticles were injected IA. The particles were tracked via fluorescence to monitor cell invasion and ability to infiltrate the cartilage. The WYRGRL-PPS nanoparticles were able to infiltrate both the cartilage and chondrocytes. The WYRGRL-PPS particles compared to a control peptide were also retained within the ECM at higher concentrations than the non-targeted PPS particles. A 44.8 fold increase and a 71.7 fold increase at 24 and 48hrs respectively was seen in the ratio of ECM to intracellular fluorescence of the targeted particles compared to the non-targeted particles.[89] This study specifically highlights the functional benefits of incorporating appropriate targeting ligands into polymer drug delivery systems.

There is an abundance of studies that have used polymeric microparticles for arthritis drug delivery. Rather than summarize every study in text, the following table highlights some of the more recent studies of polymer-drug systems and their outcomes in various arthritis models.

Table 1: This table highlights some of the polymer-drug combinations as they have been applied to OA and their results.

POLYMER TYPE	DRUG	MODEL/ROUTE OF DELIVERY	OUTCOME	REFERENCE
POLY(L-LACTIC ACID) (PLA)	Paclotaxel	carrageenan induced rabbit model of arthritis/IA Injections	20% paclitaxel loaded PLA microspheres in the 35-100 um size range delivered intra-articularly reduced all measure of inflammation	[115]
PLA	Methotrexate (MTX)	Rabbit induced arthritis model/IA Injections	10 fold increase in MTX retention in joint compared to free MTX after intra articular injection	[116]
PLGA-PEG	methacrylic derivative of ibuprofen	Ex vivo sheep joints	Decreased burst release of drug and prolonged sustained release for up to 3 months	[117]
PLGA	Clonidine	In vitro drug release	Achieves controlled release for up to 30 days of hydrophilic drug	[118]
PLGA	Lornoxicam	Rat induced arthritis model/IA Injections	Reduced drug plasma levels compared to free drug, retention time increased after intra-articular injections	[119,120]
PLGA	Naproxen Sodium	Rabbit induced arthritis model via intra-articular injection of ovalbumin and Freud's Complete Adjuvant/IA Injection	Improved cure of articular arthritis when treated with PLGA loaded particles compared to BSA microspheres.	[121]
PLGA	ibuprofen/ Labrafil	In vitro drug release	Prolonged drug release with addition of labrafil	[122]

PLGA	Methylprednisolone (MP)	Rat induced arthritis model/IA Injection	Rapid increase in MP concentration in plasma at 30 minutes compared to MP suspension	[123]
PLGA	Dexamethasone	mouse dorsal air pouch model/local Injection	Similar DXM release with varying polymer molecular weights	[99]
PLGA	Betamethasone sodium phosphate (BSP)	rat air-pouch model/antigen-induced arthritic rabbit model/local or IA Injection	joint swelling significantly decreased at 21 days with administration of PLGA drug loaded particles	[124]
PLA AND PLGA	hyaluronate	Arthritis and osteoarthritis rat models/IA Injection	administration of particles did not worsen already altered articular tissues and did not cause inflammation in healthy rat knees	[125]
PLGA	PTH(1-34)	Papain-induced OA rat model/IA Injection	Effect of PTH/PLGA microspheres on suppressing the OA progression was similar to that of a once-every-three-day injections	[126]
PLGA	Sulforaphane (SFN)	Surgically induced OA (ACL transection) in rats/local delivery (or injection)	Treatment with SFN-PLGA microspheres inhibited the mRNA and protein expression of COX-2, ADAMTS-5 and MMP-2 induced by LPS in articular chondrocytes. Intra-articular SFN-PLGA microspheres delayed the progression of surgically induced OA in rat.	[127]
PLGA	siRNA against TNF-a	preclinical model of RA/local injection	PLGA microspheres slowly released siRNAs effectively inhibited the expression of TNF-a in arthritic joints	[128]

CHITOSAN	Flurbiprofin	Rat knee joints/IA Injection	significant extended release of flurbiprofen from microspheres in comparison with its solution	[109]

Overview of Post-Traumatic Osteoarthritis

Post-traumatic osteoarthritis (PTOA) is OA that occurs following an injury to the joint. These injuries can be traumatic injury to the bone or the surrounding soft tissue and include ligament or meniscal tears, fractures, or dislocations. People who suffer from PTOA are largely much younger, healthier, and generally more active than general idiopathic OA patients. [129] Because there are currently no disease modifying OA drugs, these younger patients require surgical intervention 7 to 9 years earlier than idiopathic OA. [130] PTOA account for almost 12% of all OA cases in the United States which is a population of roughly 12 million people, making PTOA a huge financial and physical burden, totally healthcare cost close to $3 billion annually in the US. [131]

Following a joint injury, chemical and mechanical stimulators activate chondrocytes, the primary cell type that comprises the articular cartilage. These activated chondrocytes propagate inflammation through the production of inflammatory cytokines that cause heightened local inflammation that affects surrounding soft tissue and synoviocytes. Chondrocytes also increase production of matrix metalloproteases (MMPs) which directly contribute to matrix disruption and cartilage breakdown. [132,133] Early intervention after a joint injury to disrupt this inflammatory process could prevent early onset of PTOA. Such intervention can be accomplished through the use of the various drug delivery systems listed previously. By targeting sites of early cartilage

degradation, a disease modifying OA drug can be delivered locally, avoiding systemic complications.

Role of Reactive Oxygen Species in Inflammatory Disease

Reactive oxygen species (ROS) are an important aspect of normal cell growth and metabolism, but when oxidant levels exceed that of cellular antioxidant potential, tissue-damaging oxidative stress occurs.[134] Some key ROS are hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH^{\cdot}$), hypochlorous acid (HOCl), and superoxide anions (O_2^{-}). Reactive nitrogen species such as nitric oxide ($NO^{\cdot}$) and highly reactive peroxynitrite anion ($ONOO^{-}$) are also important reactive species that impact pathogenesis of chronic inflammatory diseases. Oxidants can be generated exogenously (i.e. by ultraviolet light or chemotherapeutics) or produced intracellularly through cytosolic enzyme systems such as NADPH oxidases (NOX) and uncoupled nitric oxide synthase (NOS), and through normal metabolism in mitochondria and peroxisomes. ROS levels are kept in balance through an enzymatic antioxidant defense system composed of superoxide dismutase, catalase, glutathione peroxidase, and peroxiredoxins in combination with non-enzymatic scavengers such as flavonoids and glutathione. If ROS levels are too low, cellular proliferation and host defense against microbial invasion are impaired. Conversely, excessive ROS can damage proteins, lipids, and DNA, as well as activate redox-sensitive signaling pathways with potentially damaging downstream effects. $OH^{\cdot}$ is capable of oxidizing most biological molecules, and O_2^{-} can either react directly with biomolecules or produce other ROS (H_2O_2 and $OH^{\cdot}$).[135] H_2O_2 is not highly reactive itself, but it is an intermediate to both $OH^{\cdot}$ and hypochlorite radical production.[136] Similarly, $NO^{\cdot}$ alone is not very reactive to non-radical species, but it can generate other reactive

nitrogen species or combine with O_2^- to form $ONOO^-$, which strongly oxidizes DNA, proteins, and lipids. Furthermore, $ONOO^-$ can nitrosylate tyrosine residues in proteins, a marker of cell damage.

Oxidative stress exacerbates many inflammation-associated diseases through DNA damage, protein modification, lipid peroxidation, and disruption of cell signaling. Lipid peroxidation products from the reaction of hydroxyls with lipids in cell membranes can accumulate in cells and cause cell death through direct membrane damage or through apoptosis induced by caspase activation.[136] DNA damage also promotes apoptosis, alters gene expression, and causes mutations in expressed proteins. Modification of proteins can lead to dysregulation of transcription factors such as nuclear factor kappa B (NF-κB), resulting in propagation of pro-inflammatory signaling cascades, and other transcription factors that induce pro-inflammatory adhesion molecule, cytokine, and chemokine expression.[136] These consequences of oxidative stress make ROS a potentially valuable therapeutic target for osteoarthritis and post-traumatic osteoarthritis (PTOA) whose progression is tightly tied to oxidative stress.[137-142]

Conclusion

As surveyed herein, particle based drug delivery systems show promise for improving the pharmacokinetic and pharmacodynamics of OA drugs, including providing means for sustained therapeutic action with fewer side effects and longer-term benefits. Materials that have made it to clinical trial are of particular interest for future studies. Many uses of liposomes for human application in other fields such as cancer treatment have been highlighted.[61] Due to their high biocompatibility, development of liposomes for OA applications looks very promising not only for drug delivery but also for OA detection and targeting.[2,3] Polymeric nano- and microparticles have high potential for development for use in OA applications. Several of the common polymers

used for the production of these particles are FDA approved for other biomedical applications, so the potential for these particles to progress into clinical trial is high. Polymeric delivery systems have the capacity to provide much needed extension for targeted delivery and prolonged release of drugs for OA prevention, treatment, and detection. However, significant challenges remain for clinical translation of these polymeric delivery vehicles within the pharmaceutical industry. Many of the drug delivery systems pair drugs that are already on the market or in use for treatment of OA with new polymers to improve either route of delivery or reduce off target side effects. Marketing these polymeric drugs delivery systems remains a challenge for the OA industry. Continued evaluation of advanced, targeted polymeric drug delivery systems through robust preclinical studies will be necessary to optimizing their ability to improve pharmacokinetics and reduce side effects. These studies will be key for justifying the progression of these technologies from pre-clinical testing to clinical trials and bringing them to market. ROS are a target of interest for the prevention of PTOA following joint injury. ROS regulate many downstream inflammatory mediators and pathways. By controlling ROS through the use of antioxidant therapy delivery by a local drug delivery system, there is strong evidence that OA progression can be slowed or even stopped.

CHAPTER II

DRUG FREE ROS SPONGE POLYMERIC MICROSPHERES REDUCE ARTICULAR DAMAGE FOLLOWING MECHANICAL INJURY

Text adapted from:

O'Grady, K.P.*; **Kavanaugh, T. E.*;** Cho, H*.; Ye, H.; Gupta, M.K.; Madonna, M.C.; Lee, J.; O'Brien, C.M.; Skala, M.C.; Hasty, K. A.; Duvall, C. L. Drug Free ROS Sponge Polymeric Microspheres Reduce Tissue Damage from Ischemic and Mechanical Damage. *ACS Biomat. Sci. & Eng.* **Revisions Under Review**.

Introduction

In PTOA, osteoarthritis develops following joint injuries such as dislocation, ligament/meniscal tears, and fractures that trigger inflammation. The presence of lipid peroxidation products and nitrotyrosine in biological fluids and tissue of patients with OA suggests that ROS play a role in cartilage degradation and may be a viable therapeutic target.[143,144] The role of ROS in PTOA progression is also supported by the observation that chondrocytes, the primary cell type that comprise articular cartilage, produce abnormal levels of ROS in response to mechanical stress and to stimulation with inflammatory cytokines and chemokines, such as interleukins and monocyte chemoattractant protein-1, as well as lipid-derived inflammatory mediators including prostaglandins and leukotrienes.[145,146] In addition to classical signaling molecules, chondrocytes produce inflammation-propagating ROS such as nitric oxide and superoxide.[147] While $NO^{\cdot}$ and O_2^{-} are the primary ROS produced by chondrocytes, these radicals generate derivative radicals including $ONOO^{-}$, H_2O_2, and hypochlorite (OCl).[138] Recent studies

highlight the importance of H_2O_2 in the onset of OA[148], where high levels of H_2O_2 cause the hyperoxidation of peroxiredoxins. Peroxiredoxins are major intracellular antioxidants, and their oxidation by H_2O_2 leads to inactivation and inhibition of peroxidase function. This loss of function allows H_2O_2 to accumulate, further exacerbating ROS-induced tissue damage and inflammation. In addition to causing cell death, oxidative stress can contribute to the breakdown of extracellular components such as proteoglycans and collagens by increasing production of proteases[145,146] as well as the direct de-polymerization of hyaluronic acid. In the setting of OA, ROS are also utilized as a "secondary messenger" in pro-inflammatory signaling pathways involving receptor tyrosine kinases, MAP kinase (ERK1/2, JNK, p38), lipid pathways (PI3-kinase/Akt), and transcription factors (NF-κB, p53, and AP-1). Redox signaling from excessive ROS, in particular H_2O_2, can result in the formation of cysteine sulfenic acid residues (Cys-SOH) as the ROS species react with protein thiols. Cys-SOH can directly regulate the activity of signaling molecules such as the protein kinase C family. Similar redox signaling has been confirmed in the production pathway for matrix metalloproteinase (MMP)-13 through the oxidation/reduction of cysteines involved in the MAP kinase JNK-2 pathway.[147] The role of ROS in many inflammatory pathways that propagate PTOA has been confirmed, which indicates the potential for therapeutic scavenging of ROS to halt PTOA progression.

Previously, we demonstrated the utility of an ROS-responsive, H_2O_2-scavenging microsphere system for delivery of hydrophobic drugs such as the anti-inflammatory and antioxidant molecule curcumin from a local depot. [149] This system is based on poly(propylene sulfide) (PPS), which undergoes a phase change from a hydrophobic to a hydrophilic state upon oxidation,[76] permitting on-demand release of encapsulated drug.[150,151] Blank PPS microspheres (PPS-MS) containing no drug scavenged ROS *in vitro* and in ischemic muscle, although unloaded

PPS-MS did not functionally improve the vascular response to ischemia in young mice with short-term hyperglycemia.[149] The antioxidant properties of PPS have also been explored in a hydrogel formulation, where PPS served as an ROS sink and protected cells from cytotoxic levels of H_2O_2.[151] In the present work, we more comprehensively define the antioxidant properties of PPS for a range of ROS types and therapeutically test blank PPS-MS in a model of mechanically induced PTOA.

Materials and Methods

Materials

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) except the following. Propylene sulfide (>96%) was purchased from Acros Organics through Fisher Scientific (Pittsburgh, PA, USA) and was purified by distillation over CaH_2 powder just before polymerization. Amplex Red Hydrogen Peroxide/Peroxidase Assay kit was purchased from Thermo Fisher Scientific (Molecular Probes, Waltham, MA, USA). Peroxynitrite was purchased as a solution in 0.3 M sodium hydroxide from Cayman Chemical (Ann Arbor, MI, USA). Amplite Fluorimetric Hypochlorite Assay kit was purchased from AAT Bioquest (Sunnyvale, CA 94085). Hypochlorite Detection Kit was purchased from Cell Technology (Fremont, CA 94538).

Microsphere Synthesis

Synthesis of poly(propylene sulfide) (PPS)

PPS was prepared as previously described[151] by anionic ring opening polymerization of propylene sulfide using DBU/1-buthane thiol. Briefly, in a hot air dried and nitrogen flushed 100 mL flask, 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) (4.5 mmol, 0.673 mL) in dry tetrahydrofuran (THF) (25 mL) was degassed for 30 minutes, and the reaction mixture was cooled to 0°C. To this

flask, a previously degassed solution (30 minute) of 1-butane thiol (1.5 mmol, 0.161 mL) in THF (20 mL) was added drop wise and allowed to react for 30 minutes. Later, freshly distilled and degassed propylene sulfide (120 mmol, 9.39 mL) was added to the reaction mixture, and the temperature was maintained at 0°C for 30 minutes. The polymerization was carried out for another 1.5 h at room temperature and quenched by addition of 2-iodoethanol (2 mmol, 0.40 g). On the next day, the polymerization mixture was filtered to remove precipitated salt, and the filtered polymer solution was concentrated under vacuum. The crude polymer in dichloromethane (5 mL) was purified by three precipitations into cold methanol and dried under high vacuum to yield a colorless viscous polymer. ^{1}H NMR (400 MHz; $CDCl_3$, δ): = 1.3-1.4 (s, CH_3), 2.5-2.8 (s, -CH), 2.8-3.1 (s, CH_2), 3.72 (t, CH_2-OH).

Characterization of PPS

PPS was characterized for structure, molecular weight, and polydispersity as described previously.[149,151] The number average molecular weight (M_n) and polydispersity (PDI) of PPS were assessed by gel permeation chromatography (GPC, Agilent Technologies, Santa Clara, CA, USA) using dimethylformamide (DMF) + 0.1 M lithium bromide mobile phase at 60°C through three serial Tosoh Biosciences TSKGel Alpha columns (Tokyo, Japan). An Agilent refractive index (RI) and Wyatt miniDAWN TREOS light scattering (LS) detector (Wyatt Technology Corp., Santa Barabara, CA, USA) were used to calculate absolute molecular weight based on dn/dc values experimentally determined through offline injections into the RI detector. The chemical structure of the PPS was confirmed by ^{1}H nuclear magnetic resonance (NMR) recorded in $CDCl_3$ with a Brüker 400 MHz spectrometer.

Microsphere fabrication and characterization

PPS-MS were prepared using the oil-in-water (O/W) emulsion solvent evaporation method[152,153] as described previously.[149] Briefly, PPS (60 mg) was ultrasonicated (Cole-Parmer, USA) in chloroform (1 mL) until completely dissolved to form the oil (O) phase. The O phase was then added drop-wise into 1% (w/v) aqueous poly(vinyl alcohol) (PVA) solution (7 ml) and emulsified using an Ultra-Turrax TP 18-10 homogenizer (Janke and Kunkel KG, IKA-WERK) at 20,000 rpm for 1 minute. The emulsion was transferred to a round-bottom flask and subjected to high vacuum (~635 mm Hg) using a rotary evaporator (Rotavapor RII, BUCHI, Switzerland) for one hour to remove the chloroform. Microspheres were then recovered by centrifuging the remaining aqueous solution at 7500xg for 8 minutes. The microspheres were then washed once with deionized water to remove excess PVA. Lastly, the microspheres were lyophilized (Labconco Freezone 4.5, USA) prior to storage. Microspheres were characterized for size and morphology by scanning electron microscopy (SEM, Hitachi S-4200, Hitachi Ltd, Tokyo, Japan). The microspheres were suspended in a water drop and placed on a double sided carbon tape attached to an aluminum stub, air dried, then sputter-coated with gold for 60 s. Microsphere size was quantified from SEM images using ImageJ 1.43u software (Freeware, NIH, Bethesda, MD) to measure diameters of >600 microspheres.

Degradation and ROS Scavenging Activity of PPS *In Vitro*

PPS degradation by ROS in vitro

The degradation of PPS with various ROS was characterized using GPC. PPS was dissolved in DMF + 0.1M lithium bromide at a concentration of 10 mg/mL. Hypochlorite and

H_2O_2 were added to polymer solutions at 1, 2, and 5 equivalents of total sulfur molecules. 10 mM SIN-1 was added at 5, 10, and 15 μL per mL solution. ROS-treated PPS samples were incubated 24 hours on a shaker and then filtered and assessed by GPC using the system described above.

PPS-MS degradation by ROS in vitro

Degradation of PPS-MS with exposure to different ROS was visualized using microscopy. PPS-MS were suspended at a concentration of 1 mg/mL in PBS in chambered cover glass. Microsphere samples were incubated with PBS, 15% H_2O_2, 15 μL 30% NaOCl, or 5 mM SIN-1 for 24 hours. Following this incubation, microspheres were visualized using microscopy on a Nikon Eclipse Ti inverted microscope (Nikon Instruments Inc., Melville, NY). Further ^{1}H-NMR characterization of the treated PPS-MS samples was performed with a Brüker 400 MHz spectrometer after the samples were lyophilized and reconstituted in $CDCl_3$.

Hydrogen peroxide scavenging in vitro

H_2O_2-scavenging activity of the PPS-MS was verified *in vitro* using an Amplex Red Hydrogen Peroxide/Peroxidase Assay kit from Thermo Fisher Scientific (Molecular Probes, Waltham, MA, USA) according to the manufacturer's instructions. 100 μM H_2O_2 was prepared as the reaction solution. H_2O_2 was added to wells in a black-walled, 96-well plate containing either PBS or PPS-MS (final PPS concentration of 1 mg/mL). The samples were incubated at 25˚C for 1 week. The Amplex Red working solution was freshly prepared as described by the manufacturer using the included Amplex Red, horseradish peroxidase, and 1x reaction buffer. The working solution was added to the wells and fluorescence was measured in a plate reader at 30 minutes with an excitation of 530 nm and an emission of 590 nm.

Peroxynitrite scavenging in vitro

The ability of PPS-MS to scavenge peroxynitrite was tested *in vitro* using a Pyrogallol Red (PGR) bleaching assay.[154] Treatment groups consisting of PBS, PPS-MS (0.5-1.5 mg/mL), and ascorbic acid (positive control, pH adjusted to 7.4) were prepared in a 48-well plate with 500 µL volume per well. A PGR stock solution was prepared in PBS (0.025 µM), and 10 µL was added to each well. Peroxynitrite stock solution was thawed on ice and diluted to a concentration of 1 mM in 0.3 M NaOH. After baseline absorbance of the dye was measured in a plate reader at 540 nm, 5 µL of peroxynitrite was added to each well (final concentration of 10 µM) and absorbance measurements were collected for one hour.

Hypochlorite scavenging in vitro

PPS-MS scavenging of hypochlorite was measured *in vitro* using a fluorimetric hypochlorite assay kit. 100 µL of PPS-MS in PBS were added to a 96-well plate at various concentrations ranging from 0.25-1 mg/mL. 100 µL of 10 mM NaOCl was added to wells containing PPS-MS or PBS. The plate was incubated for 10 minutes at 25˚C on a shaker. Following the incubation, 50 µL of each solution was transferred to a black-walled 96-well plate. 50 µL of hypochlorite assay mixture (200 x Oxirite^{TM} Hypochlorite Sensor + 5 mL Assay Buffer) was added to each well. Fluorescence intensity in the wells was measured in a plate reader with an excitation of 540 nm and an emission of 590 nm.

Superoxide scavenging in vitro

In vitro superoxide scavenging activity of the PPS-MS was evaluated using a dihydroethidium (DHE) fluorescence assay. The scavenging of superoxide by PPS-MS (1, 0.5,

and 0.1 mg/mL) was investigated using a superoxide-generating, cell-free enzymatic system containing 0.046 U/ml xanthine oxidase and 0.2 mM xanthine in a black-walled, 96-well plate containing 10 μM DHE (final concentration). The fluorescence intensity was measured in a plate reader (Tecan Group Ltd., Mannedorf, Switzerland) over a time frame of 1 hour with an excitation of 405 nm and an emission of 570 nm. The reaction of DHE with superoxide forms a specific fluorescent product, 2-OH-ethidium (2OH-E), and the selected excitation/emission wavelengths provide optimal specificity for measuring superoxide.[155,156] Specificity of the assay for superoxide detection was confirmed using bovine SOD (20 U/ml) as a positive control treatment in a well containing xanthine / xanthine oxidase and DHE.

PPS-MS Scavenging of ROS *In Vitro* in LPS-Stimulated Macrophages

PPS-MS scavenging of cellular H_2O_2

RAW 264.7 cells were seeded at 500,000 cells/well in 24-well plates in phenol red-free DMEM supplemented with 10% FBS and ciprofloxacin and were allowed to adhere overnight. Cells were treated for 1 h with PPS-MS in fresh DMEM, and 1 μg/mL of LPS was then added prior to an additional 24 h of incubation. Control groups consisted of cells without LPS stimulation and stimulated cells with no microsphere treatment. After 24 h of stimulation, 50 μL of cell supernatant was collected and H_2O_2 levels were measured with an Amplex Red assay using the manufacturer's instructions as described above.

PPS-MS scavenging of cellular hypochlorite, hydroxyl radicals, and peroxynitrite

RAW 264.7 cells were seeded at 50,000 cells/well in 96-well plates in phenol red-free DMEM supplemented with 10% FBS and ciprofloxacin and were allowed to adhere overnight. Cells were treated for 1 h with PPS-MS in fresh DMEM, and 1 μg/mL of LPS was added prior to an additional 24 h of incubation. Control groups consisted of cells without LPS stimulation and stimulated cells with no microsphere treatment. After 24 h of stimulation, cells were washed with PBS and then incubated with 5 mM Aminophenyl fluorescein (APF) in phenol red-free, serum-free DMEM for 25 min. Cells were washed with PBS, and fresh phenol red-free, serum-free DMEM was added to the cells. APF intracellular fluorescence (linked with presence of hypochlorite, hydroxyl radicals, and peroxynitrite) was measured on a plate reader at 30 minutes following media exchange (Ex/Em 488/550 nm).

PPS-MS scavenging of multiple cellular ROS

RAW 264.7 cells were seeded at 75,000 cells/well in 12-well plates in DMEM supplemented with 10% FBS and ciprofloxacin and were allowed to adhere overnight. Cells were treated for 1 h with PPS-MS in fresh DMEM medium, and 1 μg/mL of LPS was then added prior to an additional 24 h of incubation. Control groups consisted of cells without LPS stimulation and stimulated cells with no microsphere treatment. After 24 h of stimulation, cells were washed with PBS and then incubated with 5 μM H2-DCFDA in phenol-red free, serum-free DMEM for 25 min. Cells were washed with PBS and harvested in PBS. Intracellular fluorescence, which corresponds to levels of peroxynitrite, hydroxyl radicals, and several other ROS,[157] was measured via flow cytometry (FACSCalibur, BD Biosciences) and analyzed using FlowJo software.

In Vivo Experiments

Post-traumatic osteoarthritis model

For PTOA experiments, 6 C57BL/6 mice (Jackson Laboratory) at 9-10 weeks of age were divided into two different treatment groups: Saline control and PPS-MS (n=3). Animals received an intra-articular injection of 10 µL of Saline or PPS-MS suspension (1 mg/mL) in both knee joints. The injections were given 24 h prior to exposure to mechanical loading. The PTOA model of noninvasive repetitive joint loading was induced by subjecting the both knee joints of mice (anesthetized with 3% isoflurane) to 50 cycles of compressive mechanical loading at 9 N. This procedure was repeated three times per week over a period of two weeks using conditions adapted from previous studies.[158,159] All procedures in this PTOA study were performed according to protocols and experimental procedures approved by the Institutional Animal Care and Use Committee of Vanderbilt University.

ROS measurement in knee joints in OA model

The Amplex Red assay was used to confirm the presence of oxidative stress (specifically H_2O_2) in the PTOA model in 6 C57BL/6 mice. Three mice received mechanical loading on both knees, and three mice received no loading. After 14 days, the knee joint was excised immediately post-mortem and excess muscle was removed (6 knee joints per group). The joint was cut at the distal femur and proximal tibia to yield ~200 mg of tissue. Upon excision, knee joints were placed in Kreb's HEPES Buffer (pH 7.35) on ice until assayed. Amplex Red working solution was prepared as described above (*2.5.3*). Knee joints were transferred into the Amplex Red solution (500 µL of working solution per well) and incubated in the dark for 1 hour at 37 °C. A series of

dilutions of hydrogen peroxide from 15 μM to 0 μM was prepared in a black-walled 96-well plate at the same time that the tissue incubation began. After 1 hour, 150 μL of solution from each sample was transferred to the black 96-well plate and Amplex Red fluorescence was measured on an IVIS imaging system (Lumina Series III, PerkinElmer) with 530/590 nm excitation/emission filters.

Assessment of cartilage damage and matrix metalloproteinase (MMP) activity in PTOA

At the completion of the two-week loading period, mice in the PTOA experimental groups were injected I.V. (tail vein) with a 100 μL composite mixture of 50 μL of monoclonal antibody to type II collagen (MabCII680) labeled with XenoFluor 680 fluorescent dye (XF680; Perkin-Elmer, Waltham, MA), and 50 μL of 1 nM MMPSense® 750 FAST Fluorescent Imaging Agent (MMP750) (Perkin-Elmer, Waltham, MA), a substrate that fluoresces when enzymatically cleaved by MMPs. After 24 hours, the mice were imaged for MabCII and MMP750 fluorescence using an In Vivo Imaging System (IVIS) (Perkin Elmer, Waltham, MA). MabCII680 detects cartilage damage due to selective binding to exposed CII (which is not accessible in healthy articular surfaces), and MMP750 was used to assess MMP activity *in vivo.*[160] The fluorescence in each knee joint was quantified using Living Image 4.0 software to calculate the region of interest (ROI) and graphed as radiant efficiency (photons/sec/cm^2/str)/(μW/cm^2). Previously, our collaborators have shown that ROIs measured with this method also correspond to the histological score for OA.[159]

Statistical Analysis

All data are reported as mean ± standard error of the mean (SEM). An Analysis of Variance (ANOVA) with a post-hoc Tukey test for multiple comparisons was used to determine treatment effects for comparisons between three groups (PTOA model).

Results

H_2O_2 is Elevated in Mechanically-Induced PTOA

Oxidative stress was confirmed in the PTOA model using the Amplex Red assay. Tissue H_2O_2 was measured in excised knee joints with and without OA. In the PTOA model, H_2O_2 is significantly increased in injured knees after 2 weeks of loading compared to control knees (Fig. 8).

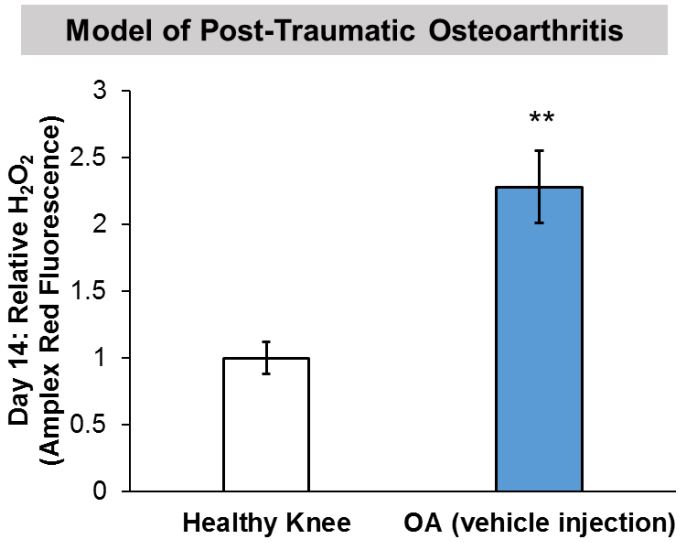

Figure 8: H_2O_2 is elevated in a mechanical model of PTOA. Tissue H_2O_2 levels in knee joints from mice with and without osteoarthritis. H_2O_2 levels were significantly elevated in knees with OA compared to healthy knees. n=6 knees/group (3 mice), mean ± SD, **p<0.01 for OA vs. healthy knees.

Microsphere Synthesis and Characterization

Synthesis and characterization of PPS

PPS was synthesized by anionic polymerization of propylene sulfide as described previously[151] and depicted in Fig. 9A. The polymer structure was confirmed by ^{1}H NMR spectra recorded in $CDCl_3$ (Fig. 2B): 1.3-1.4 (s, CH_3), 2.5-2.8 (s, -CH), 2.8-3.1 (s, CH_2), 3.72 (t, CH_2-OH). The molecular weight and polydispersity of PPS as determined by GPC were M_n = 6700 g/mol and PDI = 1.1, respectively (Fig. 9C).

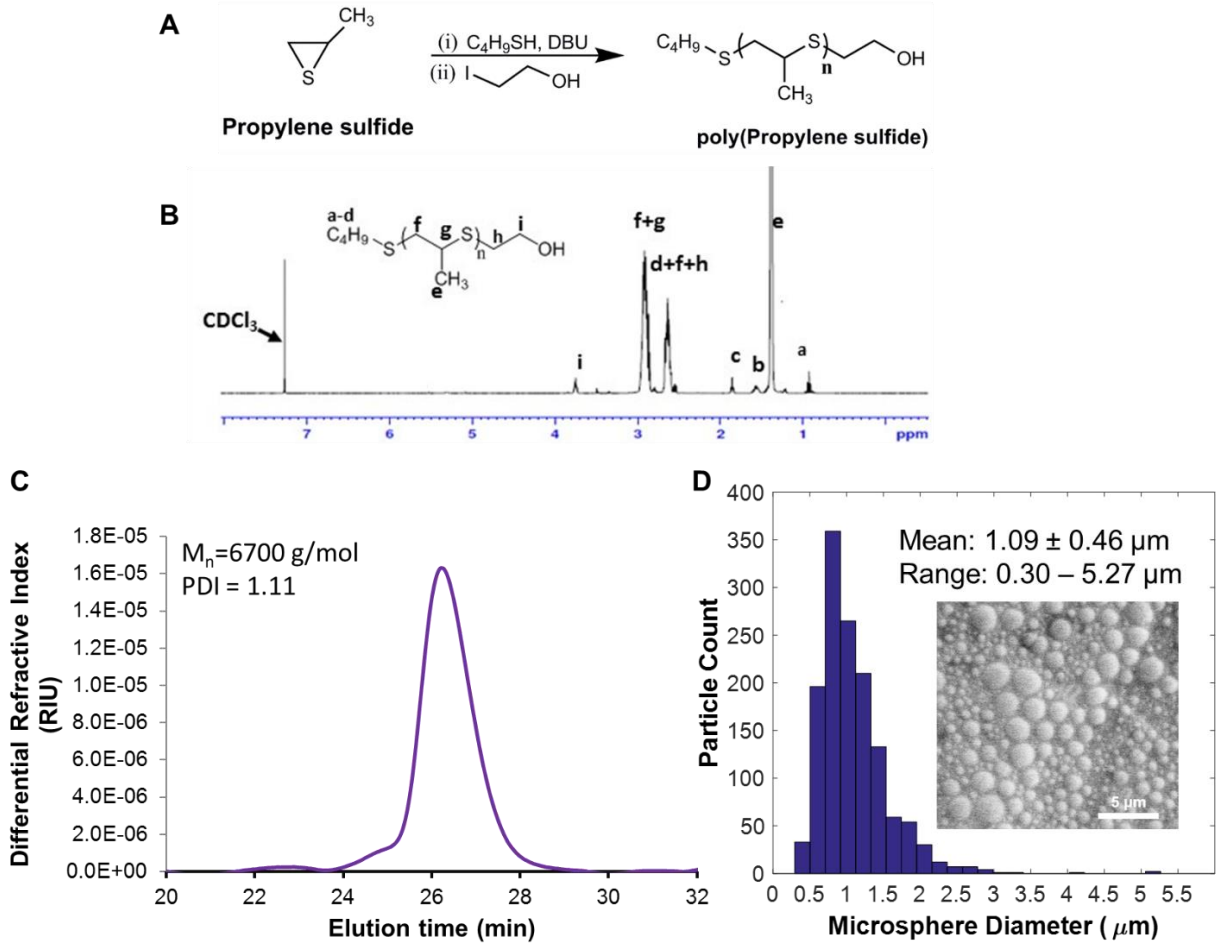

Figure 9: Characterization of the PPS polymer and microspheres. A) Synthesis of poly(propylene sulfide) by anionic polymerization. B) ^{1}H NMR spectrum for PPS polymer. C) The average molecular weight (M_n = 6700 g/mol) and polydispersity (PDI = 1.1) of the PPS were assessed by GPC. D) The size distribution of the PPS-MS was measured with SEM, and the mean diameter and diameter range were 1.09 ± 0.46 µm and 0.30 – 5.27 µm, respectively.

Microsphere characterization

PPS-MS were characterized for size and morphology by SEM (Fig. 9D). Measurements of microsphere diameters in SEM images resulted in an average diameter of 1.09 ± 0.46 µm (mean ± SD, n > 600) and a diameter range of 0.30 to 5.27 µm.

Oxidative Degradation of PPS and ROS-Scavenging *In Vitro*

PPS-MS are degraded by multiple ROS in vitro

The degradation of PPS-MS was assessed upon exposure to H_2O_2, hypochlorite, and peroxynitrite (SIN-1) (Fig. 10A-B). Compared to a control microsphere sample (Fig. 10A i), H_2O_2 and hypochlorite cause complete dissolution of the microspheres (Fig. 10A ii-iii). Peroxynitrite also significantly disrupts the microsphere structure, but what appear to be highly swollen microspheres or aggregates of the polymeric byproduct remain visually apparent in the samples (Fig. 10A iv). These observations were complemented with ^{1}H-NMR analysis (Fig. 10B). ^{1}H-NMR of PPS-MS exposed to H_2O_2 shows a shift in PPS peaks that correlates with oxidized PPS (Fig. 10B ii). Hypochlorite completely degrades PPS and no PPS peaks remain in the NMR spectrum (Fig. 10B iii). SIN-1 does not shift NMR peaks for PPS to the oxidized peaks of PPS (Fig 10B iv).

PPS polymer is oxidized by H_2O_2, hypochlorite, and peroxynitrite in vitro

The oxidation of PPS in polymer form was observed using GPC to detect changes in molecular weight and polydispersity after incubation of the polymer with H_2O_2, hypochlorite, and peroxynitrite (generated by SIN-1). The chromatograms in Figure 10C show shifts in the molecular weight of PPS incubated with these ROS in comparison to a control polymer sample (Fig. 10C i). H_2O_2 oxidizes PPS to form sulfoxides and sulfones, thereby increasing the overall molecular weight (left shift, Fig. 10C ii). Hypochlorite breaks sulfur-carbon bonds in the PPS, resulting in a disappearance of larger PPS polymer chains visualized by a lack of elution peak at high equivalents of hypochlorite (Fig. 10C iii). However, at lower equivalents of hypochlorite, there is a decrease

in molecular weight and an increase in polydispersity of the polymer chains (data not shown). Peroxynitrite has little detectable effect on PPS molecular weight as measured by GPC (Fig. 10C iv).

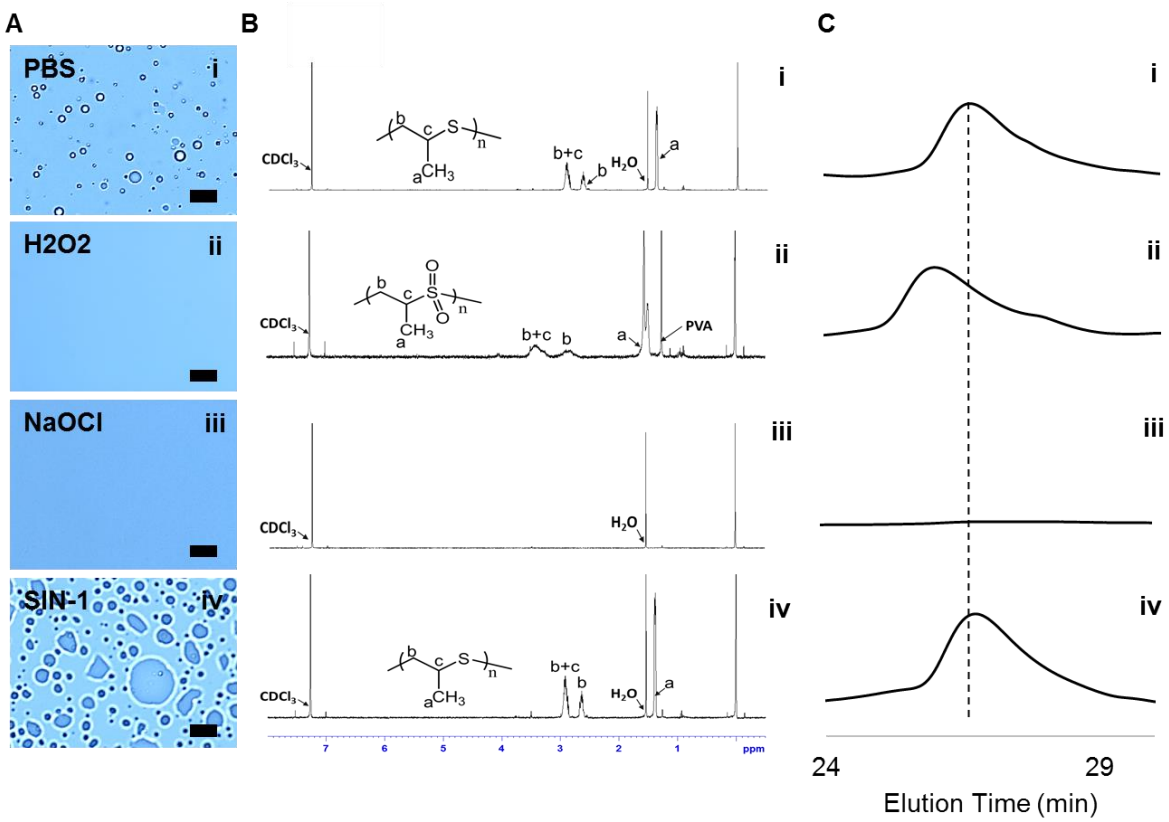

Figure 10: PPS is oxidized and degraded in the presence of various ROS species. A) Visualized degradation of PPS-MS in the presence of H_2O_2, hypochlorite (NaOCl), and peroxynitrite (SIN-1). Scale bar = 10 um. B) ^{1}H-NMR of oxidized PPS-MS. C) GPC traces of oxidized PPS polymer. For columns A-C, row (i) represents PBS control, (ii) is treatment with H_2O_2, (iii) is treatment with hypochlorite, and (iv) is treatment with peroxynitrite.

PPS-MS scavenge H_2O_2, peroxynitrite, and hypochlorite but not superoxide in vitro

The H_2O_2-scavenging activity of PPS-MS was confirmed using Amplex Red as a hydrogen peroxide reporter molecule. Treatment of a 100 μM H_2O_2 solution with PPS-MS (1.5, 1.0, 0.5, and 0.25 mg/mL) significantly reduces Amplex Red fluorescence (Fig. 11A) (one-way ANOVA, $p<0.001$). Pyrogallol Red is bleached in the presence of peroxynitrite, and this bleaching effect can be reduced or inhibited by antioxidant compounds such as ascorbic acid.[154] In the presence of PPS-MS, PGR is protected from bleaching in a dose-dependent manner (Fig. 11B, one-way ANOVA $p<0.0001$). Ascorbic acid was tested as a positive control and resulted in only 2% relative bleaching (nearly complete protection for PGR from peroxynitrite) (data not shown). The hypochlorite-scavenging activity of PPS-MS was confirmed using a fluorimetric hypochlorite assay. Treatment with PPS-MS significantly reduces hypocholorite levels *in vitro* after 10 minutes of incubation (Fig. 11C, $p<0.001$). A DHE assay was used to determine whether PPS-MS scavenge superoxide. The assay results show no change in DHE fluorescence with PPS treatment in comparison to an untreated superoxide solution (Fig. 11D). A decrease in DHE fluorescence was observed with treatment with SOD as a positive control, confirming the veracity of the assay and that superoxide is not scavenged by PPS.

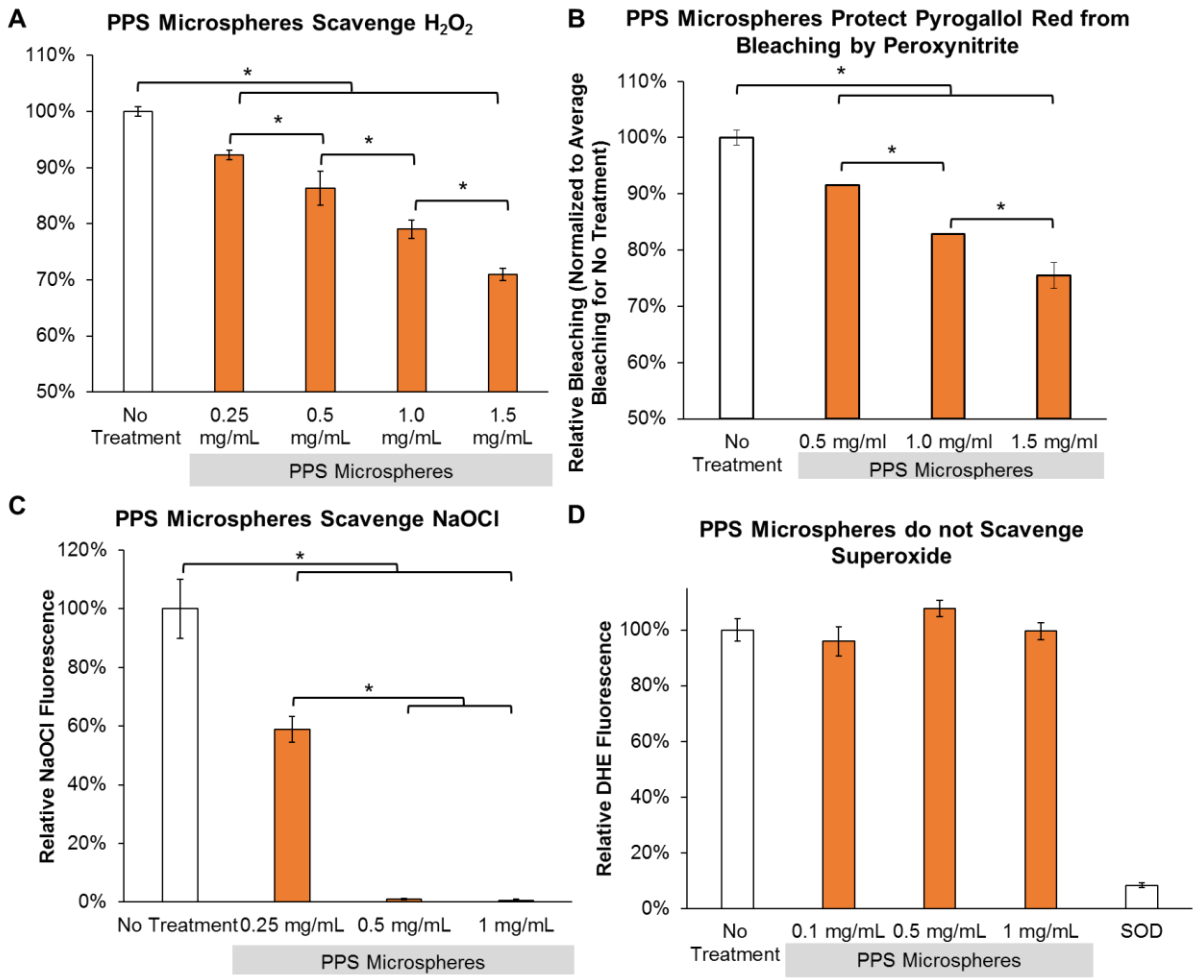

Figure 11: PPS-MS exhibit antioxidant properties for multiple, but not all, types of ROS. A) PPS-MS significantly reduce Amplex Red fluorescence following incubation with 100 µM H_2O_2 (unpaired t-test, $p<0.0001$). B) PPS-MS protect PGR from bleaching by peroxynitrite in a dose-dependent manner (one-way ANOVA $p<0.0001$, *significant post-hoc comparisons). C) PPS-MS scavenge hypochlorite dose-dependently (one-way ANOVA $p<0.001$, *significant post-hoc comparisons). D) PPS-MS do not scavenge superoxide produced by a xanthine/xanthine oxidase system. Data presented as mean ± SD.

PPS-MS Reduces ROS in LPS-activated Macrophages *In Vitro*

PPS-MS reduce H_2O_2 levels in activated macrophages

An Amplex Red assay was used to measure extracellular H_2O_2 secreted by RAW cells activated with LPS. Treatment of activated RAW cells with PPS-MS significantly reduces H_2O_2 levels at doses ranging from 100 to 400 μg/mL ($p<0.001$) relative to activated, untreated cells (Fig. 12A). Furthermore, the H_2O_2 levels in PPS-treated cells are statistically equivalent to ROS levels in non-activated RAW cells ($p>0.05$).

Measurement of PPS-MS effects on cellular hypochlorite, hydroxyl radicals, and peroxynitrite

APF, a derivative of fluorescein that is sensitive to hypochlorite, hydroxyl radicals, and peroxynitrite, was used to measure the effect of PPS-MS on intracellular ROS. PPS-MS doses ranging from 100-400 μg/mL significantly reduced APF fluorescent signal compared to the LPS stimulated, no treatment control (Fig. 12B, $p<0.001$).

PPS-MS effects on multiple ROS

Flow cytometry quantification of general ROS detected by the DCFDA dye confirmed that PPS-MS reduce intracellular ROS levels in activated RAW cells (Fig. 12C). DCFDA fluorescence was significantly reduced in activated RAW cells treated with PPS-MS in a dose-dependent manner for doses of 200 to 400 μg/mL ($p<0.05$). DCFDA reacts with a variety of ROS, including peroxynitrite and hydroxyl radicals.[157]

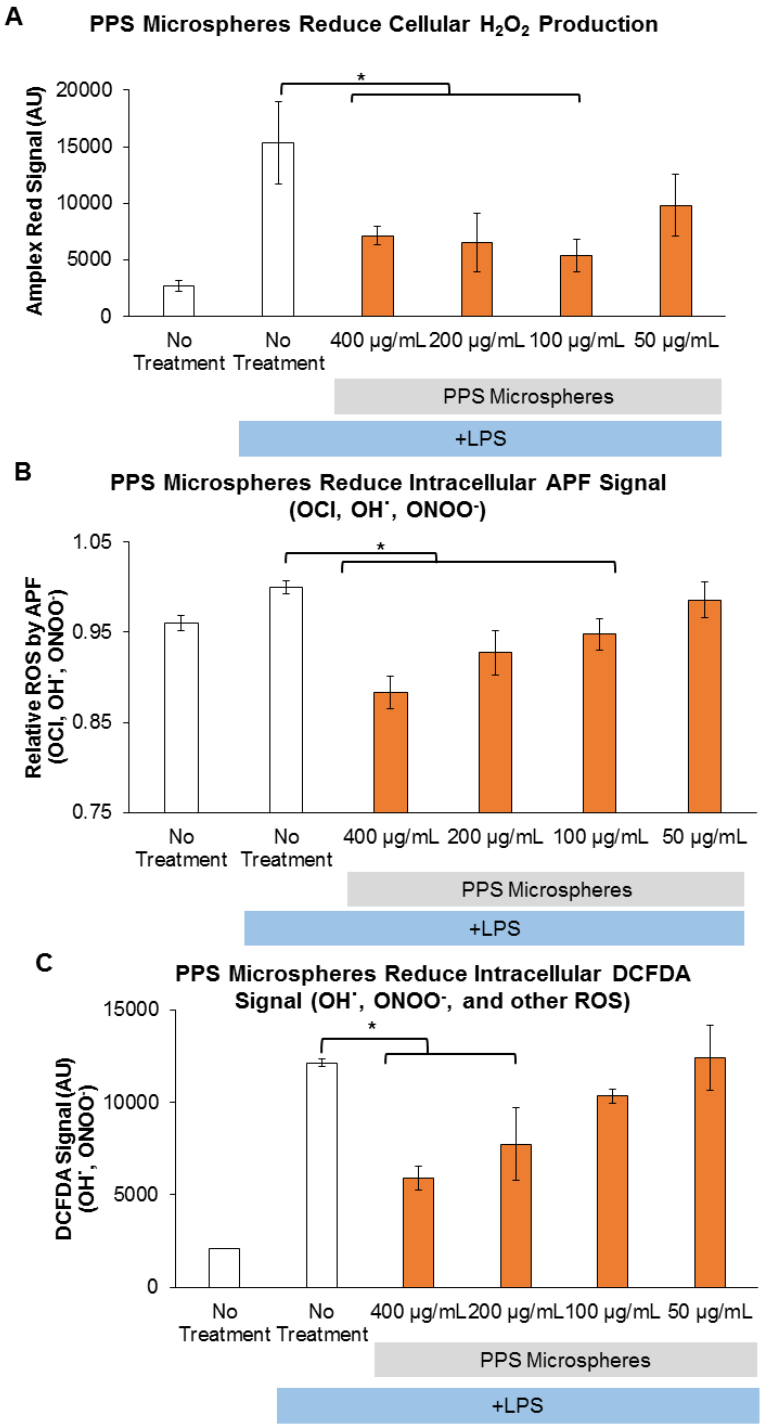

Figure 12: PPS-MS reduce ROS in LPS-stimulated RAW macrophages. A) PPS-MS significantly reduce excreted H_2O_2 in stimulated RAW macrophages measured by Amplex Red (one-way ANOVA $p<0.05$, *significant post-hoc comparison). B) PPS-MS scavenge intracellular

hypochlorite and other ROS in a dose-dependent manner (one-way ANOVA $p<0.05$, *significant post-hoc comparisons). C) PPS-MS reduce multiple ROS species as measured by DCFDA and the response is dose dependent (one-way ANOVA $p<0.05$, *significant post-hoc comparisons). Data presented as mean ± SD.

PPS-MS treatment reduces MMP activity in PTOA

IVIS imaging was used to analyze the effects of PPS-MS treatment on the PTOA mouse model as the fluorescence signal for MabCII680 and MMP750 correspond directly to cartilage damage and MMP activity, respectively (Fig. 13A). MMPSense is an MMP cleavable probe that uses a quenched fluorophore that emits fluorescence after cleavage. MabCII is a collagen II-specific, fluorescently tagged antibody that preferentially binds to exposed collagen II on damaged articular cartilage. No fluorescence signal was observed for the normal animals without mechanical loading (Fig. 13A&C). Intense fluorescence signal from both reporters was observed for mice that received mechanical loading and treatment with saline. Significant decreases in fluorescence signal MMP750 (Fig. 13D) was observed for the mechanically loaded mice treated with PPS-MS ($p<0.05$). No significant decrease in MabCII680 was observed, however, the data trend to show decreased MabCII680 binding, suggesting a larger sample size may result in significant differences. These results suggest treatment of mice with PPS-MS attenuated MMP activity as compared with the group treated with saline alone.

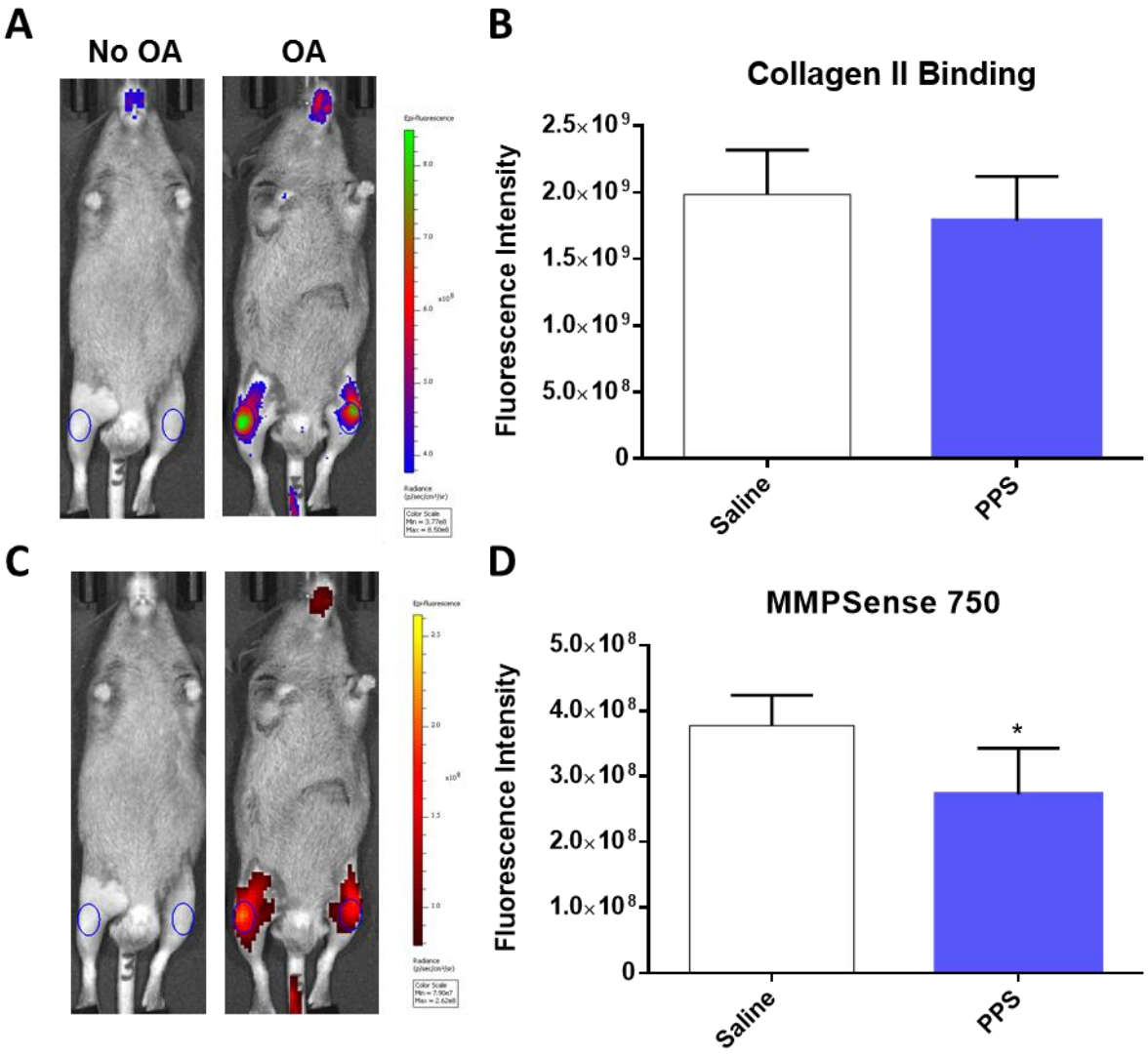

Figure 13: PPS-MS reduce MMP activity in PTOA. Mice were mechanically loaded both knees and then imaged *in vivo* for binding of MabCII680 and evaluation of MMP activity. A) MabCII680 imaging of normal control mice and PTOA mice. B) Quantified MabCII680 signal bound to damaged cartilage. C) MMPSense 750 imaging of normal control mice and PTOA mice. D) Quantified MMPSense 750. MMP activity significant decreases with PPS-MS treatment. (Data is expressed as mean ± SD, *$p<0.05$, n=6/group.)

Discussion

Transient or low levels of ROS contribute to cell signaling that promotes both homeostasis under normal conditions and repair following injury. However, chronic or high levels of ROS can be detrimental to vascular function[163-165] and preservation of tissue such as articular cartilage following injury.[145,166] Therefore, we have explored the therapeutic effects of a local depot of antioxidant PPS-based microspheres in a mechanical model of PTOA. PPS was selected as the polymer for the microspheres because it scavenges H_2O_2 (Fig. 11A), peroxynitrite (Fig. 11B), and hypochlorite (Fig. 11C), protects cells from H_2O_2-induced toxicity,[149,151] and can be used to deliver hydrophobic therapeutic molecules via ROS-responsive drug release.[149] PPS-MS treatment also reduces H_2O_2 secretion (Fig. 12A) and intracellular hypochlorite, hydroxyl radicals, and peroxynitrite (Fig. 12B-C) in LPS-stimulated macrophages. The concept of targeting multiple ROS has been previously demonstrated *in vitro* in a hybrid polymer-enzyme nanocarrier system.[167] However, micron-sized particles are large enough to form a stable depot that is retained in the joint without significant diffusion away from the injection site. Furthermore, targeting a particle size of approximately 1 μm enables preferential uptake by phagocytic immune cells, targeting the antioxidant effect to the relevant inflammation-associated cells.[149] Therefore, fabrication of particles in this size range was targeted in order to achieve a combination of both intracellular and extracellular ROS scavenging, which is important for optimal cell and tissue protection from oxidative stress.[168] Additionally, as we have previously established, PPS-MS can also be extended to achieve locally-sustained drug release.[149]

PPS-MS have promise for localized oxidative stress reduction in PTOA. OA is a complex disease process that occurs over a long period of time, but it begins with early focal, superficial

lesions in the articular cartilage followed by the loss of proteoglycans and breakdown of cartilage collagen. Type II collagen is present in meniscal and articular cartilages but is inaccessible to antibodies in the normal, healthy joint. Therefore, IVIS imaging of the binding of type II collagen antibody in the mechanically loaded knee confirms that significant degradation of knee joint cartilage occurs in the context of mechanical loading, leading to the unmasking of the type II collagen (Fig. 13A). This is in agreement with our previous observations that MabCII680 binding correlates with the histological score for OA.[159] The presence of active MMP levels in the joint is suggestive that the MMP enzymes secreted by the synovium or the chondrocytes themselves might play a role in this unmasking. A member of the MMP family, MMP-13 (collagenase-3), is the primary enzyme responsible for the degradation of type II collagen in the cartilage matrix in osteoarthritic cartilage.[169]

The ROS generated in response to mechanical injury regulate expression of numerous genes involved in immune and inflammatory responses including MMP-13.[166] Our results showed that mechanical loading causes cartilage destruction, increased MMP activity (Fig. 13), and oxidative stress (Fig. 1). These results show that treatment of knee joints with an intra-articular injection of PPS-MS significantly reduced MMP activity in the PTOA model (Fig. 13D). Impeding OA at its early stages is crucial in building an effective therapeutic regime against OA,[170] and the results of this study suggest that targeting early oxidative stress in PTOA with a local depot of chondroprotective PPS-MS can attenuate further cartilage degradation by decreasing MMP activity.

Conclusions

There is a significant need for long-lasting and well-controlled therapies for inflammatory diseases such as PTOA. There are currently no pharmacological treatments for OA that treat the underlying molecular cause of disease.[171] In this work, oxidation-sensitive PPS was used to formulate microspheres for local antioxidant therapy. The microspheres are capable of scavenging multiple ROS including H_2O_2, hypochlorite, and peroxynitrite which are implicated in disease progression. *In vivo* studies demonstrated that PPS-MS impede progression of cartilage damage in a PTOA model. These collective results suggest that targeting ROS in these models is an effective therapeutic strategy. In sum, the results establish PPS-MS as a promising stand-alone therapy or as a drug delivery vehicle that may synergize with PTOA.

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