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A Local, Sustained Delivery System for Zoledronic Acid and RANKL-Inhibitory Antibody as a Potential Treatment for Metastatic Bone Disease

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A LOCAL, SUSTAINED DELIVERY SYSTEM FOR ZOLEDRONIC ACID AND RANKL-INHIBITORY ANTIBODY AS A POTENTIAL TREATMENT FOR METASTATIC BONE DISEASE

THESIS

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science
in Biomedical Engineering in the College of Engineering
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By

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ABSTRACT OF THESIS

A LOCAL, SUSTAINED DELIVERY SYSTEM FOR ZOLEDRONIC ACID AND RANKL-INHIBITORY ANTIBODY

AS A POTENTIAL TREATMENT FOR METASTATIC BONE DISEASE

Cancerous solid tumors can migrate and lead to metastatic bone disease. Drugs prescribed to reduce bone resorption from metastasis, such as zoledronic acid and the RANKL-inhibitory antibody Denosumab, cause side effects such as osteonecrosis of the jaw when delivered systemically. This project used two biocompatible materials, acrylic bone cement (PMMA) and poly(lactic-co-glycolic acid) (PLGA), to incorporate and sustain release of anti-resorptive agents. Results showed similar mechanical properties for acrylic bone cements loaded up to 6.6% drug by weight. Results showed sustained zoledronic acid release for 8 weeks from both systems, with PMMA releasing up to 22% of loaded drug and PLGA films releasing over 95%. The antibody release rate was lower, with the majority of antibody still inside the PLGA films after 8 weeks. In vitro bioactivity remained above 50% for zoledronic acid eluted from both materials at early, middle, and late time points. This study sheds light on the behavior of these biocompatible polymers at high drug weight percent loadings compared to previous studies. PLGA demonstrated superior release kinetics but inferior bioactivity of eluted drug. By incorporating anti-resorptive drugs into locally implantable materials, this work could lead to a treatment offering improved quality of life for cancer patients.

KEYWORDS: PMMA, PLGA, bisphosphonate, drug delivery, bone

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Among the complications from many types of cancers is metastasis into bone tissue, which is known as metastatic bone disease (MBD). Metastatic bone disease has been linked to increased pain, increased incidence of skeletal fractures, hypercalcemia, and decreased patient mobility.[1] Current treatments for MBD include surgery to remove the diseased tissue, radiotherapy, medication, or some combination of the three.[2]

Bisphosphonates are one common medication prescribed in these circumstances.[1] Bisphosphonate drugs including zoledronic acid (ZA) have been linked to improved bone mineral density (BMD) and decreased bone pain for patients with MBD.[3] The monoclonal antibody denosumab has shown similar results.[4] However, bisphosphonate intravenous injections have also been linked to osteonecrosis of the jaw (ONJ), a painful condition in which bone in the maxillofacial region is exposed after the death of the surrounding gum tissue.[5],[6] Denosumab injections have also been linked to ONJ.[4]

The objective of this work was to develop a surgically deliverable vehicle to release antiresorptive agents at the sites of MBD without traveling through the bloodstream. This was done in order to avoid side effects such as ONJ. However, as bioavailability of both agents from injections falls below 75%, the implant could potentially improve efficiency in that area as well.[7],[8]

Chapter 2 details the background of metastatic bone disease and the treatments for it, as well as the history of using acrylic bone cement and poly(lactic-co-glycolic acid) (PLGA) as biomaterials for drug delivery. Chapter 3 covers the methods and results of using acrylic bone cement as the delivery vehicle, due to its popular use in treatment of MBD already. Chapter 4 covers the methods and results of PLGA films as the delivery vehicle. Chapter 5 contains the conclusions of the study.

Chapter 2: Background and Significance

2.1. Incidence of Cancerous Tumors

After heart disease, cancer is the USA's second leading cause of death, responsible for nearly 25% of all deaths as of 2013. Over 1.5 million cases were diagnosed in that year in the USA. For women, it is estimated that over 230,000 cases of breast cancer were diagnosed in 2014. For men, it is estimated that over 230,000 cases of prostate cancer were diagnosed.[9]

2.2. Incidence and Physiology of Metastatic Bone Disease

The adult human skeleton is comprised of over 200 bones of different shapes and sizes. Bone with high surface area to volume ratio is considered trabecular, and more compact bone is cortical. Healthy adult bone tissue features a matrix of protein, mainly collagen I, supplemented with minerals such as hydroxyapatite (HA). Interacting with this protein matrix are osteoblastic cells which generate bone material and osteoclastic cells which resorb it, although some osteoblasts are integrated into the bone matrix and known as osteocytes. At the core is a spongy bone marrow filled with cells. These cells include blood cells, adipose cells, stem cells, and the progenitors of osteoblasts and osteoclasts.[10]

Bone is continually remodeled in order to heal and grow, but also as part of a larger physiological regulation of serum phosphate and calcium concentrations, as those ions are stored as minerals in bone tissue. The process of osteoclasts and osteoblasts working in conjunction to maintain skeletal health is known as coupling. The osteoclasts resorb bone by secreting acid to dissolve the minerals and secreting enzymes to lyse the protein matrix. In healthy tissue, the osteoclasts and their chemical secretions are cordoned off into select lacunae by a barrier of podosomes. Upon completion of resorption, osteoblasts enter the lacunae and lay down the protein matrix anew. To facilitate coupling, there are multiple chemical methods of communication and

stimulation between osteoclasts and osteoblasts. Growth factors embedded in the bone matrix, such as transforming growth factor β1 (TGF-β1) and insulin-like growth factor 1 (IGF1), are thought to stimulate osteoblasts upon liberation from the bone matrix during remodeling. There are also signaling molecules secreted by osteoclasts themselves, such as sphingosine-1-phosphate (S1P) which can promote osteoclast differentiation or chemotaxis towards certain lacunae. Osteoclasts can also communicate with osteoblasts via binding of membrane molecules. One example is the presence of ephrins on osteoclast membranes and ephrin receptors on osteoblast membranes. Another is the presence of receptor activator of nuclear factor κB (RANK) receptors on osteoclasts and the RANK ligand (RANKL) on osteoblasts.[11] Still another example is osteoprotegerin (OPG), a molecule produced by stromal and osteoblastic cells which binds to RANKL in competition with RANK and decreases bone resorption. The local concentration of OPG thus forms a balance with the local concentration of RANKL in healthy bone tissue to control bone remodeling.[12]

Bone tissue can play host to a variety of tumors, some malignant and others not. Giant cell tumor is one example of a tumor that is not cancerous, but leads to bone pain and damage.[13] The two most prevalent types of primary bone cancers are multiple myeloma (cancer of bone marrow) and osteosarcoma (cancer of osteoblasts).[14] Uncontrolled malignant solid tumors, although they begin in one organ system, are also prone to metastasizing into the skeleton. In prostate cancer patients, a postmortem study by Galasko et al. showed 68% had bone metastases.[15] In women with advanced stage breast cancer, estimates range from an average of 65 to 75% developing bone metastases.[16] In lung cancer patients, estimates are that MBD affects between 30 and 40%.[17]

The presence of tumor cells in the bone environment can lead to an increase in the numbers and activity of either osteoblasts or osteoclasts in the vicinity, depending on the tumor.[1],[17] However, patients with multiple myeloma or metastatic breast carcinoma have predominantly osteoclast-mediated osteolysis.[1] Solid tumors secrete growth factors such as parathyroid hormone-

related peptide (PTHrP), interleukin-1 (IL-1), interleukin-6 (IL-6), and prostaglandin E2 among others. These chemicals upregulate the differentiation of osteoclasts, disrupting the delicate balance of coupling.[12] In such cases, increased resorption of the bone leads to increased incidence and growth of metastases in the bone environment, resulting in a positive feedback loop. In addition to physically providing spaces in bone for tumor cells to grow in, resorption also causes the release of growth factors such as TGF-β1 which are normally used for coupling, but can be usurped by tumors.[16],[18],[19]

In the case of multiple myeloma, the condition has been linked to increases in blood levels of RANKL, IL-6, and macrophage inflammatory protein 1a. All three compounds are also linked to osteoclast differentiation from progenitor cells.[20] In the cases of breast and prostate tumors, the common markers are PTHrP levels in blood serum and n-telopeptide of type-I collagen (NTX) levels in urine.[15],[21] Elevated RANKL and IL-6 levels have also been observed in biopsied tissue samples from bone metastases of these tumors.[22]

Every 3 to 6 months, the average patient with metastatic bone disease will experience a skeletal-related event (SRE). In fact, a study by Brown et al. showed that the rate of bone resorption, measured by NTX in urine, correlated to increased risk of SRE's and death in patients with metastases.[15] The SRE umbrella includes conditions from fracture to spinal cord compression to hypercalcemia and bone pain. Pain or skeleton structural compromise that is significant enough to be prescribed surgery is also considered an SRE.[17] For bone pain, the common sites include the base of the skull, the vertebrae, the pelvis and femur.[15] For lung cancer patients with SREs who survived 36 months, the average cost of SRE treatment was $11,979 per patient.[17]

2.3. MBD Treatments

Depending on location, severity and symptoms, metastatic bone disease has various treatments available. It is most commonly treated using radiotherapy, analgesics, surgery, bisphosphonates, or some combination.[17]

Palliative radiotherapy is a localized radiation treatment for sites of bone metastasis. One study by Zhu et al. found up to one-third of patients reporting full pain relief at the treated site. The team also found that 50-80% of patients experienced moderate to significant pain relief.[23] However, radiation therapy had side effects including disturbed sleep and fatigue on patients, as well as radiation toxicity and tissue damage to areas surrounding the tumor.[7]

Analgesic treatments for bone pain include opioids and non-steroidal anti-inflammatory drugs (NSAIDs). For mild bone pain, World Health Organization recommendations are to use acetaminophen or NSAIDs to provide relief. For mild-to-moderate and moderate-to-severe pain, opioid drugs are recommended for stronger effect.[24]

Bisphosphonate treatments have been linked to decreases in bone turnover in patients with cancer-induced MBD. Bone resorption markers such as bone-specific alkaline phosphatase (produced by osteoblasts during osteogenesis, and increasingly so during high bone resorption) have been measured at lower concentration in the serum of patients who were on regular bisphosphonate treatments.[25],[26]

Surgery of MBD-affected sites is in most cases intended for pain relief rather than curing the cancer. However, when surgery is used for curative or palliative purposes, the entire tumor is removed from the site and the bone is reconstructed with prostheses. In extremely serious cases, amputation may be required.[2],[14],[27] In MBD-mediated bone lesions with painful symptoms, it

has been argued that surgery can decrease the need for opioid painkillers and potentially improve patient mobility and quality of life.[27]

One supplementary treatment used in conjunction with conventional therapies is bone cement injection, known as vertebroplasty when applied to the spine and osteoplasty when applied elsewhere in the skeleton. The bone cement hardens in the damaged area to stabilize the structure.[2],[28],[29] However, two caveats as listed by Anselmetti et al. is that a patient should not have systemic or local infections, or blood vessels or vital organs within 10 mm of the injection site. Infections have been a historical concern with application of bone cement, while thermal and chemical necrosis of surrounding cells have been previously studied for bone cements as well.[2]

2.4. Zoledronic Acid

Zoledronic acid (ZA) is a third-generation bisphosphonate, prescribed as Reclast® or Zometa® (Novartis Pharmaceuticals). In cancer patients, it is given as a 4 mg (dissolved in 5 mL saline) dose every 4 weeks via an intravenous injection.[30] Bioavailability of ZA to the entire skeleton from IV dosages is estimated at 60%, with the remainder excreted via urine within one day of injection.[31] ZA has been approved as a treatment to increase bone mineral density and reduce blood hypercalcemia in conditions of multiple myeloma, solid tumor metastases, osteoporosis, and Paget's disease.[30]

As the structure of bisphosphonates all involve a central carbon with two phosphonate groups on opposite sides, the resulting polar molecule forms ionic attractions to minerals in bone such as HA.[7] Specifically, there is ionic attraction between the calcium cations of HA and the oxygen atoms bonded to the phosphorous atom on each phosphate group. The fact that both phosphates have three atoms enables bisphosphonates to foster strong interactions with multiple cations around them.[32]

After binding to the surface of the bone matrix, ZA is endocytosed by osteoclastic cells as they lie on the bone surface.[22] This has been suggested as one reason why ZA affects osteoclasts much more than osteoblasts, as osteoclasts more commonly perform phagocytosis and are thus more prone to absorb bisphosphonates into their cytosol.[33] The mechanism of action of ZA, and all nitrogen-containing bisphosphonates, is the binding of the compound to farnesyl diphosphate (FPP) synthase. The binding spot on the enzyme that ZA occupies is the traditional binding site for geranyl diphosphate (GPP). By slowing down the kinetics of the reaction to produce FPP, ZA causes a cascade effect in the mevalonate pathway which decreases the osteoclast's rate of production for important cholesterols and prenylated guanosine triphosphatase enzymes, leading to apoptosis.[32],[34],[35] Additionally, the inhibition of FPP leads to excessive concentrations of isopentenyl pyrophosphate (IPP), which is an intermediary product of the mevalonate pathway. IPP is normally converted to geranyl diphosphate, but without FPP synthase it accumulates and is converted to ApppI, and ApppI causes apoptosis by inhibiting the adenine nucleotide translocase.[36]

As one of the newest bisphosphonates, it should come as little surprise that ZA is one of the most effective. One study by Berenson et al. showed that a 4 mg dose of ZA was at least as effective as a 70 mg dose of pamidronate in preventing SRE's in patients.[3] In a 2007 study by Black et al. on bone mineral density in osteoporosis patients, a once-yearly dose of ZA led to higher lumbar spine BMD increase (6.7%) than a once daily dose of alendronate (6.2-6.6%) or risedronate (5.4-5.9%).[7]

Additionally, ZA has been shown to prevent cancer treatment-induced bone loss. A study by Gnant et al. found that 4 mg intravenous ZA every 6 months effectively inhibited bone loss from endocrine treatment for breast cancer patients.[37]

Kimachi et al. also showed anti-differentiation effects of ZA on osteoclast precursors. Their in-vitro study showed that 30 μM ZA in solution significantly decreased the migration of RAW 264.7 murine macrophage cells, when migration was induced by differentiating agents such as TNF-α. As little as 10 μM ZA after 24 hours incubation significantly decreased expression of mRNA linked to differentiation-mediating receptors in RAW cells.[38]

However, bisphosphonates are not perfect. After IV doses, acute phase reactions such as fever, headache, and/or bone pain are considered common. ZA has also been linked to renal failure, and therefore is not recommended for patients who show low creatinine clearance or other signs of kidney malfunction.[7] It has also been linked to ONJ, which some studies have shown to vary between 1.3 and 1.4% of patients. However, for cancer patients suffering from certain cancers in particular, and receiving high doses of intravenous ZA, some studies have shown risk of ONJ to be as high as 12%.[5],[6],[39] Additionally, Rizzioli et al. reported that the patient population in the HORIZON-PFT trial for ZA showed statistically significant increase in serious atrial fibrillation when compared to patients taking placebo.[7]

2.5. Denosumab

An alternative strategy to combat excessive bone resorption is prevention of osteoclast differentiation from progenitor cells. Increased serum levels of various biomolecules have been linked to higher presence of osteoclasts.[20]

Nakagawa et al. first showed via DNA analysis in 1998 that there were membrane receptors expressed on osteoclasts and osteoclast progenitors which were identical to RANK.[40] Upon activation, the RANK receptor binds to TRAF6, which activates the TAK1 protein kinase complex, triggering multiple signaling pathways including JNK and MAPK. Another triggered pathway, the NF-kB pathway, begins with the phosphorylation of IκB kinase complex. The NF-kB proteins ultimately

enter the nucleus and bind to specific regions of DNA, leading to increased expression of certain genes. The JNK and MAPK pathways meanwhile produce compounds that bind to transcription factor proteins such as c-Fos and NFATc1, altering their activity and changing gene expression in that manner. The alteration of gene expression changes the cells' biochemistry and causes differentiation.[41],[42],[43]

The molecule that binds to this receptor is RANKL, also referred to as osteoclast differentiation factor (ODF) and TNF-related activation-induced cytokine (TRANCE), and it has been characterized as "an essential signal for osteoclastogenesis."[40] RANKL is embedded in the cell membrane in osteoblasts and stromal cells, however some RANKL isoforms can dissolve and travel through human serum. Suzuki et al. classified 3 isoforms: hRANKL1 containing intracellular, transmembrane, and extracellular domains; hRANKL2 containing only transmembrane and extracellular domains, and hRANKL3 containing only extracellular domains.[44]

One proposed solution to unhealthy osteoclast activity has been binding other ligands to RANKL in the bone environment, in order to prevent RANKL from activating pre-osteoclasts. Lin et al. produced an immunoglobulin G (IgG) antibody which could bind to murine RANKL. When incubating pre-osteoclastic RAW 264.7 mouse macrophages with 100 ng/mL murine RANKL and either 5 or 10 μg/mL anti-RANKL antibody, they found that there was a statistically significant decrease in the number of osteoclasts. When the team artificially induced resorption in rat jaws, they found that injecting anti-RANKL antibody into the jaw on days 1, 2, and 8 of an 11-day study could significantly (at higher concentrations) decrease levels of soluble RANKL and amount of bone resorption.[45]

One human antibody which competitively binds to RANKL is the immunoglobulin G2 denosumab, formulated as Prolia® and Xgeva® (Amgen Inc.). It is delivered via subcutaneous

injection, with a dose of 120 mg per month for adults with bone metastases and a bioavailability of 62%. Denosumab has been shown to decrease resorption of bone in osteoporosis patients.[4],[8]

Stopeck et al. reported a study of 2049 women internationally with metastasis from bone cancer, with half of the patients treated with 4 mg ZA every 4 weeks, and the other half treated with 120 mg Denosumab every 4 weeks. Denosumab led to a statistically significant 18% delay in time until the patients' first SRE compared to ZA. It also caused a statistically significant 23% reduction in likelihood of the patient developing more than one SRE, again compared to ZA. When patients' serum NTX levels were measured, Denosumab also caused a decrease of 80% versus placebo, which was statistically significant when compared to the 68% decrease from ZA.[46]

2.6. Acrylic Bone Cements

Bone cement is used as a grout to fix prosthetic implants. Among the scenarios for its use are degenerative disorders and fractures.[27],[29],[47] In conjunction with surgical procedures that remove diseased tissue, bone cements are also used as a temporary filler.[13]

Acrylic bone cements are sold as a powder plus a liquid, to be mixed. The powder is mostly poly(methyl methacrylate) or PMMA, along with other compounds such as the radiopaque barium sulfate for easier x-ray detection and benzoyl peroxide as a polymerization initiator.[12],[48] The liquid is predominantly methyl methacrylate (MMA) monomer, with some accelerator such as dimethyl-p-toluidine (DMPT).[47] The benzoyl peroxide forms free radicals upon reacting with the DMPT. These free radicals then bind to MMA molecules, severing the carbon-carbon double bonds and producing MMA radicals. The reaction propagates via chain addition, in which the radicals attack the double bonds on a nearby MMA molecule, combining the molecules into ever larger species, but maintaining an unpaired valence electron at one end of the carbon backbone. The polymerization has two termination processes that eliminate radical species. During combination, the radicalized

termini of the polymers connect to each other, eliminating the radicals. During disproportionation, a polymer radical acquires a hydrogen molecule off the penultimate carbon in the backbone of another polymer radical, producing a terminal double bond in the latter species.[28],[29],[46],[49] This reaction is exothermic, with an estimated 52 kJ released per mole of monomer.[29] In fact, a study by Anselmetti et al. found that peak bone cement internal temperatures during vertebral surgery range between 40.3 and 75.8 degrees Celsius, depending on the cement formulation and the geometry of the volume it must occupy.[50]

PMMA bone cement is primarily used during surgery to coat the perimeter of the mating surfaces of the implant and the bone. The cement has the ability in its initial doughy form to penetrate into small pores and crevices in bone tissue and harden there. This anchors the implant to the bone so that loads borne are evenly transferred from one to the other.[28],[51] In low-viscosity formulations, PMMA bone cement is also injected into areas of damaged or fractured bone for the aforementioned vertebroplasty and osteoplasty procedures.[29]

When implanted following surgery and curettage (removal of diseased tissue with a specialized scoop), bone cement has been found to reduce rates of tumor recurrence. One hypothesis is that this is due to the heat generated from the bone cement polymerization reaction causing necrosis in the remaining tumor cells in the region.[13] The cytotoxicity of released monomer has also been cited as a possible reason for reduced recurrence.[13]

However, PMMA particles have also been reported to cause osteoclast differentiation, which would give rise to increased resorption. A study by Sabokbar et al. added PMMA particles to murine blood monocytes in vitro and found that, when the cells were placed in an environment replicating that of cortical bone, tartrate-resistant acid phosphatase (TRAP) assays showed the macrophages

maturing into osteoclasts. The same study also found that loading a bisphosphonate into the bone cement decreased the osteoclastic presence.[52]

2.7. Drug Loading and Release From Bone Cements

Bone cements have a history of drug loading for different purposes. One example, when used to attach implants to bone tissue, is loading the bone cement with antibiotics.[53],[54],[55] This is done to prevent infection after surgery, and has risen greatly in popularity since H.W. Buchholz et al. first tested the clinical effects of addition of antibiotic powder to PMMA powder in the late 1960's.[47],[56] This method has not only been applied in vivo, but tested in vitro for quantifying release profiles for different drugs in different cements.

One study by Penner et al. on thick 2.5 w/w% vancomycin in Palacos-R bone cement discs, of 28 mm diameter and 20 mm thickness, showed that 6.1 mg was released per disc over a 5-week incubation. The release rate decreased with time, from 0.673 mg released on the first day down to less than 0.05 mg released over the final week. 6 w/w% tobramycin-loaded samples also displayed high levels of initial release followed by exponential decay, with each disc releasing 20 mg tobramycin. However, there were statistically significant increases in released mass of both drugs when the two were loaded together to a total 8.5 w/w% antibiotic loading.[54]

Another study by Chang et al. compared 2.5, 10, and 16.7 w/w% loadings of daptomycin, vancomycin, or teicoplanin into Simplex bone cement for release and activity. Samples of all loadings would decrease in rate of release over time. However, 2% loaded samples eluted drug for just two days, 10% samples eluted for 40 days with teicoplanin and 21 days for the other antibiotics, and 16.7% loaded samples eluted for 60 days. Teicoplanin had the highest release efficiency for all loadings, while there was no statistically significant difference between vancomycin and daptomycin. When supernatants were tested against methicillin-resistant staphylococcus aureus, 10 w/w%

sample supernatants all showed measurable antibacterial activity up to the last time point of detectable release.[55]

Bisphosphonates have been previously loaded into bone cements, and studied via in vitro incubation and in vivo. Zhu et al. injected titanium particles into rat femurs to promote osteolysis and test the effectiveness of applied PMMA with 0.5 w/w% alendronate at improving local bone health.[57] Matuszewski et al. tested drug elution from their small PMMA cylinders loaded with 0.15 w/w% pamidronate, over 6 weeks of saline incubation.[58] Healey et al. tested elution from their 5 w/w% pamidronate in PMMA study using disc-shaped samples, while Yu et al. tested elution of radiolabeled ZA in their 0.004 w/w% samples.[53] The team of Zwolak et al. loaded cylindrical samples of Simplex P bone cement with between 0 and 1 mg of ZA per 1.5 mL bone cement volume, equivalent to less than 0.1 w/w%.[59]

2.8. Mechanical Effects of Drug Loading on Bone Cements

Studies of drug addition to bone cement have shown that there is a point of mechanical decline if the drug is loaded in excessive amounts. However, the point at which drug loading becomes excessive may vary depending upon many factors. The weight percent (w/w%) of loaded drug is one important variable. One study by Davies et al. showed that 1.25 w/w% loadings of erythromycin or colistin did not significantly affect fatigue strength compared to the control. However, another study by Nelson et al. with 3.3% weight loadings of gentamycin or Keflin into Simplex P did show decreases in yield strength in static compression.[60]

The composition and properties of the drug used to load the bone cement is another important variable. Duey et al. performed compression tests on ASTM-F451 standard PMMA cylinders with different antibiotic loadings, 24 hours after polymerization by hand mixing. The team found that there was no statistically significant difference between blanks and samples loaded with

2.5% tobramycin, but there was a significant decrease in ultimate compressive strength for samples with 2.5% total antibiotic, as a mixture of 1.25% tobramycin and 1.25% vancomycin.[61]

The manner of drug addition also affects mechanical properties. A study by Lautenbacher et al. dissolved gentamicin in water and mixed the solution in with MMA monomer just prior to polymerization. Comparing equal w/w% loadings of gentamicin, the team saw a significant decrease in mechanical properties with dissolved drug.[60]

Bisphosphonates have also been studied for their effects on bone cement mechanical properties. Yu et al. loaded up to 0.004 w/w% ZA or 0.04 w/w% pamidronate into Antibiotic Simplex: Radiopaque bone cement, testing 4-point bending and static compression.[53] Calvo-Fernandez et al. hand-mixed PMMA beads with 1.5 w/w% alendronate and polymerized with methyl methacrylate into cylindrical samples, testing static compression after incubation for 1 month in saline.[62] The team of Matuszewski et al. tested samples of DePuy's CMW1 with 0.15 w/w% pamidronate into PMMA powder, studying static compression and 3-point bending.[63] Lewis et al. loaded 0.42 w/w% alendronate in tablet form into CEMEX XL acrylic bone cement powder and tested fatigue at 2 Hz, 15 Mpa amplitude tension-compression.[64] Healey et al. tested tension and compression of Simplex bone cements loaded with doxorubicin or pamidronate, including tests after 6 months of incubation in fluid.[53]

2.9. Poly(lactic-co-glycolic acid)

Poly(lactic-co-glycolic acid) (PLGA) is a heavily studied polymer commonly used in drug delivery applications. Unlike PMMA, it is a biodegrading polymer.[65],[66],[67] It hydrolyzes into lactic and glycolic acid, both of which are natural metabolites of the human body.[68] Systems employing PLGA in use with various drugs can be produced in a number of ways.[69],[70],[71] PLGA is used medically for suturing wounds, and is being studied for delivery of biomolecules to target

cells as well as production of structures to promote cell growth.[72] It has been loaded or conjugated with antibiotics, proteins, other peptides, nucleic acids, steroids and more.[68]

Drugs incorporated into PLGA can be released by either diffusion through the polymer, especially at locations near pores, or by erosion of the polymer itself.[69] Early stages of degradation are characterized by water cleaving PLGA chains to decrease their molecular weight.[68] However, PLGA is classified as a bulk-degrading polymer. Therefore, an aqueous solvent can penetrate a PLGA matrix, not only into the surface region of rampant hydrolysis and dissolution of monomers and oligomers, but seeping even deeper.[65],[67] Consequently the next stages of degradation are characterized by mass loss due to water dissolving the polymer from the inside and out. The freed monomers' carboxylic acid groups autocatalyze PLGA degradation in these bulk regions to speed up the process.[72],[73] At the final stages, mechanical ruptures at the surface are prone to occur due to stress concentration from zones of higher mass loss.[68],[69]

One common method of micro-scale drug encapsulation by PLGA is emulsified microspheres. For hydrophilic drugs, this formulation is produced by a water/oil/water double emulsion. PLGA is first dissolved into an organic solvent, the oil phase. An aqueous solution containing dissolved drug is added into the oil phase and broken into droplets by sonication. The oil phase is then added to a larger volume of water and stirred.[66],[74] The microspheres can be isolated after hardening, then lyophilized and stored in a freezer until use.

The drug elution from these particles over time is affected by many factors including microsphere size, microsphere surface area to volume ratio, pore density and size distribution, the chemical properties of the encapsulated compound, the ratio of copolymers such as lactic versus glycolic acid, and the protocol used to prepare the microspheres.[66],[68] For instance, typically a higher weight percentage of glycolic acid monomer in the PLGA relative to lactic acid leads to faster

degradation of polymer in an aqueous environment.[67],[72] Higher molecular weights for PLGA of the same copolymer ratio will also tend to slow degradation rate.[65],[75] The end groups of PLGA molecules also impact degradation; all else being equal, ester-terminated PLGA degrades more slowly than carboxyl-terminated PLGA.[72]

PLGA has been employed as a delivery system for bisphosphonates as well, with different teams attempting drug incorporation, prolonged drug elution, and bioactivity against cells and in vivo from different systems. For instance, the team of Samdancioglu et al. tested 50:50 PLGA microspheres with encapsulated alendronate at a theoretical loading of 10 w/w%. (Theoretical loading represents the highest possible amount of drug in the microspheres, in experiments in which drug loading is not measured, by assuming the entire mass of drug added during emulsion was encapsulated and loaded into the material.) The microspheres were prepared by water/oil/water emulsion at neutral pH and oil/water emulsion at different aqueous pH values.[76] Shi et al. loaded alendronate at a theoretical 4.7 w/w% loading into 50:50 PLGA via double emulsion, although they also added powdered HA to the outer water phase at 0, 30 or 50 w/w% of PLGA. The team also tested single emulsion microspheres in which dissolved alendronate was bound to powdered HA in aqueous solution, dried, and added to dichloromethane (DCM) along with PLGA.[77] Long et al. prepared alendronate-loaded PLGA films at multiple loadings as high as a theoretical 0.5 w/w%. The films were prepared by adding alendronate powder directly into DCM with dissolved PLGA, distributing the drug via vortex mixing and sonication, and then cooling and solvent casting.[78] Nafea et al. tested 50:50 PLGA microspheres loaded with alendronate, prepared via water/oil/water and oil/water emulsion to a theoretical 10 w/w% loading. However, drug loading via the water/oil/water emulsion proved so low that a release study could not be performed.[79] Perugini et al. loaded clodronate into different molecular weights of 50:50 PLGA, 75:25 PLGA, and poly(D,L-lactic acid) (PDLLA) microspheres via double emulsion. They also tested the effects of adding

carboxymethylcellulose in the drug-solution inner water phase and adding Span 20 into the DCM phase, and their two theoretical drug loadings were 9 and 18 w/w%.[80] Sharma et al. loaded clodronate at a theoretical 22.2 w/w% into 50:50 PLGA microspheres via double emulsion, and also tested subsequent conjugation of the microspheres to the peptide LyP-1 to improve microsphere targeting in vivo into 4T1 tumors in mice.[81]

In addition to controlled delivery applications, bisphosphonates have also been conjugated with PLGA nanoparticles in order to direct and retain those nanoparticles in bone tissue. Choudhari et al. used ZA as a chemical conjugate to direct PLGA nanoparticles with the chemotherapeutic docetaxel. When tested on MDA-BO2 cells in vitro, they found that there was a significantly higher percentage of late apoptotic cells upon exposure to docetaxel-loaded ZA-treated nanoparticles compared to loaded nanoparticles without ZA.[82]

Loading of proteins in microsphere systems has also proven successful. Clark et al. showed that insulin growth factor (IGF) bioactivity was retained after double emulsion into 50:50 PLGA microspheres and subsequent sintering at 49^{o}C. Release continued for 120 days.[83] A study by Cleland et al. loaded human growth hormone into PLGA microspheres, finding that the majority of HGH maintained structural integrity. The same study showed that less than 75% of loaded protein was released over 4 weeks.[84] The team of Cho et al. tested encapsulation of bovine serum albumin in PLGA prepared as water/oil/water double emulsion microspheres, and found that for 34 kDa PLGA they could encapsulate as much as 66% of protein if they added surfactant to the outer water phase.[85]

Antibodies specifically have been loaded into PLGA systems as well. Joung et al. loaded anti-rabbit 3D8 scFv antibody into PLGA microspheres and tested their ability to combat the effects of the vesicular stomatitis virus on HeLa cells in vitro.[86] Gdowski et al. encapsulated Anti-AnxA2

antibodies within 50:50 PLGA nanoparticles, controlled for size to a diameter under 250 nm.[87] Ma et al. encapsulated scFv-pDL10 anitbody into PLGA microspheres controlled for smaller size to facilitate prolonged release, injecting them into mouse models to study cellular and immune responses.[88] Son et al. also used 3D8-scFv encapsulated into 50:50 PLGA microspheres by double emulsion, and studied its activity in vitro upon release.[89]

2.10. Study Aims

The aim of this study was to produce an implantable system for locally releasing ZA or Denosumab, which may be useful for future applications in order to decrease bone resorption and associated pain while avoiding side effects of systemic delivery. These drugs were incorporated into polymers that gradually released the compound of interest into the surrounding fluid after incubation. They were intended to be inserted initially during bone curettage or resection procedures on patients, and to deliver drug doses in a manner less likely to incur systemic side effects as seen in the conventional drug delivery methods. The project attempted to show the successful loading, sustained release profile, and a reasonable level of in vitro bioactivity of both drugs delivered from their respective systems.

Chapter 3: Materials and Methods

3.1. Production of PMMA samples

The form of PMMA used was C~ment 1 (Aap Implantate AG, Germany). The powder contained 70% PMMA, 15% barium sulfate, 15% benzoyl peroxide. The monomer ampoule contained 84.4% methyl methacrylate (MMA), 13.2% butylmethacrylate, 2.4% dimethyl-p-toluidine, and 0.00002% hydroquinone. Drug was added and loaded into the PMMA powder.

Zoledronic acid monohydrate (Medkoo Pharmaceuticals, Charlotte, NC) was mixed into PMMA and stirred for 5 minutes with a spatula prior to polymerization with MMA. Figure 3.1 illustrates an example of this process, with the ZA dyed red for visibility purposes. PMMA powder was weighed out to 1.1 grams, and 425 µL of MMA fluid was pipetted into the powder, to keep roughly in accordance with the original packaged ratio of 40 g powder and 15.475 mL MMA. Polymerization occurred in a shallow ceramic bowl. As soon as the monomer was pipetted onto the powder, the bowl was transferred to the bottom shelf of a freezer, with the door kept ajar during the rest of the process. The dough-like substance was mixed for 2 minutes by spatula, kneaded by hand for 3 minutes, and pressed flat onto the bowl surface. The dough was cut with a razor blade into approximately equal-sized pieces, 8 if used for mechanical testing and 5 if used for release studies. Each piece was hand-rolled into a thin strand and pressed into a hole in a PTFE mold, the dimensions of which also differed for mechanical versus release studies. The weight of drug powder mixed into bone cement was a variable, as the weight percent ratios were 0, 1, 2, 5, and 10% ZA. However, the weight percentages were not formulated as a ratio of powder weights, but rather took into account the weight of the MMA fluid. To keep in accordance with literature, the weights were subsequently reported in terms of ZA powder to PMMA powder, meaning the percentages tested were 0%, 1.3%, 2.6%, 6.6%, and 12.7%.

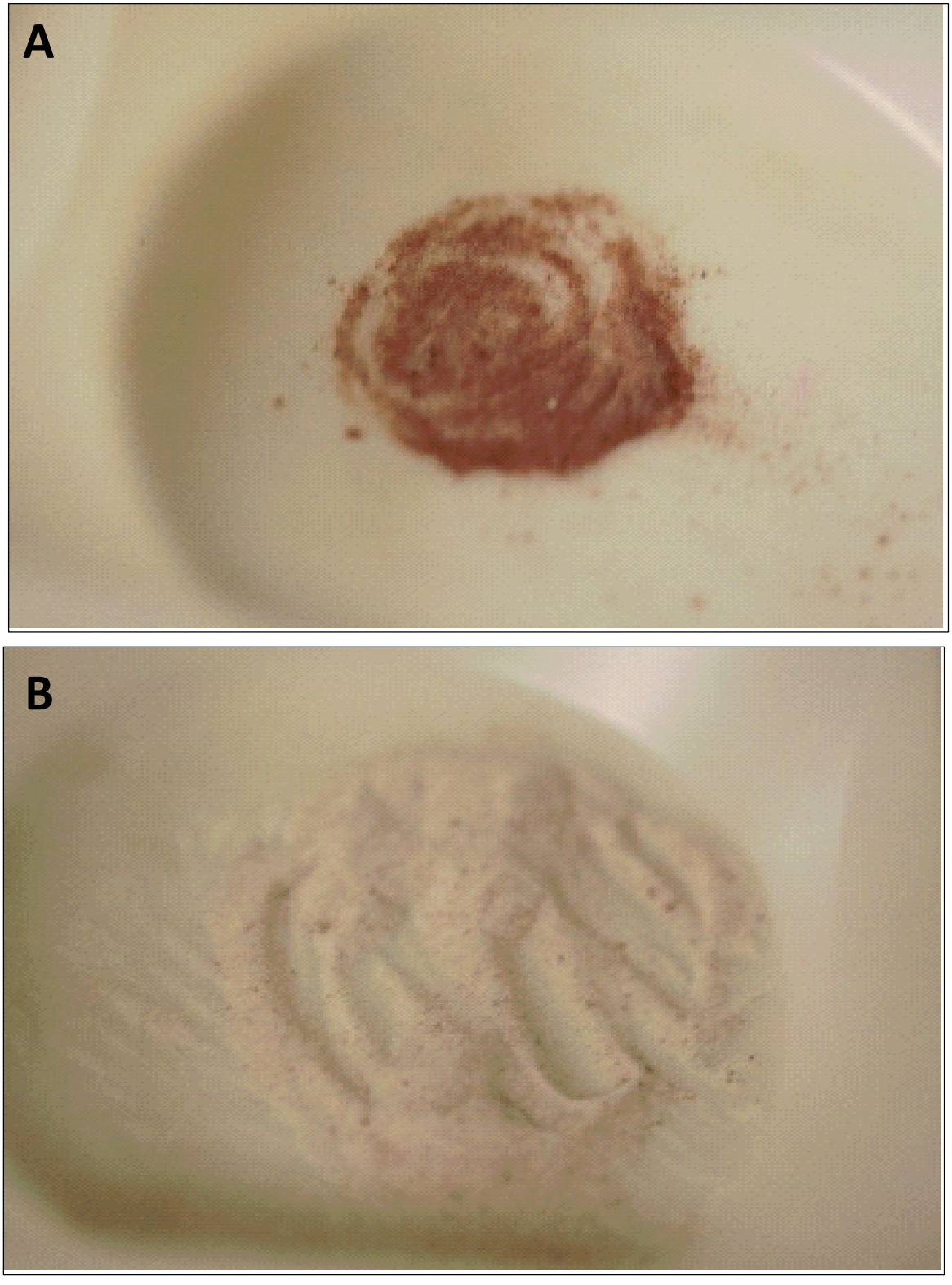

Figure 3.1: Zoledronic acid (A) after mixing with food coloring and (B) after further mixing into PMMA.

3.2. Mechanical Testing Protocol

ASTM F451, the ASTM standard specification for acrylic bone cement, calls for a compression test of PMMA using 6 mm radius and 12 mm height cylindrical samples, at a displacement rate of between 19.8-25.4 mm/minute.[51] In accordance with that ratio, the samples used in this compression test were pressed into a PTFE mold at room temperature and allowed to set for 24 hours. The mold produced cylinders of roughly 9 mm height and 4.5 mm diameter. Samples with uneven edges were trimmed with a razor blade, as were samples for which the edge region was discolored. The samples were subsequently compressed on a Bose ElectroForce 3300 uniaxial testing machine (Bose Corporation, Framingham, MA) at room temperature, using a ramp function with a 25 mm/min displacement rate. Figure 3.2 shows an example of an original 2.6% PMMA cylinder before and after trimming.

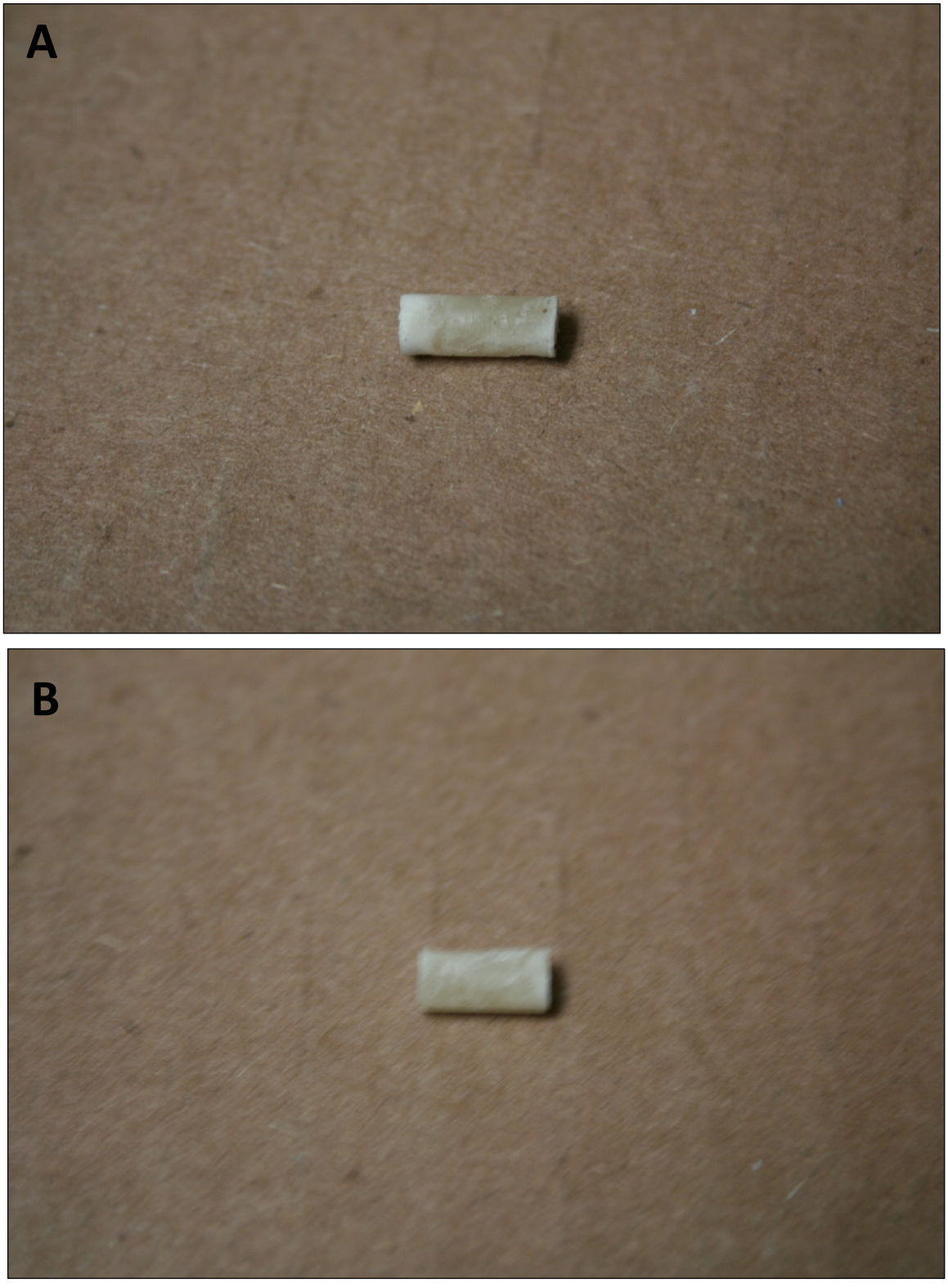

Figure 3.2: Sample PMMA cylinder (A) after removal from mold and (B) after trimming prior to compression.

3.3. Release Profile of ZA from PMMA samples

Cylinders for ZA release studies were prepared in the same manner as cylinders for compression testing. However, in accordance with a ZA in PMMA study by Zwolak et al., the samples' dimensions were 6 mm radius by 10 mm height.[59] Average sample mass was 303.9 mg. Samples were removed from molds after 24 hours and placed into scintillation vials containing 5 mL phosphate-buffered saline (PBS), at pH 7.4. The vials were then placed onto a plate shaker and incubated at 37°C. After 1, 2, 3, 5, 7, 14, 21, 28, and 56 days, time points were collected. The PBS in the tubes was withdrawn via pipette, stored in separate vials in a -20^{o}C freezer, and refilled, and the tubes restored to the plate shaker.

ZA release was measured via high-performance liquid chromatography (HPLC) on a Primaide (Hitachi Ltd., Japan). The UV detection wavelength was 220 nm, for a peak eluting between roughly 4.2 and 4.8 minutes, using a hydrophilic Luna HILIC column. The mobile phase used was a gradient between acetonitrile (ACN) and a solution of 0.000188% formic acid, titrated to pH 3.5. The gradient began at 40% ACN and 60% formic acid, run for 2 minutes. Subsequently there was a 5-minute transition period which ended at a 5% ACN and 95% formic acid ratio by the 7 minutes mark. The last 3 minutes were a transition back to 40% ACN and 60% formic acid for the next sample run.

Stocks for running standards were prepared at 400 μg/mL ZA in pH 10 PBS, titrated back to pH 7.5, and stored in a refrigerator for up to 3 weeks prior to remaking. Standards for each time point were prepared by mixing equal volumes of fluid from the three blank cylinder supernatants, then mixing in ZA stock solution to reach dilutions of 0, 2, 5, 10, 20, and 50 μg/mL ZA. This was done in order to account for any absorption of other solutes in the release supernatant at 220 nm wavelength. For time points showing peaks higher than 50 μg/mL ZA, tests were rerun with

standards of 100 and 300 µg/mL. Standard curves were prepared by plotting standard ZA concentration versus peak area.

3.4. Loading of ZA into PLGA Films

Zoledronic acid was added and loaded into PLGA microspheres using a water/oil/water double emulsion. The details of the process were as follows.

The ZA solution was prepared by dissolving 160 mg ZA into 4 mL deionized (DI) H_2O in a 15 mL centrifuge tube. This solution was then titrated with NaOH to pH 10. The ZA solution was placed on a plate shaker, at room temperature for 24 hours, and then checked again to ensure a pH of 10.

Films were prepared from ester-terminated 75:25 PLGA, 0.55-0.75 dL/g inherent viscosity (Lactel Polymers, Birmingham, AL). The oil phase was prepared by dissolving 350 mg of PLGA into 5 mL dichloromethane (DCM) in a 15 mL centrifuge tube. The outer water phase was prepared by adding 25 g of NaCl and 1.75 g of methyl cellulose into 1 L DI water. The solution was stirred overnight and vacuum filtered to 0.45 µm in order to remove large undissolved particles of methyl cellulose. The first emulsion began with pipetting 330 mL of ZA solution into the oil phase, and mixing in an S/P Vortex Mixer (American Scientific Products, Portland, OR) for 60 seconds at setting 9. The solution was subsequently sonicated in a GE 50 (Markson Science, Henderson, NC) sonicator in pulse mode at 45% amplitude and 20 kHz frequency, for 45 seconds. In order to prevent the risk of heat damage to the ZA molecules from the sonicator, the centrifuge tube was positioned inside an ice bath. The oil phase was poured slowly into 125 mL saline solution and homogenized in an Omni PDH homogenizer (Omni International, Kennesaw, GA) at 4000 rpm for 5 minutes.

After homogenization, the outer water phase volume was increased to 200 mL and stirred at 500 rpm with a magnetic stir rod for 90 minutes. The microsphere suspension was then centrifuged in 50 mL centrifuge tubes at 2500 rpm for 5 minutes in a Sorvall Legend Mach 1.6 (Thermo Electron,

Waltham, MA), and the supernatant poured out. The tubes were refilled with 10 mL DI water, and the microspheres were resuspended using a vortex mixer. The microsphere suspensions were combined into one tube, which was brought up to 50 mL volume with DI water. The microspheres were centrifuged at 4000 rpm for 7 minutes.

Following this, the microspheres were resuspended in 25 mL water, vortexed for mixing, and centrifuged at 2500 rpm for 5 minutes. This sequence was repeated two more times to wash the microspheres. Singh et al. designed a method of fabricating scaffolds out of PLGA microspheres without using heat or dichloromethane vapor, but simply by soaking in ethanol.[90] Influenced by that protocol, the washed microspheres were soaked in ethanol for 5 minutes to soften, vortexed at setting 8, and centrifuged once more at 4000 rpm for 7 minutes. The supernatant was poured out of the tube, and the film was placed in a 43^{o}C oven for 60 minutes to evaporate the remaining ethanol. The film was then compressed in a Carver 3851 hydraulic press (Carver, Wabash, IN) at 3000 psi for 5 minutes, with the intent of producing a flat oval shape of increased surface area in order to speed up drug release.

Final films had a central thickness around 1.3 mm and peripheral thickness around 0.2mm, with a major axis diameter of 17.5 mm and minor axis of 13.2 mm. In order to obtain consistent drug loading for all films tested, the films were cut with a razor blade into 3 pieces of approximately equal mass. The 3 films were again compressed at 3000 psi for 5 minutes. The films were then frozen at -20^{o}C for at least 24 hours, and dried in a Freezone lyophilizer (Labconco, Kansas City, MO) for 48 hours to remove residual water. Finally they were stored at -20^{o}C until incubation. The final film mass averaged 94.3 mg. Figure 3.3 shows a sample film.

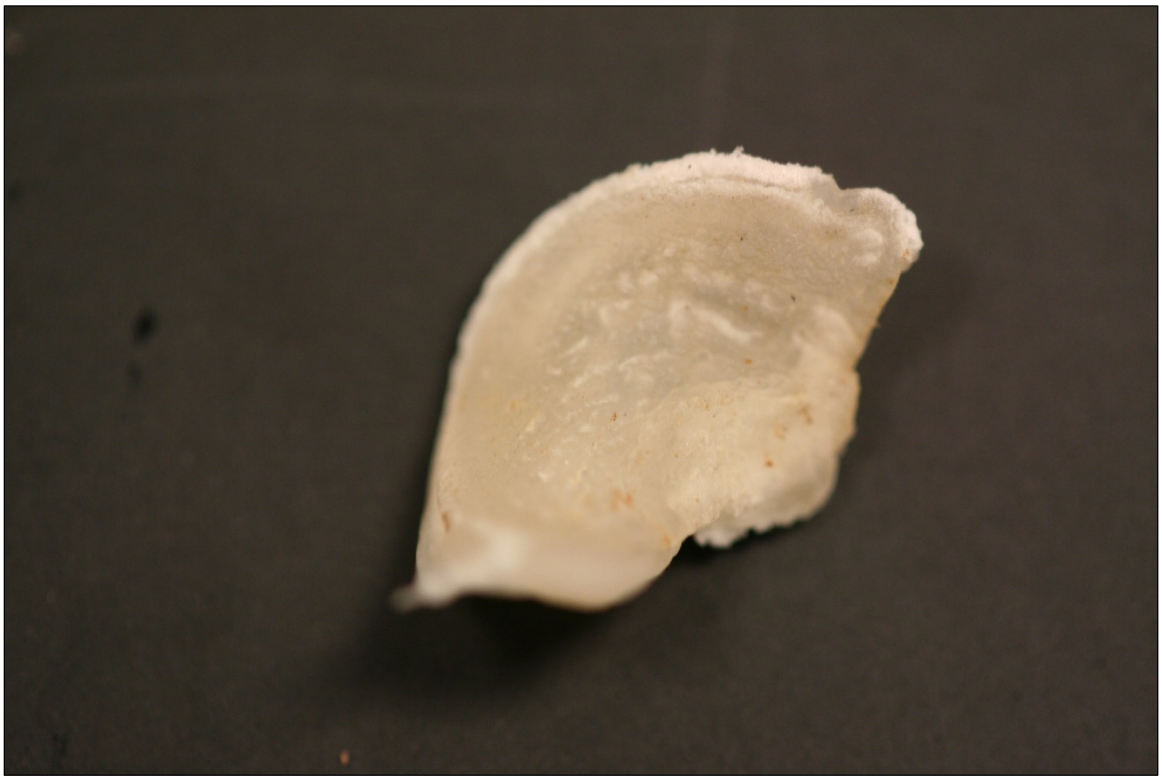

Figure 3.3: Blank PLGA film sample prior to PBS incubation.

These films, like the PMMA cylinders, were incubated in scintillation tubes on a plate shaker in a 37^{o}C oven. The volume of PBS was again 5 mL per tube. Time points were taken at 1, 2, 3, 5, 7, 14, 21, 28, 35, 42, 49, and 56 days. ZA content was measured using the same HPLC method described previously.

3.5. Loading of anti-RANKL Mab626 into PLGA Films

The films for the antibody study were prepared in virtually the same manner as the ZA films. However, the inner water phase for double emulsion was prepared from Mab 626 (R&D Systems, Minneapolis, MN), an anti-human RANKL monoclonal antibody dissolved to 1 mg/mL in sterile PBS. The antibody solution was added at 320 µL into 320 mg of 75:25 PLGA in 5 mL DCM.

In order to ensure high concentrations of antibody in the release supernatants, the films were not cut into 3 pieces, but rather separately prepared three times. Following compression, the films' diameters were trimmed by about 1 to 1.5 mm all around, removing the yellow edges. The peripheral thickness of the films doubled to about 0.4 mm following this. This helped the film to fit inside the scintillation tube, due to its increased size compared to the ZA-loaded predecessors. The average starting mass for antibody-loaded films was roughly 257 mg. Figure 3.4 below shows an antibody study film before and after trimming, but prior to lyophilization.

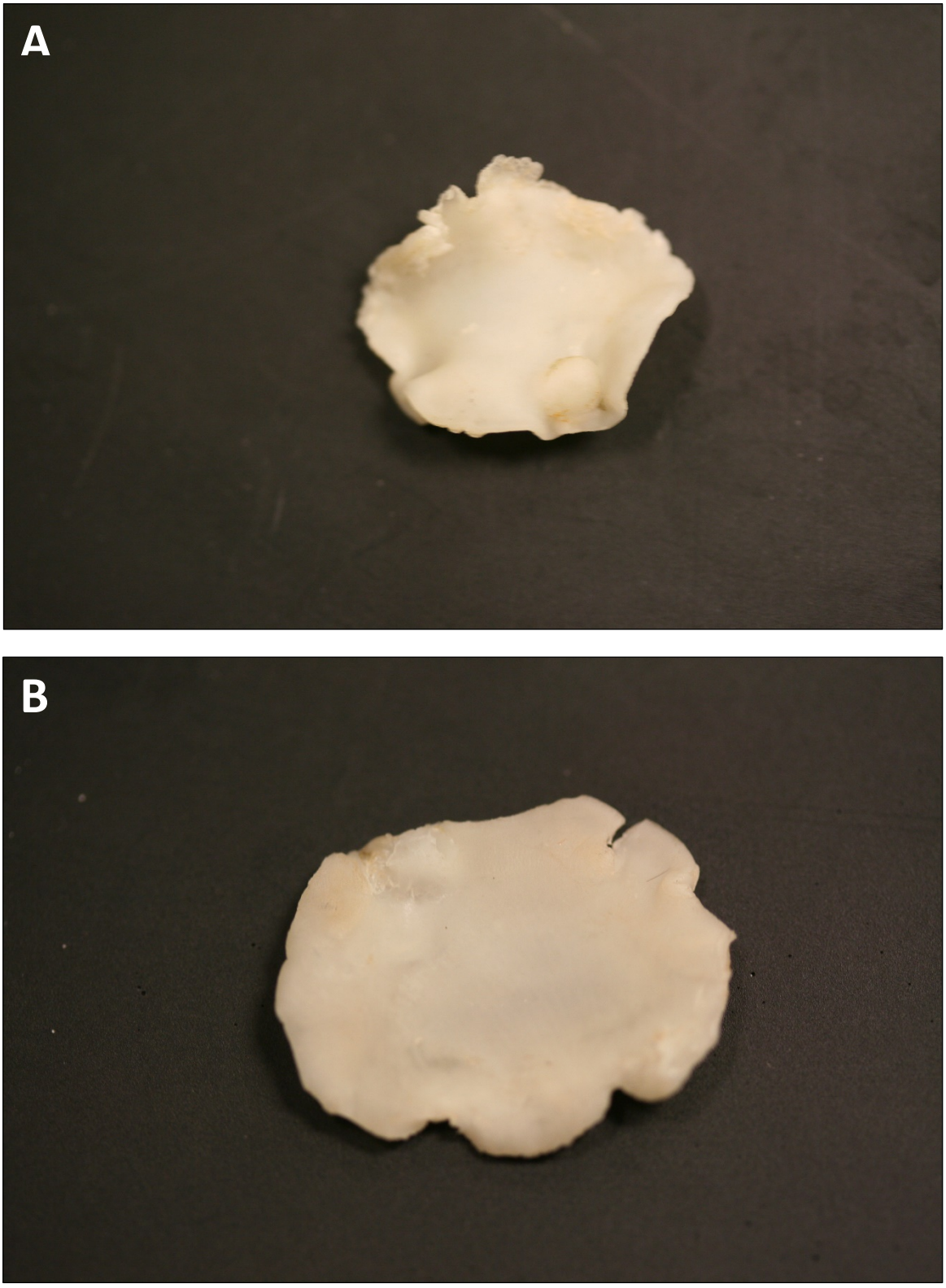

Figure 3.4: Pre-lyophilized blank PLGA film (A) prior to and (B) after trimming.

Films were incubated in 5 mL PBS at 37°C for 56 days. To further increase the amount of antibody detected at each time point, the number of time points was shortened compared to the ZA-loaded films. Time points were taken at 1, 3, 7, 14, 28, 42, and 56 days after incubation. This schedule intentionally provided more time in between release points than the ZA study did, to ensure the concentration of antibody in supernatant would be high enough to reliably detect.

Antibody release was detected using a Micro BCA Protein Assay Kit (Thermo Fisher Scientific, Pittsburgh, PA). Samples and blanks were thawed for 3 hours on a plate shaker, along with a 500 µL aliquot of Mab 626, diluted from stock to 20 µg/mL in sterile-filtered PBS. Then the samples and blanks were each mixed by a 1 mL pipette, and 500 µL from each was transferred to a microcentrifuge tube. The tubes were centrifuged at 5000 rpm for 5 minutes in a Sorvall Legend Micro 21R (Thermo Electron, Waltham, MA). 150 µL was removed from the top of each tube and pipetted into a 96-well plate. 150 µL from the antibody stock was pipetted into a well with 150 µL PBS and mixed to create a standard of 10 µg/mL, and this process was repeated to create two standard wells. Both standards were serially diluted down to 0.625 µg/mL, and the last standard was blank PBS at 0 µg/mL, creating two columns of standards with 150 µL volumes. BCA reagent was prepared in a 6 mL glass bottle, the reagents mixed in accordance with kit instructions. 150 µL was pipetted into each standard and sample well. After 2 hours incubation in a 43°C oven, with a Parafilm covering to limit evaporation, light absorbance was measured at a wavelength of 570 nm in a PowerWave HT spectrophotometer (BioTek, Winooski, VT) .

3.6. Encapsulation Efficiency Testing of Loaded PLGA Films

Although drug powder mixing for PMMA samples allowed nearly the entire amount of added ZA powder to load into the bone cement material, there were concerns about the percentage of added drug which would successfully load into PLGA microspheres during PLGA emulsion. PLGA

films were prepared, dissolved and tested for drug content in order to produce a ratio: the mass of drug loaded into the films, against the mass of drug initially added to the emulsion inner water phase. Levels of 100% encapsulation efficiency were designated as the theoretical loadings, representing the maximum amount of potential drug loaded into the materials.

ZA-loaded PLGA films were freshly prepared, lyophilized and dissolved in DCM to 100 mg/mL in 1.5 mL microcentrifuge tubes. The films which had been already used in release studies were also tested, although they were dissolved in DCM only following the end of the release study. The films were all rotated for 12 or more hours until no visible large particles were noticeable. During testing of freshly prepared ZA-loaded films, 75:25 PLGA crystals were dissolved at 100 mg/mL in DCM to serve as a blank and control. During encapsulation testing of the release study films, the unloaded blank PLGA films were used as a control, and dissolved under the same circumstances as the loaded films. Following rotation and visual confirmation of film dissolution, 100 µL of PLGA-in-DCM was pipetted into a new microcentrifuge tube, and 900 µL DI water at pH 10 was then added to prepare a 1:10 dilution. The mixture was vortexed in the S/P Vortex Mixer for 1 min at setting 9, and then rotated for 6 hours to facilitate dissolution of ZA into DI water. Periodically during the mixing process, the tube was removed, vortexed, and returned to the rotary mixer. The tube was centrifuged for 3 min at 5000 rpm to separate the DCM from the DI water. 100 µL of the DI water was pipetted out into a microcentrifuge tube and further diluted 1:10 to decease ZA concentration and keep it in line with the standards. The ZA stock for standards was prepared and stored in the same manner as for release study tests. After titrating stock to pH 7.4, the ZA standards were prepared at 0, 10, 20, 50, 100, and 300 µg/mL into fresh PBS. ZA content was evaluated via HPLC using the acetonitrile/formic acid gradient described earlier.

Antibody-loaded PLGA films, and their respective blank films, were dissolved in dimethyl sulfoxide (DMSO) to 100 mg/mL in 15 mL centrifuge tubes. They were shaken for 12 hours until no

large particles were visible. Subsequently 30 µL were withdrawn and pipetted into a microcentrifuge tube. 970 µL DI water was added to the tube, leaving a polymer precipitate and a fluid of 3% DMSO and 97% water by volume. The precipitate was agitated with a spatula and the mixture was briefly vortexed. The mixture was centrifuged at 5000 rpm for 5 min. 150 µL was removed from each tube and tested for protein content in a MicroBCA assay as before, using blank films as controls. Well plate standard columns of Mab 626 in PBS were prepared in the same manner as for release testing.

3.7. Bioactivity of Zoledronic Acid

An in vitro test was performed to compare effects of ZA released from PMMA and PLGA samples to those of freshly prepared ZA stocks. In accordance with the bioactivity tests performed by Zwolak et al, an MTT test was selected.[59] The cells tested were RAW 264.7 mouse macrophages (ATCC TIB 71). Cells were grown in a 37^{o}C incubator with a 5% CO_2 internal atmosphere. Medium used was Dulbecco's Modified Eagle Medium (DMEM; Thermo Fisher) with 10 volume percent (v/v%) fetal bovine serum (Thermo Fisher). The cells were grown in 48-well plates at an original density of 5000 cells per well in 144 µL media. After allowing 6 hours of incubation for the cells to settle, 16 µL of the ZA standards and supernatants were added to their respective wells. There were 3 replicates for each standard, each blank sample supernatant, and ZA-loaded sample supernatant. The plates were then incubated for 48 hours.

The negative controls were wells with DMEM but no cells, treated with 16 µL sterile PBS. The standards were cell-seeded wells treated with 0, 6.25, 12.5, 25, 50, and 100 µg/mL ZA. There were 3 different time points tested from both the PMMA and PLGA release studies, making 6 time points total. 6 wells were tested for each time point, 3 with supernatants from the ZA-loaded samples and 3 with supernatants from the blanks. This was done in order to allow 1 well each for the 3 replicate supernatants per time point, plus 1 well each for the 3 blank replicate supernatants per time point.

Supernatant samples from PMMA and PLGA alike were thawed on a plate shaker for 24 hours. PH was adjusted for all samples with NaOH to between 7.2 and 7.5. The samples were then filtered through sterile 0.22 µm syringe filters and into sterile microcentrifuge tubes. They were then centrifuged at 10,000 rpm for 10 minutes prior to addition to media, to remove particulate matter.

After 48 hours incubation, methylthiazolyldiphenyl-tetrazolium bromide or MTT (Sigma-Aldrich, St Louis, MO) was dissolved in DI water at 5 mg/mL and rotated in a centrifuge tube covered with foil to prepare the stock. Under a sterile hood, 38 µL were pipetted into each well of the cell culture plate. Following 2 hours incubation at 37^{o}C, the wells were exposed to 160 µL lysis buffer. The buffer consisted of equal volumes of N,N dimethyl formamide (Sigma) and DI water. Sodium dodecyl sulfate (Thermo Fisher) was dissolved in the solution at 200 µg/mL. After overnight incubation, 150 µL were pipetted out of each well and placed into a corresponding well in a 96-well plate. The 96-well plate was inserted into a plate reader and tested for absorbance at 570 nm.

Percent activity of a sample was generated by comparing the viability of RAW 264.7 cells incubated in DMEM with 3 different categories of diluent, all at 10 v/v%: blank sample PBS supernatants from multiple time points, ZA-loaded sample PBS supernatants from those same time points, and ZA in PBS standards. Formazan absorbance, from wells incubated with the ZA standards, was plotted on a standard curve and characterized via a regression fit. Formazan absorbances from samples were calculated into concentrations using that function. Wells incubated with supernatants from blank cylinders showed cell viability equal or higher than wells incubated with blank PBS, and so were not factored into calculations. The calculated effective concentrations for each tested time point were divided by the mean HPLC-measured ZA concentration at that time point, and the results were expressed as percentages.

3.8. Bioactivity of Released Mab 626

The procedure for mouse anti-RANKL antibody was to determine effectiveness at preventing osteoclast differentiation of RAW 264.7 cells in medium with RANKL. First, RAW cells were pipetted into 24 well plate at 5000 cells/well, using 200 µL of Minimum Essential Media (Thermo Fisher) with 10% FBS. The cells were incubated for 6 hours at 37^{o}C. Stock solutions of PBS containing human soluble RANK ligand (Peprotech, Rocky Hill, NJ) and Mab626 were thawed, diluted, and added to their respective wells at a combined 50 µL or 20 v/v% to produce standards. There were 3 replicates for each standard, and the well plates were further incubated for 5 days prior to testing. The negative controls were wells with Minimum Essential Media but no cells, diluted with 50 µL sterile PBS. The standards were wells containing 0 ng/mL RANKL with 0 µg/mL Mab626, 50 ng/mL RANKL with 0 µg/mL Mab626, and 50 ng/mL RANKL with 7.5 µg/mL Mab626.

After 5 days, the wells were tested for tartrate-resistant acid phosphatase (TRAP) content, in an adaptation of an assay used by Suzuki et al.[91] The media was pipetted out of the wells, and the wells were rinsed once with sterile PBS. The PBS was replaced with 250 µL of 8.2 mg/mL sodium acetate buffer with 0.2 v/v% Triton X-100 at pH 4.5. The wells were placed on a plate shaker for 20 minutes. All wells were then pulse sonicated on an ice bath at 45% amplitude and 20 kHz frequency for 15 seconds, then left to sit and cool for 60 seconds, and then sonicated for an additional 15 seconds. Lysates were pipetted into 500 µL microcentrifuge tubes and centrifuged at 10,000 rpm for 5 minutes. 50 µL of cell lysate from each well was pipetted into 130 µL TRAP assay buffer in a 96 well plate. The TRAP assay buffer used 8.2 mg/mL sodium acetate, 0.9 mg/mL ascorbic acid (Cayman Chemical, Ann Arbor, MI), 2.2 mg/mL sodium tartrate (Sigma), and 2.1 mg/mL para-nitrophenylphosphate (pNPP; Thermo Fisher) in DI water at pH 4.5. Absorbance at 400 nm wavelength was measured in the spectrophotometer after 4 hours incubation at 37^{o}C.

Chapter 4: Results

4.1. Effects of Drug Loading on Mechanical Properties of PMMA samples

Figures 4.1 and 4.2 show the mechanical testing data for ZA in PMMA at 0%, 1.3%, 2.6%, 6.6%, and 12.7% w/w loading. Values of ultimate strength were obtained from the highest measured compressive load on each sample prior to failure. Values of compressive modulus for each sample were measured from the slope of the linear elastic region of the stress-strain curve, prior to failure, with a cutoff of $R^2 = 0.990$ for linearity. A Grubbs outlier test was performed on samples that appeared to be potential outliers. However, no sample was discarded from these tests.

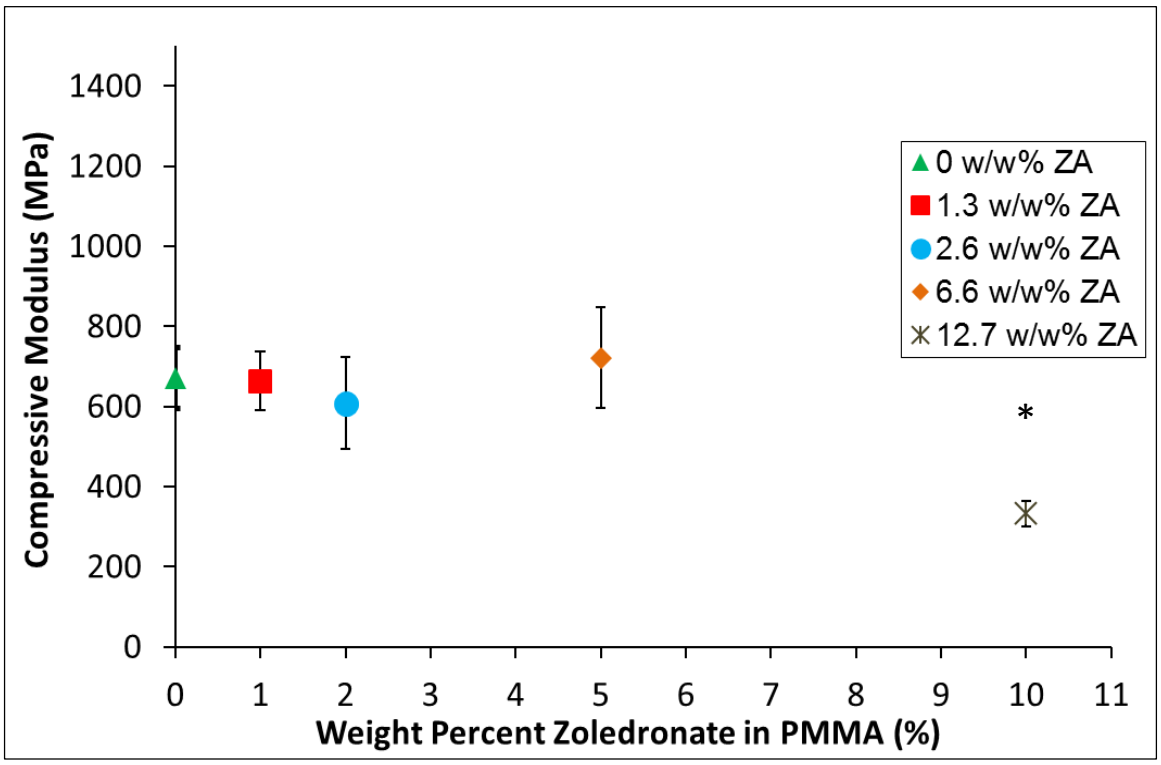

Figure 4.1: PMMA ultimate compressive strength vs. w/w% zoledronic acid (Data are mean ± SEM, n=5: '*' signifies statistically significant ($p<0.05$) difference from 0%).

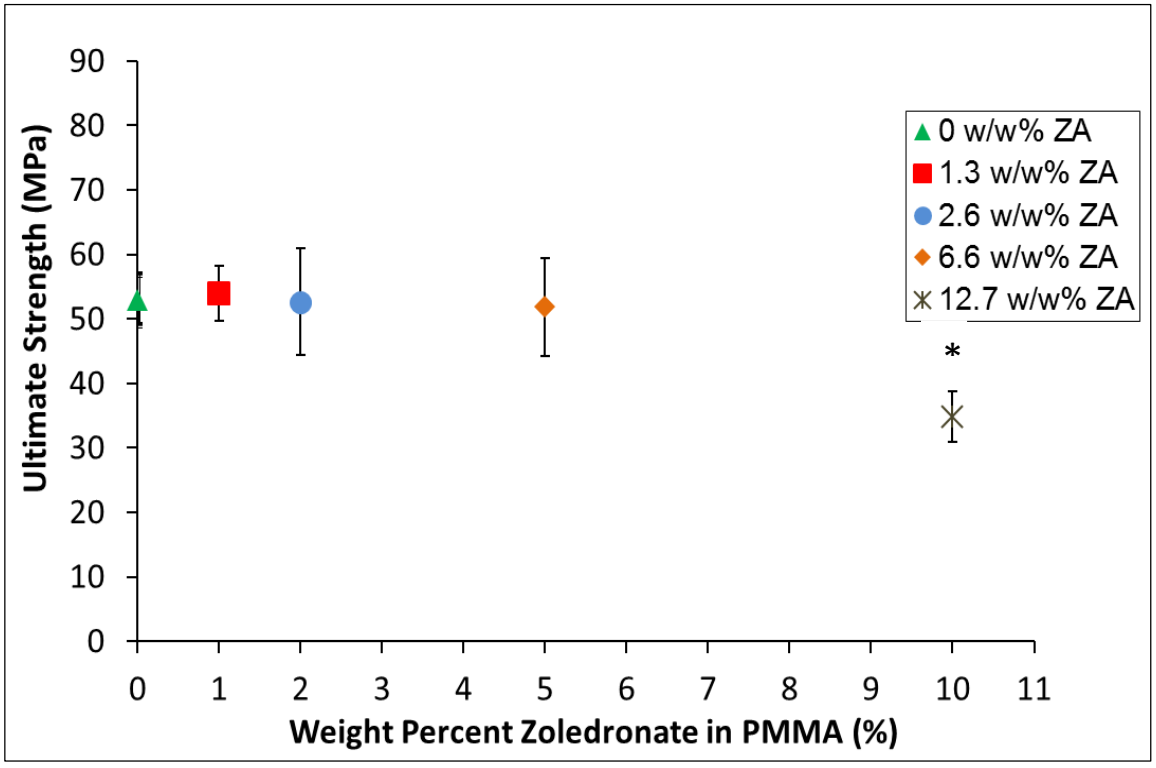

Figure 4.2: PMMA compressive modulus vs. w/w% zoledronic acid (Data are mean ± SEM, n=5: '*' signifies statistically significant ($p<0.05$) difference from 0%).

The mean compressive modulus of unloaded cylinders was about 671.0 MPa, with a standard deviation of 183.2 MPa. The ultimate strength was 52.9 MPa with a standard deviation of 9.6 MPa. For 12.7% ZA cylinders, the mean compressive modulus was 333.1 MPa, or roughly half the modulus of unloaded cylinders, and the standard deviation was 71.6 MPa. The 12.7% cylinder ultimate strength was 34.9 MPa with a standard deviation of 8.8 MPa. A one-way ANOVA showed a statistically significant ($p<0.05$) change in mean compressive modulus and ultimate strength with increased drug loading. When samples of each weight percent loading were compared in a post-test against the 0% ZA controls, the 12.7% ZA samples alone showed statistically significant differences in both categories. The ANOVA did not disprove the null hypothesis between 0 and 6.6 w/w% loading, which was the reason for using 6.6% as the maximum loading for release studies. It was only with addition of the 12.7% data set that ANOVA revealed a statistically significant trend.

4.2. Release of ZA from PMMA samples

Figure 4.3 shows the release profiles of the PMMA samples. In all cases the highest ZA release rate was recorded on the first day of incubation, with the 6.6% cylinders releasing on average 1.22 mg, 2.6% cylinders releasing 0.43 mg, and the 1.3% cylinders releasing 0.29 mg. However, the release rate on the second day dropped to less than half that amount for all cylinders. A subsequent downward trend in release rate continued until all cylinders reached a nadir of release on day 21, with 12 μg from the 6.6% cylinders, 0.1 μg from the 2.6% cylinders, and 1 μg from the 1.3% cylinders. This marks the only point when the 2.6% release was lower than the 1.3% release. On day 28 the ZA release increased to 19 μg for 6.6% and 2.6% cylinders, and 6 μg for 1.3% cylinders. The final time point at day 56 marked a decline to 12 μg for 6.6% cylinders, 6 μg for 2.6% cylinders, and 4 μg for 1.3% cylinders.

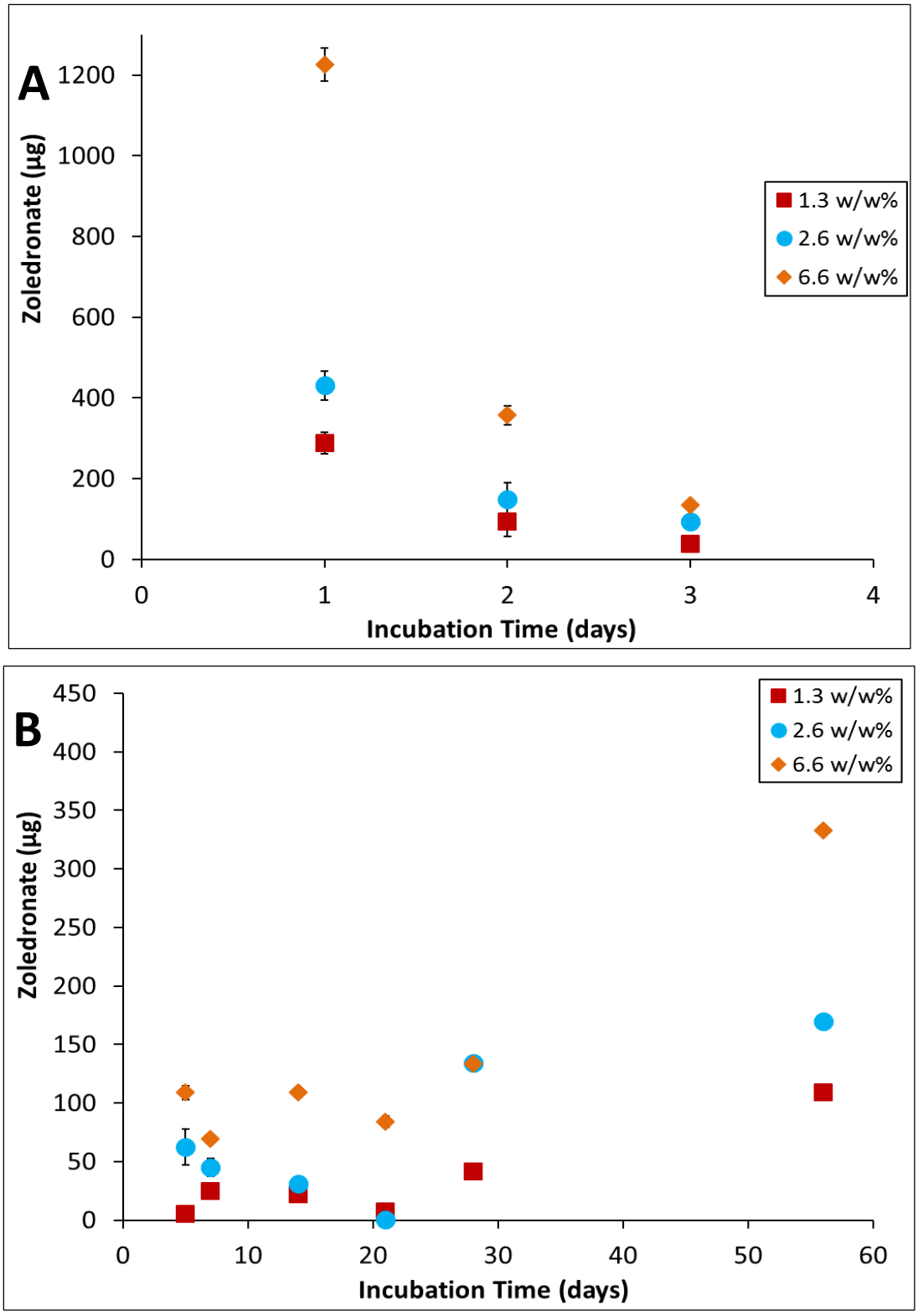

Figure 4.3: ZA release from PMMA vs. incubation time during (A) days 1-3 and (B) days 5-56 (Data are mean ± SEM, n=3).

Figure 4.4 shows the cumulative release from PMMA cylinders of average mass 303.9 mg, demonstrating a burst in week 1 followed by a sustained release for an additional 7 weeks. The amount released varied based on the w/w% loading. The 6.6% cylinders released a total of 2.24 mg over 4 weeks and 0.33 mg over the next 4 weeks. The 2.6% cylinders released a total of 0.96 mg over 4 weeks and 0.16 mg over the next 4 weeks. The 1.3% cylinders released a total of 0.55 mg the first 4 weeks and 0.11 mg over the next 4 weeks. At the day 1, 28 and 56 day points, ANOVA showed a statistically significant increase in total ZA release with increased w/w% loading ($p<0.05$). A significant linear trend ($p<0.05$) between released concentration and weight percent loading was observed for all 3 time points.

Assuming even powder distribution within the polymerized bone cement, each cylinder should have contained approximately 1, 2, or 5% of its total weight as ZA. The ZA release efficiency, defined as the percentage of loaded drug mass released over 8 weeks incubation, decreased as w/w% drug loading increased. The 1.3% cylinders released about 21.6% of loaded ZA, the 2.6% cylinders released 18.7%, and the 6.6% cylinders released 16.9%.

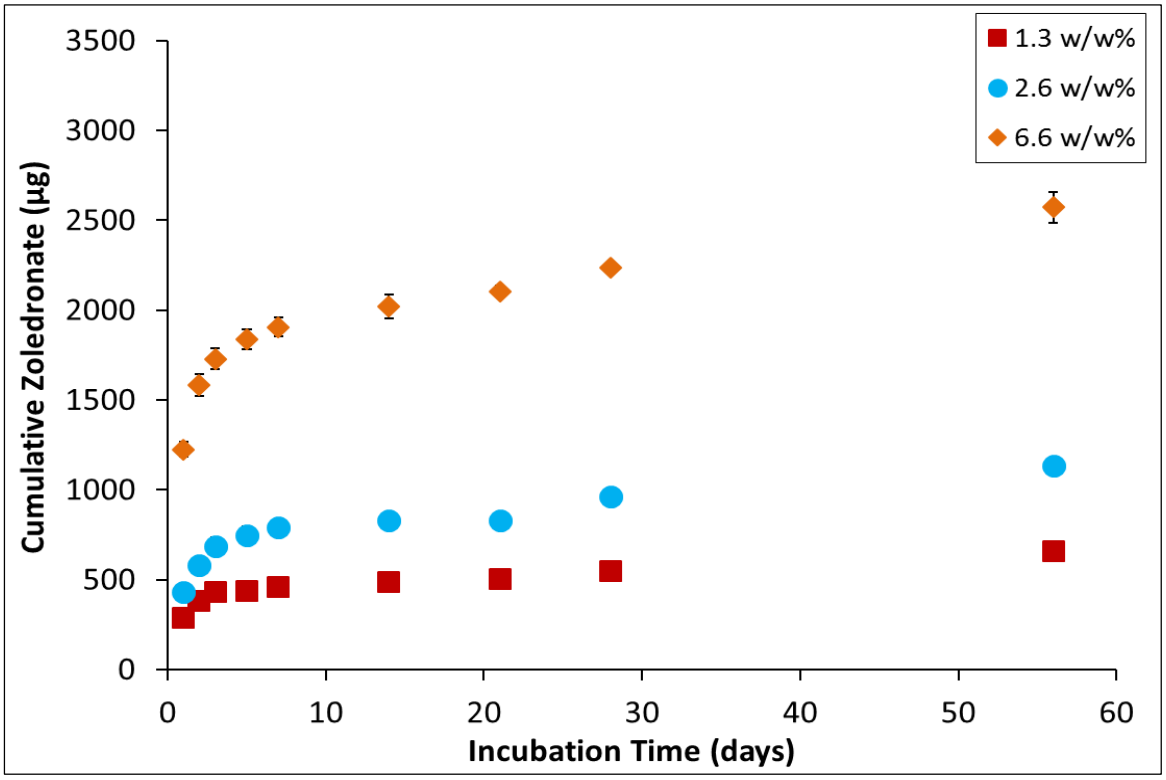

Figure 4.4: Cumulative release of ZA from PMMA vs. incubation time (Data are mean ± SEM, n=3).

4.3. Release of ZA from PLGA Films

The results of encapsulation efficiency tests on ZA-loaded PLGA films varied among the 4 sample films, one of which had been subdivided and used for the release study prior to testing. The mean encapsulation efficiency was 69.5%, while the standard deviation was 33.4% (data not shown). Mean ZA-loaded film mass was 293.7 mg, with a standard deviation of 45.7 mg. Compared to the theoretical maximum film mass, defined as PLGA mass plus mass of drug added, this meant the yield was 80.8%. For release studies, mean ZA loaded film mass was 94.3 mg initially, with a standard deviation of 7.8 mg. Final analysis revealed that the initial loading of ZA in the film was 3.75 w/w%. The films' mass loss over 8 weeks was 11.7 mg on average, dropping final film mass to 82.6 mg. Blank control film mass was 111.7 mg initially, with a standard deviation of 8.1 mg. The mass loss for blank films was 10.5 mg over 8 weeks, dropping the average blank film mass to 98.2 mg.

Figure 4.5 shows the release profile of ZA after averaging results from the three PLGA films. The drug mass released on the first day was 0.80 mg per film. By the second day it was down to 0.41 mg, approximately half that of the previous day. The release rate continued to drop down to a nadir of 4.1 μg/day, recorded on day 21. The day 28 release rate was a comparable 5 μg/day. However, on day 35, the ZA release had increased to 125.1 μg/day, which was roughly 2500% of the rate of the previous week and 16% of the release rate during the first day. The next three weeks saw declining releases of 36, 31.4 and finally 22.3 μg/day ZA.

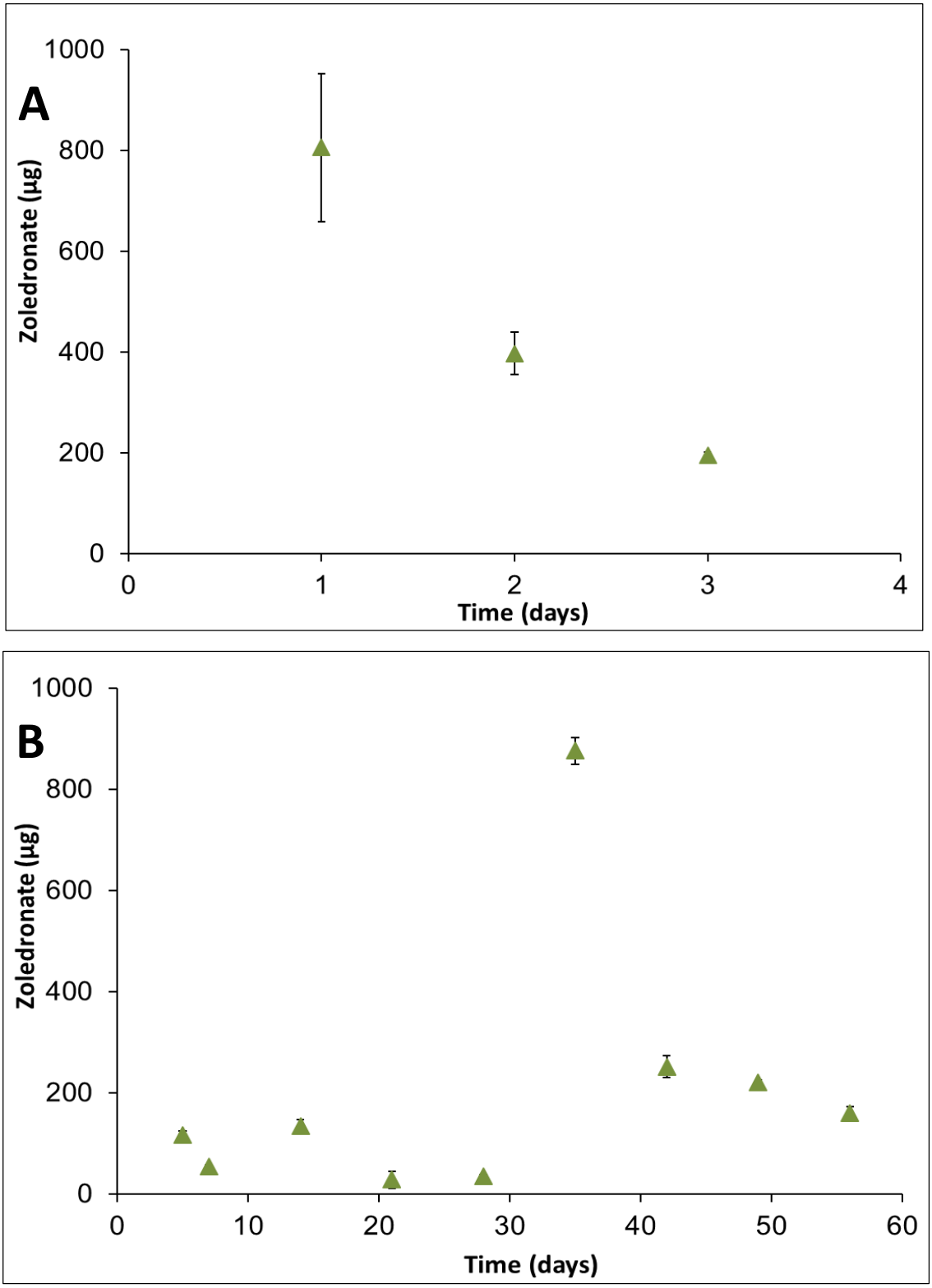

Figure 4.5: ZA release from PLGA thin film during (A) days 1-3 and (B) days 5-56 (Data are mean ± SEM, n=3).

Figure 4.6 shows the cumulative plot of ZA release from PLGA films versus incubation time, which was on average 1.94 mg during the first 4 weeks and 1.52 mg during the last 4 weeks. The ZA release efficiency over 8 weeks totaled 96.6% of the loaded drug, with standard deviation 6.2%.

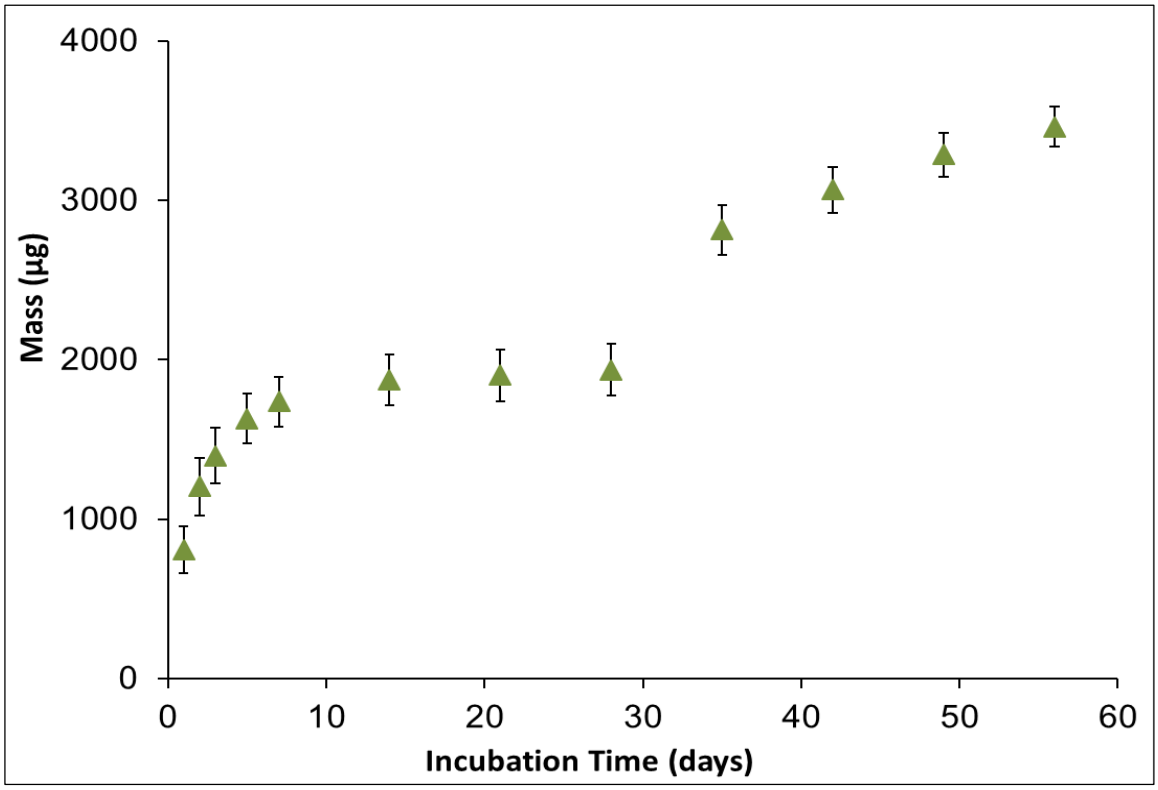

Figure 4.6: Cumulative ZA release from PLGA thin films (Data are mean ± SEM, n=3).

4.4. Release of RANKL-inhibitory Mab 626 from PLGA Films

Initial Mab 626 film mass was 256.9 mg on average, with a standard deviation of 14.3 mg. As a percentage of total theoretical Mab-loaded film mass, this gave a 73.3% yield. The mass loss over the period of incubation was 49.6 mg, leading to an average final Mab film mass of 207.3 mg. Comparatively the blank film mass was 265 mg initially, with a standard deviation of 6.9 mg. Mass loss for the blank film was 18.2 mg, leading to an average final blank film mass of 246.8 mg. The release profile of Mab 626 from PLGA films is shown in Figure 4.7.

Figure 4.8 shows cumulative antibody release over the release study. The average mass released over the first 4 weeks was 41.5 μg, and over the next 4 weeks it was 28.3 μg. The mean total release was 67.9 μg, with a standard deviation of 28.6 μg. This meant that 24.9% of the total added antibody mass was released, on average.

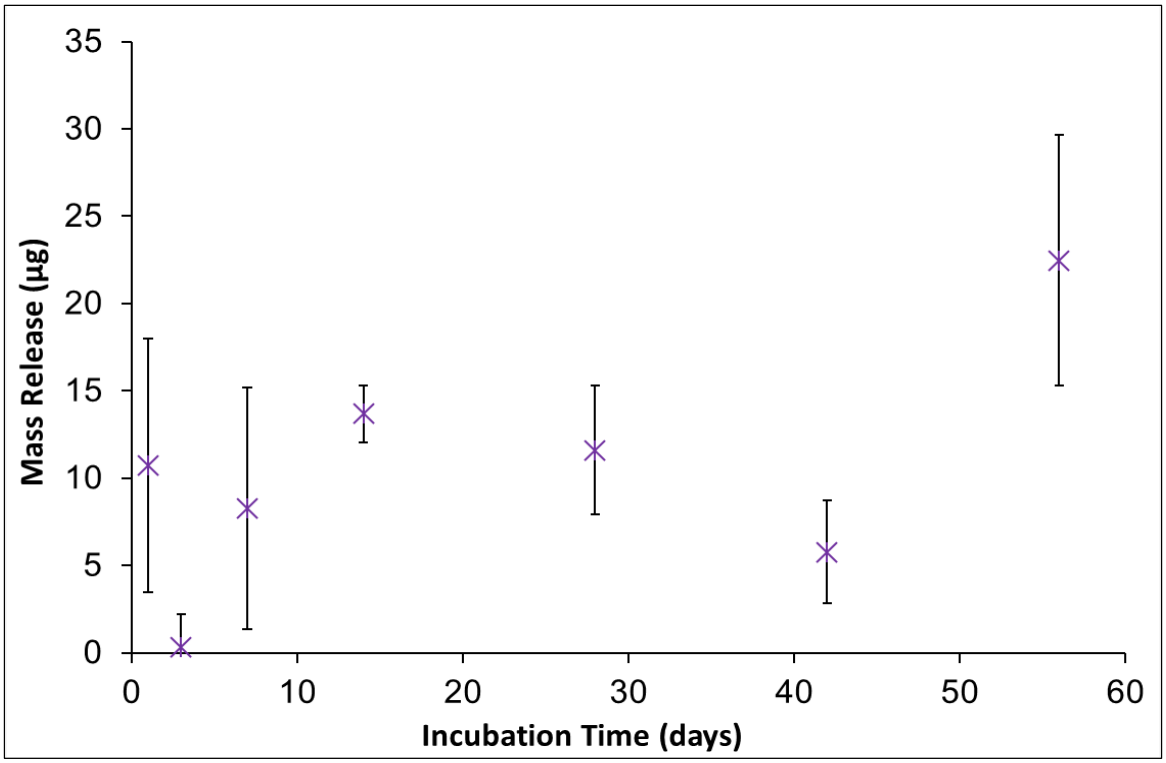

Figure 4.7: Antibody release from PLGA thin films (Data are mean ± SEM, n=3).

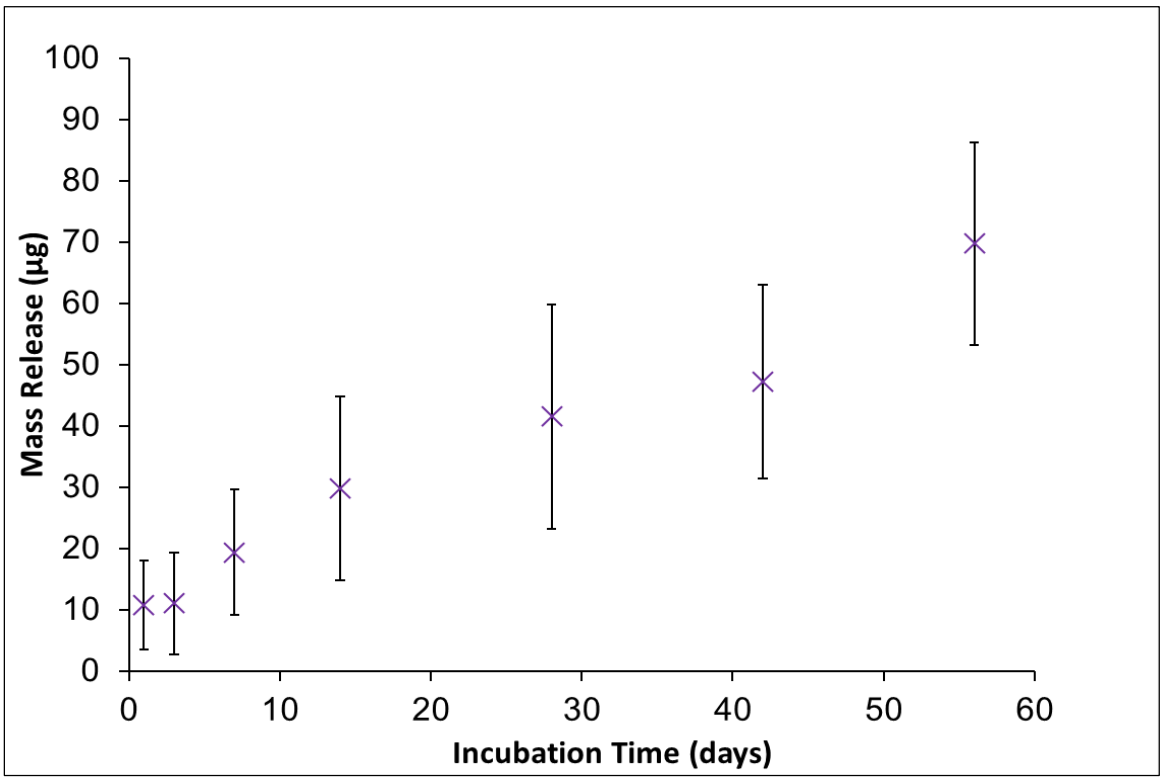

Figure 4.8: Cumulative antibody release from PLGA thin films (Data are mean ± SEM, n=3).

According to encapsulation tests performed on the films following 8 weeks of incubation, there was an average of about 112 μg of antibody remaining in the films. The retained antibody mass was 62% higher than the released antibody mass. More specifically, two of the films had 73.4 and 75.7 μg antibody remaining, while the third had 188.0 μg. Encapsulation efficiency, namely the antibody mass released plus the mass extracted from the film afterwards, averaged 56.5% of the total antibody mass originally added to the inner water phase. Standard error of the mean was 9.6% (data not shown).

4.5. Bioactivity of Zoledronic Acid

According to MTT assays, the activity of released ZA from PLGA did significantly decrease from the start to the end of the release study, whereas for PMMA the activity was retained. In the case of PMMA cylinder supernatants, Figure 4.9 below shows the percent activity of supernatants sampled from day 1, day 28, and day 56 of the 1 w/w% ZA cylinders from the release study. Notably, the cytotoxicity on day 28 was equal to 240%, which was significantly ($p<0.05$) higher than expected. The mean activity dropped to 83% by day 56, although comparing percent activities between the 3 replicates of the measured concentrations versus the 3 replicates of the calculated concentrations showed no significant difference.

For PLGA films, Figure 4.10 shows the percent activity of supernatants sampled after day 2, day 14, and day 49. Wells incubated with blank film supernatants showed viability equal or higher than those incubated with blank PBS, and again were not factored into calculations. In this case there was a statistically significantly higher percent activity during day 14, but a statistically significantly lower percent activity by day 49 ($p<0.05$). ZA activity from day 14 was 140%, while the activity from day 49 was 52%.

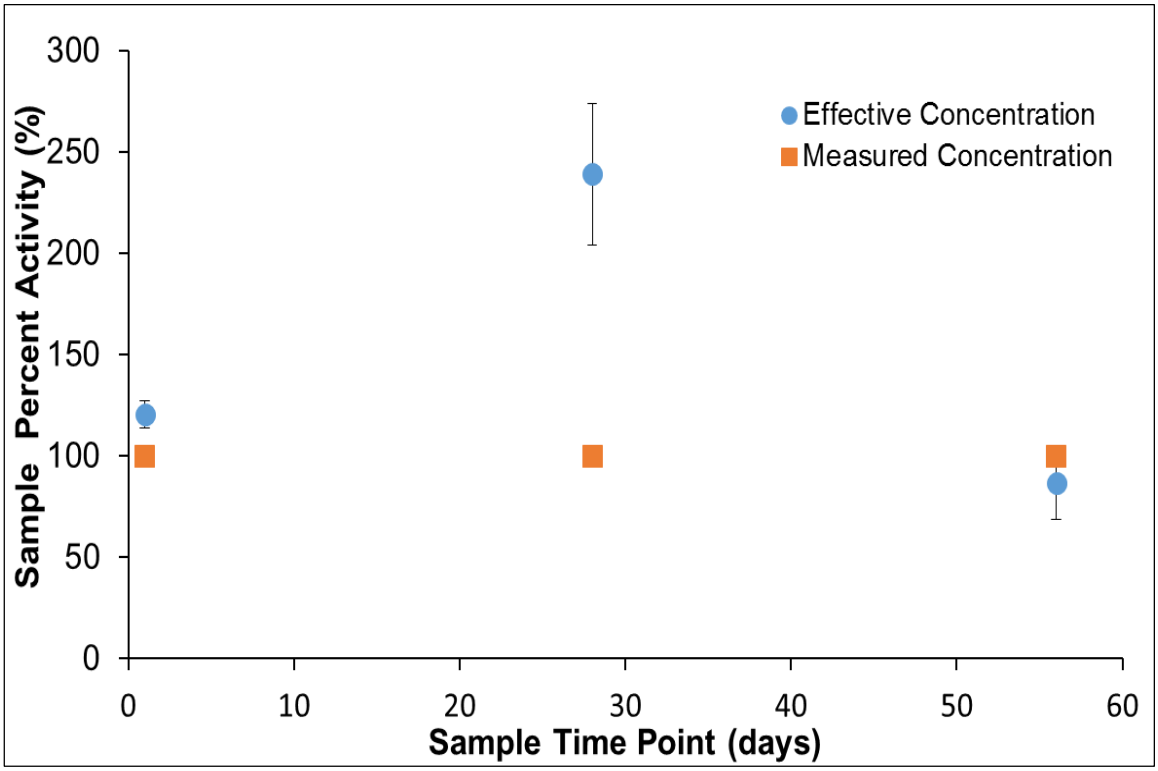

Figure 4.9: Percent activity of ZA in collected PMMA supernatant (Data are mean ± SEM, n=3).

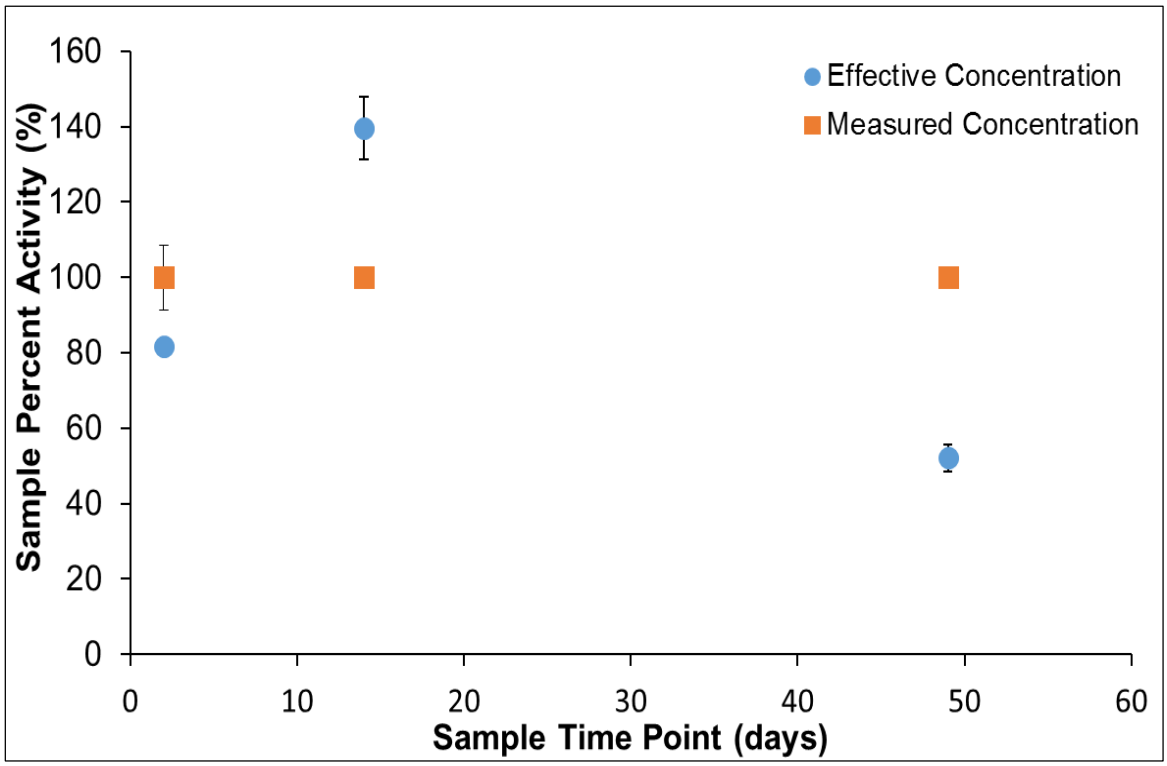

Figure 4.10: Percent activity of ZA in collected PLGA supernatant (Data are mean ± SEM, n=3).

4.6. Bioactivity of Released Mab626

Standards of Mab626, which approximated the obtainable incubated Mab626 concentrations from the release samples, were used for tests in cell studies. These standards did not cause significant effects in osteoclastogenesis. The highest average concentration of released antibody from any time point was 4.2 μg/mL. If samples at that time point were pooled together and concentrated 20x, the resulting concentrate would have 84 μg/mL antibody, which when diluted 1:10 into cell media would have meant an incubated concentration of 8.4 μg/mL. If the encapsulation and incubation were to leave the Mab626 with less than 100% activity, then the Mab626 effects on RAW 264.7 cells would be equivalent to that of an even lower concentration. In addition, supernatants from other time points would have been even lower in concentration. Figure 4.11 shows a standard curve of a TRAP assay, comparing wells without RANKL to wells with 50 ng/mL RANKL. After subtracting blank absorbance, the p-nitrophenol absorbance of wells with 50 ng/mL RANKL and no Mab626 was 0.416 absorbance units (AU), whereas for wells with the same RANKL concentration with 7.5 μg/mL Mab626, absorbance was 0.424 AU. Comparing the three replicates of both sets, there was no statistically significant difference.

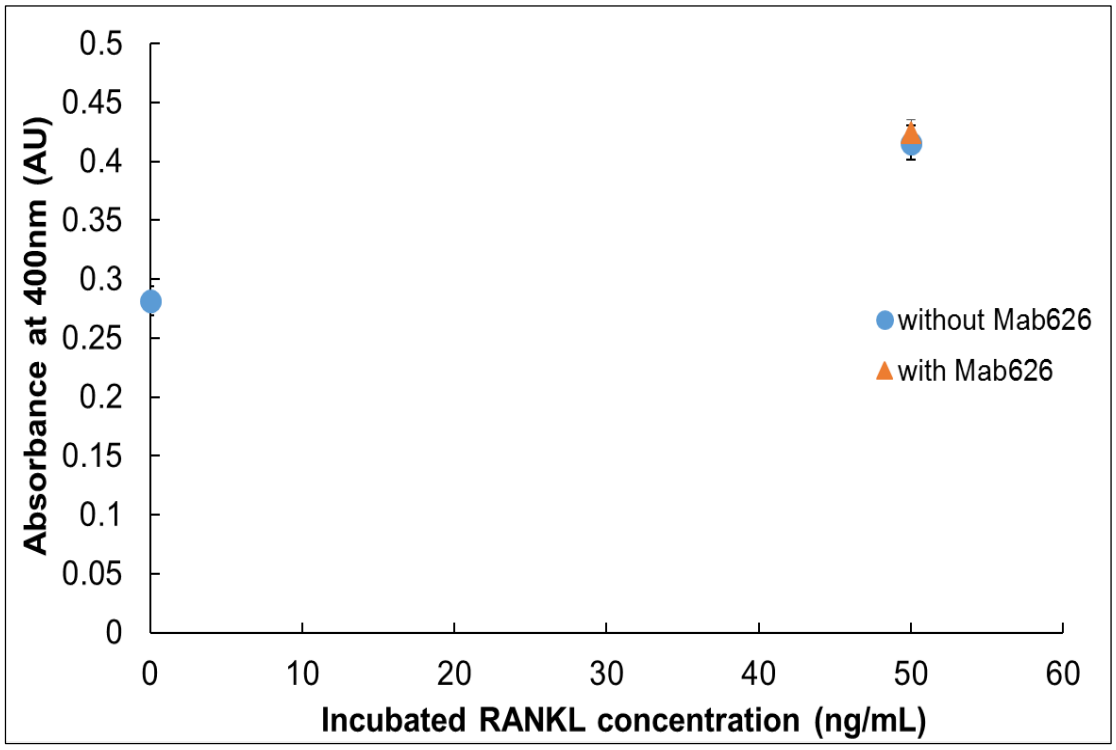

Figure 4.11: TRAP assay standard curve (Data are mean ± SEM, n=3).

Chapter 5: Discussion

5.1. Mechanical Effects of ZA Loading of PMMA

The ZA-loaded PMMA was tested for variations in ultimate compressive strength and compressive modulus, in order to record the point at which loading caused a decrease in these properties. The ASTM standard F451 recommends for commercial grade acrylic bone cements a 70 MPa ultimate strength, whereas the blank cylinders in this test were found to be 56.3 MPa on average.[61] The ISO 5833 recommendation for compressive modulus is 1800 MPa,[92] whereas the blank cylinders in this test were found to be 671 MPa.

ZA weight loadings in this experiment reached levels higher than other ZA loadings from literature, and the degree of drug loading without mechanical detriment was an important discovery. Calvo-Fernandez et al. saw roughly equal 99 MPa compressive ultimate strengths from their self-produced bone cement with and without 1.5% alendronate. Their compressive moduli only varied by 0.1 GPa between loaded and unloaded PMMA, which was not statistically significant.[62] Yu et al. loaded a bone cement packet with 0.004 w/w% ZA, and saw no significant difference in compressive or bending properties compared to the control, in spite of the ZA being added in 500 µL of aqueous solution. However, at 0.004 w/w% pamidronate in 500 µL solution, there was a statistically significant 5 MPa decrease in mean ultimate compressive strength versus the control, while the decrease jumped to 11 MPa at 0.04 w/w% pamidronate.[53] Matuszewski et al. loaded 0.15% pamidronate into PMMA and found a non-significant decrease in mean ultimate strength from 95 to 92.6 MPa, and a non-significant decrease in compressive modulus from 1.24 to 1.23 GPa.[63] Lewis et al. loaded 0.42% alendronate into PMMA and saw no significant difference in the number of 15 MPa amplitude fatigue cycles until compressive fracture.[64] Healey et al. loaded up to 5 w/w% pamidronate powder into PMMA and saw no statistically significant decreases in static

compressive properties. In fact, even after 6 months of incubation in an aqueous environment, loaded samples retained 87% of tensile and compressive yield strength.[53]

The ASTM minimum specification is 70 MPa ultimate compressive strength.[93] According to Kuhn et al, the compressive modulus of high-viscosity Aap C~Ment 1 is 89.3MPa, although our study used medium viscosity.[92] While ASTM guidelines were used for the sample sizes and static compression strain rate, the preparation of samples did depart from the recommended procedures, which we believe caused the reduction in overall mechanical strength. The location of mixing during polymerization was on the bottom shelf of a freezer surrounded by a room-temperature environment, where cold airflow from the freezer to the outside could have caused increased cement porosity. Macaulay et al. have suggested that bone cement pores are primarily caused by air trapped during mixing, and that the pores might behave as stress concentrators.[94] The lower temperature could also have played a role. Jasty et al. tested 7 brands of bone cement, finding higher mean porosity in all cases when the monomer was chilled to 0^{o}C compared to 21^{o}C. Three of those cases were statistically significant.[95]

The amount of material used to prepare sample batches was considerably lower than the material in each packet. As a cost-saving measure, the packets were opened and PMMA powder was transferred to a 50 mL plastic centrifuge tube, while the MMA fluid ampoule was broken and the fluid pipetted into a 20 mL glass vial wrapped in aluminum foil. The required masses and volumes were removed from these containers during the making of each batch of samples. This did allow for oxygen in the air to diffuse into the bottle, and oxygen could have played a role in the decreased mechanics. Bhanu et al. have argued that O_2 can react with radical initiators in place of monomer. This can produce peroxide radicals which lead to a number of unwanted species in the reaction, and compromise the polymeric products.[96] Sella et al. have also reported the oxidation of hydroquinone into quinone by O2, albeit in a controlled reaction on copper substrate.[97] If this

oxidation had deactivated a sufficient amount of inhibitor, a percentage of MMA in solution could have combined into oligomers, although no visible signs of polymerization were observed over 1 month of storage. Either of these could have decreased the speed and efficiency of polymerization enough to contribute to lower mechanical properties.

The ultimate compressive strength of the acrylic bone cement was comparable in magnitude to that of adult human cortical bone. Depending on the particular bone, longitudinal ultimate strength is estimated at between 70-280 MPa and transverse ultimate strength at around 50 MPa. However, the PMMA compressive modulus was 0.7 GPa, considerably lower than cortical bone at 11-21 GPa longitudinal and 5-13 MPa transverse.[98]

However, clinical relevance to cancer patients is also a consideration. Cancer patients are unlikely to subject their bones to high loads. Depending on the circumstances of the patient's lifestyle, there may be cases in which the 34.9 MPa ultimate strength and 333.1 MPa modulus of even 12.7% cylinders is sufficient to bear everyday loads without causing skeletal-related events.

5.2. Release of Loaded ZA from PMMA

For the release study, I used PMMA cylinders, with an average mass of about 303.9 mg for unloaded cylinders. This study used a cylindrical shape of the same dimensions as previously used by Zwolak et al.[59] The amount of drug loaded into each cylinder was approximately 15.1 mg for 6.6% ZA cylinders, 6.1 mg for 2.6% cylinders, and 3 mg for 1.3% cylinders. Initial drug mass added significantly affected the amount of drug which was released. However, the volume and mass of bone cement added per patient can vary when sizes of defects and bones themselves vary from patient to patient.

Zwolak et al. loaded less than 0.1 w/w% ZA into PMMA, in 6 mm diameter x 10 mm height cylinders in 5 mL PBS, and their release study lasted only 14 days before dropping from about 4.5

μg/mL drug concentration on the first day to nearly 0 μg/mL at the end. In total over 14 days, less than 50 μg was released out of roughly 754 μg ZA per cylinder, or less than 6.6% of the loaded mass.[59] Matuszewski et al. tested 0.15% pamidronate in PMMA, but actually released twice as much in weeks 4-6 than in weeks 1-3. That team used cylinders of 10 mm diameter and 20 mm height, or more than 8x the volume and 4x the surface area of the cylinders from this study, in 15 mL saline. Each cylinder released about 1.05 mg up to week 3 and then 2.7 mg over the following 3 weeks.[58] For roughly 6 mg loaded per cylinder, this meant that 63% of loaded drug was released. Yu et al. loaded about 0.004% ZA and ran a saline release study of 6 mm diameter x 12 mm height cylinders for 6 weeks, releasing a total of only about 131 ng ZA or 0.9% of the loaded drug.[53]

Since PMMA is a non-degrading material, drug was released either from the surface or from pores connected to the surface. Indeed, a study by Duey et al. found that for PMMA discs, loaded with vancomycin and tobramycin to a total 5% w/w, there was a positive linear relationship between surface area and drug release over 1 week.[61] A study by Baker et al. showed that PMMA loaded in vivo with 1.5 g of gentamicin released a significantly higher percentage of antibiotic than PMMA loaded with only 0.5 g. They found that this was due to an increased number of voids and cracks in the structure formed by the impurities, specifically the molecules of gentamicin.[99] The cylinders in the present study released between roughly 17-22% of their loaded drug over 8 weeks, but unlike the Baker team, the percentage of released drug decreased with loading.

ZA bioavailability to the total skeleton is approximately 60% from IV injections.[31] In contrast, the PMMA samples released less than a quarter of the dose loaded. In addition, the amount released from the cylinders from weeks 4-8 was less than 20% of the amount released between weeks 1-4, across all weight percent loadings. ZA is taken up into the skeleton from the blood, but must still be injected every 4 weeks to replenish skeletal ZA concentrations.[30] Therefore, it may be that drug locally released over the first 4 weeks would also slowly leave the

skeleton and exit the body via urine. In such an event, the drug released in the first 4 weeks may not carry over or provide benefits into the subsequent weeks. If the pattern of releasing a 5x higher drug mass over weeks 1-4 than weeks 4-8 were to continue in vivo, then in order to replace 2 IV Zometa doses instead of just 1, PMMA would need to elute 5x the required necessary dose over the first month. That could be a toxic dosage and harm healthy cells. However, the drug elution profile from PMMA differs from the delivery method of Zometa, because for the latter, the entire 4 mg dose would be infused over 15 minutes and taken up within hours.[30],[31] This slower release of ZA from PMMA might lead to ZA exiting the skeleton more slowly than it would from an injected dose. This is nonetheless one important consideration for why PMMA could be impractical for delivering ZA in the necessary time-dependent manner to reproduce the effects of multiple doses of Zometa.

Local delivery, particularly via implants, might still offer a method to increase bisphosphonate concentration in the necessary sites on the skeleton. McKenzie et al. implanted HA-coated titanium cylinders, with 100 μg immobilized ZA, into the femurs of healthy dogs, and saw high localization of ZA distribution. After 6 weeks, the ZA concentration in the implant femur bone tissue was about 732 ng/g, while testing ZA content in the other femur along with both radii and tibiae showed no more than 7 ng/g in any of them. After 52 weeks, the ZA in the implant femur averaged 377 ng/g, but did not surpass 7.1 ng/g in any other tested bone. Notably, in the jaw, ZA concentration was only 2.5 ng/g. In fact, even along the implant femur, peak ZA concentration in the femur was directly above at a portion of the implant in all cases. Only on locations 4-5 cm from the center of the implant, or less, did the 52-week ZA concentration surpass 100 ng/g. ZA concentration at both ends of the femur was under 10 ng/g.[100] In contrast, when Lin et al. injected IV alendronate at 1 mg/kg into hypercalcemic rats, or roughly 300 μg for a 300 g Sprague-Dawley rat, a study after 6 hours showed there were roughly equal ratios of alendronate to bone tissue (about 4.6 μg/g) in the femur and tibia. In the control rats the values were also similar, at 10.6 μg/g in the tibia

and 9.4 μg/g in the femur.[101] Even when ZA was locally delivered to femoral fracture sites via injection by Amanat et al., they found after a 6-week study that the ZA concentration in healthy femurs had reached 50% of the concentration in fractured femurs, at roughly 500 ng/g compared to 1 μg/g.[102]

Local delivery may also offer improvements on side effects. One cause of this may be a decreased ZA uptake in areas of the body where it is not needed. Kumar et al. showed statistically significantly lower kidney uptake (0.05% dose/gram tissue) of pamidronate from direct application compared to IV or subcutaneous (0.22% dose/gram tissue) dosing after 24 hours in adult BALB/C rats with induced femoral fractures.[103] The direct application method involved mixing pamidronate powder into hydrogel and applying it onto the fracture sites. Intravenous bisphosphonates have also been linked to renal complications, and in fact Zometa injections are not recommended to patients with kidney impairment.[17],[30] Based on evidence from the study by Kumar et al., it might be safer to give bisphosphonates locally in such cases. Such a practice might also allow for giving patients higher dosages than can be safely obtained with Zometa. For example, when Chen et al. studied the effects of different dosages of intravenous ZA on cancer patients, the patents dosed with 16 mg / 4 weeks had to be discontinued from the study after concerns about renal tolerability.[31]

Van Der Wyngaert et al. have suggested that bisphosphonate induced ONJ is caused by bisphosphonates entering the jaw, inhibiting local angiogenesis and slowing rate of jaw bone turnover.[5] When Chen et al. studied the pharmacokinetics of intravenous ZA, they found that blood serum ZA concentrations for the first dose averaged around 264 ng/mL, and they tested immediately after the 15-minute infusion of 4 mg Zometa. At the second dose, the initial serum concentration jumped to about 654 ng/mL. For all three sequential doses, roughly 40% of the injected dose reached the kidneys over the first 24 hours.[31] A study by Adriani et al., of 55 myeloma patients on bisphosphonates who developed bisphosphonate-related ONJ, showed that

82% developed the condition only after 12 months of treatment. They argued that “an important factor for BRONJ is the cumulative dosage of BPs received.”[104] Over many IV doses, ZA molecules could collect in the jaw to reach concentrations sufficient for an increased risk of ONJ. By decreasing blood concentration of ZA, a local delivery method could also reduce ZA uptake by the jaw and decrease the risk of ONJ, or at least delay it.

The data from the MTT assay shows high ZA activity for all 3 tested time points. The lack of toxic effects from blank PMMA samples would seem to suggest that byproducts of PMMA did not cause a significant difference in cell viability. However, the data from day 28, the only time point with a significant difference between expected activity and measured activity, shows a cytotoxicity level equivalent to 240% of the measured concentration. One possibility was that unreacted monomer caused a cytotoxic effect in conjunction with the released ZA. Dahl et al. showed that above 5 μg/mL concentration, MMA monomer in media showed significant levels of toxicity to endothelial and white blood cells.[105] It could be that PMMA polymerization was hindered by the ZA, causing more monomer to remain present in the loaded cylinders. It could be the voids and cracks created from water diffusion and dissolution of ZA led to deeper PBS penetration into the loaded cylinders’ volume, enabling fluid to carry away more monomer. A mass spectrometry test revealed a negligible concentration of monomer compared to the concentration of salts in all release study supernatants, but the MMA concentration necessary to cause effects on RAW 264.7 cells could have been low enough to avoid detection (data not shown).

Zhu et al. used 0.5 w/w% alendronate in PMMA and saw statistically significant BMD and push-out force improvements for 3.2 mm diameter and 15 mm height cylinders press fit into rat femoral defects compared to unloaded PMMA cylinders. Interestingly, there was no significant difference between 0.5% alendronate in PMMA and subcutaneous 1 mg/kg/week alendronate injections.[57] This was comparable in mg/kg to the prescribed oral dose of alendronate for

osteoporosis in humans (70mg/65kg/week).[106] Zhu et al. estimated their alendronate mass at 1.5 mg per cylinder, and performed the tests after 8 weeks.[57] In comparison, I prepared 6 mm diameter and 10 mm height cylinders at up to 5 w/w% and 15 mg of more potent Zoledronic acid per cylinder. Therefore it is plausible that this loading could lead to in-vivo lesion improvements comparable to systemic ZA dosages, although the Zhu team caused their lesions with titanium rather than solid tumors.

If delivering antibodies such as densoumab, however, acrylic bone cement would be a risky choice for biomaterial. Currently, antibiotics for PMMA implantation are selected partly on the basis of thermal stability, as with gentamicin and tobramycin. Buchholz et al. studied the addition of different antibiotics in PMMA in the 1970's and 1980's, and found that a few including penicillin, erythromycin, bacitracin, and fucidin were ill-suited for the application.[56],[107] Anselmetti et al. showed that bone cement can get as hot as almost 76oC during surgical applications,[50] and a study by Vermeer et al. showed that immunoglobulin G antibodies in PBS begin to denature at 61^{o}C.[108] Therefore an alternate biomaterial system was required for local delivery of denosumab.

5.3. Release of Loaded ZA from PLGA

The studied PLGA films demonstrated a double emulsion encapsulation efficiency of 69.5% and a yield of 80.8% on average. This is superior or at least comparable to values obtained from many other teams attempting to encapsulate bisphosphonates via water/oil/water emulsion, although other encapsulation techniques have yielded similar or superior values. Shmeeda et al. encapsulated ZA in liposomes of PEG and phosphatidylcholine, recording only about 5% encapsulation efficiency with a yield around 50%.[109] Cruz et al. loaded alendronate into an aqueous solution containing a polymer of methacrylic acid (Eudragit S100) and a polymer of hydroxypropyl methylcellulose (Methocel F4M), and spray-dried to generate microspheres. They

reported encapsulation efficiency of 85%.[110] Perugini et al. loaded clodronate into various polymer microspheres via double emulsion. The most successful preparation of 50:50 microspheres used 34 kDa molecular weight PLGA and demonstrated 76.1% yield and 74.9% encapsulation. They also reached 67.3% yield and 70.6% encapsulation efficiency into 75:25 PLGA at 68 kDa weight. For PDLLA, the yield was 78.2% and the encapsulation efficiency was 59.9%. Adding carboxymethylcellulose into the clodronate water phase and Span 20 into the oil phase of 50:50 PLGA formulations did not improve efficiency or yield.[80] Sharma et al. also loaded clodronate into 50:50 PLGA microspheres using double emulsion, reporting 51% encapsulation efficiency but no numbers for yield.[81] Samdancioglu et al. reported 7.3% encapsulation efficiency and 46% yield for 50:50 PLGA microspheres. At neutral pH single emulsion, encapsulation rose to 7.7% and yield to 77%. When they loaded alendronate into chitosan microspheres instead, the numbers were 3.3% encapsulation efficiency and 70% yield.[76] Shi et al. did not report yield for loading alendronate into 50:50 PLGA, but their double emulsion method gave an encapsulation efficiency of 7.1%. However, the efficiency rose to as high as 36.1% when the team added HA before emulsion. In fact, the Shi team's solid/oil single emulsion method achieved encapsluation efficiencies as high as 92.5% for alendronate in PLGA/HA.[77] Nafea et al. also noticed improvement in their formulations upon changing their emulsion method. That team reported 46.7% yield and only 0.2% encapsulation efficiency from loading alendronate into 50:50 PLGA microspheres via double emulsion. However, when they prepared microspheres via water/oil/oil emulsion, with a first oil phase of 50:50 DCM to ACN and a second oil phase of paraffin, yield rose to 84% and encapsulation efficiency to 86.1%.[79]

The films for this release study were loaded at 3.75 w/w% ZA, releasing approximately 60% of their loaded ZA within the first 4 weeks of aqueous incubation. 45% of the loaded mass was released during the first 5 days and 22% over the first day. These percentages are lower than what bisphosphonates typically display when they are delivered from encapsulated systems, which could

be beneficial as it would mean a prolonged release. Cruz et al. released nearly 100% of alendronate within 6 hours from their spray-dried microspheres.[110] Nafea et al. released over 60% of loaded drug from their water/oil/oil microspheres over the first 5 days. By 14 days, the entirety of loaded drug had eluted.[79] Sharma et al. released over 90% of loaded drug within 5 days, and all of it within 6 days.[81] Perugini et al. saw both their 75:25 and 50:50 PLGA microspheres release nearly 100% of loaded clodronate in just 48 hours, with over 50% eluting within the 24 hours. Their only exceptions were the 50:50 microspheres prepared with either CMC or Span 20, which released around 40% of loaded drug over the first 5 days. After 4 weeks, all of those microsphere batches had released at least 60-70%. The microspheres incorporating CMC had released over 95% after 7 weeks, and the ones with Span 20 had released 80-90% after 7 weeks and 95% after 9 weeks. The Perugini team's PDLLA microspheres had released about 45% after 5 days and nearly 100% after 3 weeks.[80] Samdancioglu et al. saw their chitosan microspheres release over 50% within 1 day and 80% within 3 days of incubation in sodium citrate solution. The PLGA microspheres released around 10% on the first day and 55% within 5 days, after which the release study terminated.[76] Shi et al. saw that for PLGA microspheres with 50% HA content, those prepared by double emulsion released 30% by 5 days and about 70% within 4 weeks. However, single emulsion microspheres released only 20% within the first 5 days and 60% within 4 weeks. For microspheres with 30% HA, 30% of ZA was released over 5 days and over 75% was released within 4 weeks for both single and double emulsion.[77]

Other teams tested thin coatings and films with bisphosphonate. Long et al. loaded fine ground alendronate powder at 5 w/w% directly into 85:15 PLGA films 10mg in mass and 0.1 mm in thickness. They observed 30% release within 5 days and about 45% release within 4 weeks. [78] Back et al. tested release from a coating of 2 w/w% ZA in PDLLA on titanium implants, and found over 85% released in the first day.[111] Gao et al. tested the release of ZA, pamidronate and ibandronate after

immobilization onto a 50 μm thick HA coating on a titanium implant. The team found around 50% of drug was released in the first day, and over 90% released within 2 weeks for all bisphosphonates.[112]

From the PMMA samples, the maximum amount of drug released over 8 weeks was less than 26% of what was loaded. However, for PLGA films, the amount of drug released over 8 weeks was 96.6% of the amount loaded, leaving a scant mass of drug unused. In the case of the PLGA films, the total amount of wasted drug also depends on parameters such as percent yield and encapsulation. The films yielded 80.8% of the total theoretical mass, which means that with 69.5% drug encapsulation on average and 96.6% release, the total percentage of loaded drug emerging from the films over 2 months could still average 54.2%. If delivered locally, this would compare favorably to the roughly 60% bioavailability to the total skeleton from systemic ZA injections.

In addition, PLGA would serve as a more effective vehicle than PMMA for delivering multiple ZA doses locally. The amount of drug released from the films between weeks 1-4 was only about 28% higher than the amount released from weeks 4-8. This consistency improves on the problem with PMMA, in which drug is released predominantly in one large burst. The difference between the first month dose and second month dose would shrink significantly with a PLGA system.

Modulus and strength testing were not performed on these films, because they were not intended to attach to the skeleton and serve as structural supports. However, films did remain intact after 8 weeks of agitation in PBS, losing only 11.7 mg or 12.4% of their original mass. Blank films lost even less. The durability of this 75:25 PLGA structure in PBS compares favorably to PLGA microsphere suspensions as well as films produced from other materials. Kranz et al. tested 50:50 PLGA films prepared by solvent casting, of 100 mg mass and 0.1 mm thickness. They found that those films had lost more than 40% of their initial mass within 28 days of aqueous incubation.[69]

Clark et al. tested 50:50 PLGA scaffolds prepared by fusing microspheres at 49oC and hollowing the structure using NaCl as a porogen. The scaffold mass was 41 mg, with dimensions of 2.4 mm thickness and 6 mm diameter, and it took 120 days to degrade.[83] The 75:25 PLGA films for this relese study had a triangular shape but with similar dimensions to Clark et al. around 7 mm height and 15 mm base. However, these films' masses were more than double that of Clark et al. The thickness varied at different points in the films but averaged around 0.8 mm, which was only one-third the thickness of Clark et al. but still 8 times higher than Kranz et al.

The activity of the films' supernatants was compared to the measured concentrations of ZA. Considering the variabilities of both measured concentrations and the measured toxicity values, the ZA activity at the day 2 time point was not statistically significantly different from what was expected, even though the mean was only 82%. However, even considering those variabilities, activity at the day 14 time point was a statistically significant 41% higher than expected. Other teams studying percent activity from PLGA-encapsulated systems have occasionally observed activities higher than 100%. For instance, Clark et al. saw 110% activity of IGF after studying release supernatants from 24 days after incubation.[83] The statistically significant 52% activity by day 49 could have been caused by decreased local pH during the linear phase of PLGA degradation, which harmed the ZA activity. PLGA structures have been discovered to have regions of interior autocatalytic hydrolysis, in which fluid with high acid molecule concentration is present.[67] Ding et al. loaded 50:50 microspheres with pH-sensitive dye, and estimated the internal pH of degrading microspheres via imaging, finding a range of pH values between 5 and 2.8 over a 4-week incubation in saline.[113] A previous study of ZA stability by Desphande et al. after exposure to acidic environment found no measurable amounts of any ZA degradation products after 72 hours exposure to 0.1M HCl.[114] However, the duration for which the drug in this study was exposed to low-pH regions extended for multiple weeks, which has not been previously studied in ZA. Furthermore,

Deshpande et al. did not test activity of ZA on cells, but only looked for known degradation products via HPLC.[114]

5.4. Release of Loaded Mab626 from PLGA

The films' encapsulation of Mab626 from the present study were similar to other researchers' results from antibodies, in various biomaterial systems. The studied films saw 56% encapsulation on average, with an average yield of 73.3%. Standard error of the mean for encapsulation efficiency was 9.6%, showing that films can have different loadings even when the same antibody was encapsulated via the same recipe, with the same drug mass added. Son et al. loaded 3D8 scFv anti-DNA Mab into 50:50 PLGA, up to 1 w/w% theoretical loading. They saw that doubly sonicated microspheres showed 7.7% encapsulation efficiency, for a loading of 0.07 w/w%.[89] However, with the same 3D8 scFv antibody also at 1 w/w% theoretical loading, Joung et al. saw 65% encapsulation efficiency with double emulsion. This rose to 89% when mannitol was incorporated into the antibody solution inner water phase.[86] Gdowski et al. saw 16 - 22% efficiency at 1 w/w% anti-Anx A2 antibody loading in PLGA. Ma et al. observed encapsulation efficiency of 59%, with a standard deviation of 3%, after testing three samples from the same 50:50 microsphere batch with scFv-pDL10 antibody at a theoretical loading of 0.6 w/w%.[88] Wu et al. loaded IgY antibodies into $CaCO_3$ microspheres. They found that encapsulation efficiency reached 37% for a 0.5 µg/mL IgY concentration in the inner water phase. The microspheres' drug loading was 4 w/w%. Encapsulation efficiency decreased with higher antibody concentrations, reaching a nadir of 16% when the inner water phase contained 2 µg/mL antibody. However, that formulation's antibody loading still reached 7 w/w%.[115]

The present study's antibody-loaded films released roughly a quarter of loaded antibody on average over 8 weeks. The high error bars were in many cases caused when two films would release

detectable levels of antibody whereas the third would not, or one film would release a high amount and the other two would release virtually nothing. In other words, the Mab626 release profile was actually different from film-to-film. One potential reason is that the microspheres were not controlled for size, and consequently within the films was an inhomogeneous mix of microspheres with high and low surface area to volume ratio. Different amounts of antibody loaded, as well as different microsphere yields, also affected release kinetics. Protein release kinetics from PLGA have been described as “hard to predict, ” but commonly begin with an initial burst and slower elution afterwards.[73] The films from the present study released 10.7 μg antibody in a burst over the first day, which marked the highest release rate over the course of the study. The films then released less than 0.5 μg by day 3. There was a linear release profile of around 1.7 μg/mL/day through day 14, a gradual decline in release rate until day 42, and then another increase by day 56.

The drug retention and prolonged release from these films compares favorably with other teams who encapsulated antibodies. Joung et al. incubated 2 mg/mL antibody-loaded microspheres with cells and tested release in the cytoplasmic environment after cellular uptake. They saw high intracellular levels of 3D8 scFv at 12 and 24 hours after incubation, and a steady level of the antibody in cytoplasm until it was no longer detectable by day 7.[86] Gdowski et al. ran a 12-day release study in saline buffer and saw that release was roughly linear for the first 6 days, before decreasing in rate over the next 6 days. A total of around 34% of loaded antibody was released by the end of the study.[87] Moshaverinia et al. loaded anti-BMP2 antibodies into alginate microspheres, and although they did not report w/w% loading, they found that over 25% was released within the first day and 60% within the first 2 weeks.[116] Wu et al. coated up to 10 polylysine/poly(glutamic acid) bilayers onto their calcium carbonate microspheres, and still saw 25% of loaded drug released within the first hour of incubation in simulated intestinal fluid. Within 6 hours, nearly 70% had been released.[115]

The mass of antibody released from samples proved insufficient to make a significant difference in osteoclastogenesis. A concentration of 7.5 µg/mL of Mab626 was not enough to show a statistically significant effect on cells if they were incubated with 50 ng/mL of recombinant human RANKL. Pooling all 3 release samples at each time point, and concentrating 20x, still would not have produced a solution concentrated enough to inhibit 50 ng/mL of recombinant human RANKL. In fact, if the antibodies had lost 50% of their activity by that late time point, the assay would be even less able to provide useful information. Qualitative analysis, by nuclear staining and 400x magnification optical microscopy, proved to be unreliable in determining the level of osteoclast differentiation in a cell population, even when incubated as high as 100ng/mL RANKL (data not shown). The theoretical loading of the antibody in the PLGA was only 0.16 w/w%, while the ZA film loading was almost 20x higher than that. For a test drug we used Mab626, an anti-murine RANKL antibody which came in a lyophilized form of no more than 500 µg. In order to maintain the water/oil volume ratio of 330 µL inner water phase to 5 mL PLGA in DCM, which led to high encapsulation efficiency in the ZA microspheres, the concentration of antibody stock solution was a critical parameter in antibody w/w% loading. Using one antibody container per film, the highest amount of antibody we could add was 471 µg from a 1 mg/mL solution. Tests by RND showed that Mab626 should bind to 50% of RANKL molecules, if the RANKL was at a concentration of 30 ng/mL and the Mab626 was at a concentration of between 0.5 to 2 µg/mL in solution. However, 30 ng/mL RANKL did not have sufficient differentiating effects on our RAW 264.7 cells when incubated without any Mab626, which was the reason for increasing the concentration to 50 ng/mL. RANKL and Mab626 proved less sensitive than advertised, or perhaps the binding curve simply did not scale up in a linear fashion as we hoped. Our frozen cell stocks, which started out at an age of 15 passages, were past the age range which was recommended to us by other researchers. Our lowered sensitivity could then have partially come from the RAW 264.7 cells' muted differentiation capability.

There is reason to believe that the antibody released would have retained much of its integrity and bioactivity. When Clark et al. loaded IGF into PLGA films, they found over 90% bioactivity for all time points they tested.[83] Although IGF was not an antibody, it was a protein with multiple domains. Lee et al. loaded bone morphogenetic protein (BMP) into PLGA microspheres and injected them into induced fractures in rats, and observed a larger area of bone formation and higher bone mineral density with higher loadings of BMP.[65] When Cleland et al. loaded interferon-γ into PLGA via emulsion, they saw that over 90% of released protein retained structural integrity.[84]

However, in light of the diminished bioactivity of ZA at the end of the PLGA release study, there is also reason to believe that bioactivity at later time points could diminish, possibly due to pH effects. Exposure to acids has been found to cause changes to antibody conformation. Ejima et al. tested antibody stability via differential scanning calorimetry (DSC), and found that human IgG4A was destabilized after exposure to pH 3.9 citrate buffer but did not suffer a gross conformational change compared to controls at pH 6. A conformational change was detected when the citrate buffer was dropped to pH 2.7. Human IgG4B was also found to have a conformational change by pH 2.9, while murine IgG1 showed conformational change at pH 3.9.[117] These pH's are comparable to what would be reasonably expected inside PLGA films, though it remains uncertain just how much a murine IgG2 or a human IgG2 would respond.

Chapter 6: Conclusions

The main objective of this research was to produce a system that could, in a gradual manner, release agents that reduce bone resorption. The system was intended to be locally implantable and to release drug into the immediate surroundings, while maintaining drug bioactivity. Apart from the uncertain activity of the released antibodies, this project met expectations.

Zoledronic acid proved promising as a material capable of being loaded into acrylic bone cements at weight percent loadings equal to or greater than what is currently found in commercial antibiotic-loaded bone cements. Release was observed up to 8 weeks, and although the study ended at that point, it is possible that release could have continued for longer. In-vivo testing would confirm whether cytotoxic effects from the bone cement sample supernatants were caused primarily by ZA, as opposed to other toxic materials such as MMA that could harm other cells in the body. For the potential benefits such as prolonged release, lower kidney uptake, lower jaw uptake, and higher ZA concentrations at the exact site of resection, the loading of PMMA with zoledronic acid is worth further study.

The loading of PLGA films with ZA demonstrated an improved release profile, at least in vitro, compared to PMMA. With the ability to release close to an equal amount of drug over weeks 1-4 versus weeks 4-8, it became considerably easier to replicate the effects of multiple ZA injections. The amount of drug released, compared to an equivalent w/w% loading of PMMA, is also significantly higher, leading to less drug wasted. As with PMMA, in-vivo studies for these films would confirm whether the drug release will lead to therapeutic and palliative effects lasting multiple weeks after implantation. Additionally, given some evidence of ZA's toxicity to primary breast cancer cells,[82] ZA-loaded PLGA films might one day be applicable as a therapy for not only bone, but other tissues.

Antibody-loaded films encapsulated and retained Mab626 long enough that the majority was still inside the film after 8 weeks. Although the w/w% antibody loading was low, and the release profile could turn out different in vivo, the retention of antibody compared to ZA proves promising for prolonged release. Loading a higher w/w% of antibody into microspheres, such as by addition of denosumab solution to the inner water phase, would enable higher drug mass release, and lead to concentrations high enough to dilute into cell media and test for anti-differentiation activity.

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