Effects of Therapeutic Radiation on Polymeric Scaffolds

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ABSTRACT

High levels of ionizing radiation are known to cause degradation and/or cross-linking in polymers. Lower levels of ionizing radiation, such as x-rays, are commonly used in the treatment of cancers. Material characterization has not been fully explored for polymeric materials exposed to therapeutic radiation levels. This study investigated the effects of therapeutic radiation on three porous scaffolds: polycaprolactone (PCL), polyurethane (PU) and gelatin.

Porous scaffolds were fabricated using solvent casting and/or salt leaching techniques. Scaffolds were placed in phosphate buffered saline (PBS) and exposed to a typical cancer radiotherapy schedule. A total dose of 50 Gy was broken into 25 dosages over a three-month period. PBS was collected over time and tested for polymer degradation through high performance liquid chromatography (HPLC) and bicinchoninic acid (BCA) protein assay. Scaffolds were characterized by changes in microstructure using Scanning Electron Microscopy (SEM), and crystallization using Differential Scanning Calorimetry (DSC). Additionally, gelatin 𝜖-amine content was analyzed using Trinitrobenzene Sulfonic Acid Assay (TNBSA). Gelatin scaffolds immersed in PBS for three months without radiation served as a control.

Each scaffold responded differently to radiation. PCL showed no change in molecular weight or microstructure. However, the degree of crystallinity decreased 32% from the non-irradiated control. PU displayed both changes in microstructure and a decrease in crystallinity (85.15%). Gelatin scaffolds responded the most dramatically to radiotherapy. Samples were observed to swell, yet maintain shape after exposure. As gelatin was considered a tissue equivalent, further studies on tissues are needed to better understand the effects of radiotherapy.
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1 Motivation

Approximately 230,000 women were diagnosed with breast cancer in 2013 (1). An estimated 40% of these women undergo mastectomy and may chose to undergo breast reconstruction (2). In patients with >25 - 30% chance of recurrence, radiation therapy is needed (3). Patients who chose to undergo reconstruction and need radiation therapy face many complex risks and limited reconstruction options.

For radiotherapy patients desiring breast reconstruction, the options are very few. All must undergo tissue expanders followed by either silicone or autologous tissue implants. If the patients undergo immediate reconstruction expose the implants to radiation, which can cause infection, pain, poor wound healing, flap contraction, and implant extrusion. Radiation of breast tissue while implants/tissue expanders are in place impedes delivery and lowers the quality of radiation and increases the dose of irradiation through the implant, which may increase doses to the heart and lungs (3-5). Many oncologists prefer to reconstruct after radiation therapy due to these complications. Therefore, patients are left with few alternative options and suffer multiple painful surgeries, prolonged healing times and occasionally are left with such poor skin quality (due to radiation) that they are unable to undergo any type of reconstruction (3, 6).

As this project began, research was aimed at trying to fabricate tissue that could be placed within the body to withstand and aid in healing during the radiation treatments, increasing healing time and lower the number of surgeries and complications patients face. However, as the project developed, a lack of research on material and tissue responses to X-ray radiation became apparent, and a basic understanding of the effects of therapeutic radiation became the purpose.
2 Introduction and Literature Review

2.1 Introduction

In 2006 there were approximately 377 million diagnostic and interventional radiologic examinations in the United States alone (7). In 2012 it was estimated by the International Agency for Research on Cancer (IARC) that 12.7 million new cases of cancer were reported. With increasing cases of cancer and the need for radiotherapy, it is important to the health of these patients that doses and frequencies of radiation are well developed and understood. Radiation therapy standards have been around only a short time but the use of radiation therapy continues to increase. With this increase, more knowledge on the effects of therapeutic radiation is needed.

Most research suggests that radiation targets the nuclear DNA of cells to kill cancer cells (8-11). However, exposure is not only dependent on cell radiation dose but also tissue reaction, growth factors, tumor spread, imaging technique and various biological factors (9, 11). Current research on ionizing irradiation of normal tissues focuses on cellular response in different sites of the body. Rapidly dividing cells are more radiosensitive due to the fact that cells in the G2 and mitosis phases are more susceptible to radiation which facilitates use of radiation as a cancer therapy (9, 10). Radiation can have four different effects on a cell; no effect, slight DNA damage that may not show up until later generations, severe DNA damage which may kill the cell or prevent it from dividing, and immediate cell death (10). These effects depend on the type of tissue that is being irradiated. The effects of radiation on cells have provided valuable understanding on cellular response but there is a lack of information on how tissues and materials respond to radiation therapy doses.

There are four different responses a polymer can display after undergoing radiation therapy; degradation, cross-linking, a combination of the two or no response (12). These responses are
dependent on the type of radiation, dose level, and the radiation environment (13). Studies early as the 1960’s have investigated the effects of radiation on polymers (13-15), yet there is limited data available on the effects of therapeutic radiation (X-rays). Radiotherapy is one of the main treatments for many types of cancer (16) and patients are exposed to radiation when undergoing common tests such as a CT scan (17). It is important that therapeutic radiation effects are well understood for normal tissue, tumors and candidate biomaterials in order to improve current radiation treatments and procedures. Research has shown that exposure of polymers to ionizing radiation can cause chain scission, degradation, crosslinking and hydrolysis. These studies are most often conducted using extremely high doses of gamma radiation (14, 18, 19), while the effects of X-rays on polymers and human tissues is sparse.

The International Commission of Radiological Protection (ICRP) first proposed therapeutic radiation doses in 1975 (20, 21). These doses were not based on the composition of extracellular matrix but rather on common doses that were already in use. Doses for various therapeutic radiation procedures were updated by the ICRP in 1990 and again in 2007. These new radiation doses began to take into account weight, gender, age and various other factors that can affect dosage. Current radiation fractionations range from 1.8-3 Gy\(^1\) for a total of 45-70 Gy per treatment (16). Therapeutic radiation has been an integral part of cancer therapies for many years and is still being used as an effective therapy, however much of the current knowledge is not based on empirical data and long-term effects on materials are not well documented.

\(^{1}\) Gray (Gy) is the common unit used in therapeutic radiation. For reference, 1Gy = 1 Joule in the form of ionizing radiation. Radiation is often used in units of Rad or Rem. 1 Rad = 1 Rem = 0.01 Gy (conversion only applies to X-ray radiation and can change depending on the radiation source) (22).
2.2 Development of Radiation Doses

The X-ray was discovered in November, 1895 and was rapidly utilized as a therapeutic source for treating skin lesions by early 1896 (23). With such a rapid transition from discovery to market, the effects of X-rays was not yet fully investigated, which often led to morbidity of patients as well as poor tumor control (23). Some of the first studies looking at the impact of X-ray doses on cell survival were not conducted until the late 1920s and early 1930s by two biologists, Regaud and Coutard (23). This meant that the application of X-rays as a therapeutic technique had been in use for approximately 30 years without much applied research or fundamental knowledge.

The ICRP was started in 1928 to prevent diseases and negative effects associated with exposure to radiation. This extended beyond therapeutic radiation to any type of radiation which a human being may encounter. Although the ICRP was created in 1928, the first recommended standards did not appear until 1977 through the Annals of the ICRP (24). The first paragraph of the report section states: “the recommendations [for radiation protection of patients] herein are intended to relate to exposure of patients in all types of diagnostic or therapeutic procedure where unsealed radionuclides are administered”. This is the first instance where the effects of therapeutic radiation to patients were regulated, 81 years following first time use. The report continues to state that doses and risks are proportionally related and that the higher the dose a patient receives the more risks to the patient. What this report fails to address are the differences in patients, organ or tissue composition based on dose levels. The end of this section states: “Due to lack of understanding, the interpretation of recommendations on protection of the patient has, on occasions caused unwarranted alarm and has therefore led some patients to hesitate in seeking necessary medical attentions. Every effort should be made to give the public a sound view of the general benefit from, and need for, various types of diagnostic and therapeutic use of
radionuclides in relation to the level of radiation risk involved.” This reveals the paucity of knowledge the ICRP had to create dose regulations. After 81 years there was still much to be done to improve the research aspects of therapeutic radiation.

A leap in therapeutic radiation research occurred in 1991 when a review article was generated that compiled all knowledge of therapeutic radiation doses to tissues (25). Using clinicians from four different medical universities, cancer centers and radiation therapy departments, the review article gathered together information on tolerances of normal tissues. “After eight decades of radiotherapy practice, current knowledge of both issues [tumoricidal doses and tolerance doses of various normal tissues] is imprecise.” They found very few hard data sets from clinical and laboratory studies. Often the data found was created from extrapolated whole organ data. According to the clinicians these data sets were not based on gradations of dose across the volume of an organ. Common practice at the time was a tolerance dose 5/5, based on the probability of 5% of complications within five years of treatment with a tolerance dose of 50/5 based on the probability of 50% complications within five years (25). Today, therapeutic radiation still lacks guidelines dependent on different doses and organ type.

There are many types of uncertainty when using therapeutic radiation. What kind of patient is in need, what organ is problematic, how deep within the body, what dose to use, how long to administer that dose, how often to administer that dose, what are possible risks. The list goes on and on. It is hard for clinicians to have a set protocol without some type of standards to guide them. The ICRP updated their standards in 1991 (26), but as previously stated there was still a lack of knowledge for known tissue tolerances. In 2007, the ICRP developed new standards based on weight factors which included parameters such as the age of the patient, hereditary issues, gender, external vs. internal radiation and new biological and physical research (27). Effective doses used today can be calculated based on a specific patient and case, rather than an
arbitrary dose for a certain type of disease.

Although these regulations have greatly increased survival rates and patient recovery, there is still uncertainty. Many questions remain that need to be answered about cancer and how the cells and tissues react to radiation. According to C. J. Martin the uncertainty of patients exposure dose is ± 40% (20). In Martin’s article, effective dose (E) can only be calculated using errors in x-ray measurements and calibrations as well as tissue weighing factors provided by the ICRP. Many clinicians calculate E for patients using weight factors and formulas provided by the ICRP. Martin shows that these calculations can have significant uncertainty, and it is important for clinicians to be aware of the uncertainty associated with calculated doses.

X-ray as radiotherapy has come a long way since 1896. It is an extremely valuable tool for treating malignant and benign diseases. However, the lack of basic knowledge for tolerances based on tissue type needs to be evaluated.

2.3 Radiation Doses

In 1991 when original doses were developed by the ICRP, many researchers began research into how certain doses affected patients. A conventional fractionation of doses for cancer patients in 1991 was, 1.8 to 2 Gy per day for 5 days a week (25). These does are normal for cancer patients but extremely high compared to healthy human exposure on an annual basis. To better understand these dose levels the Environmental Protection Agency (EPA) created a generic radiation diagram (Figure 1) (17). If we compare this 2013 EPA diagram with the 1991 conventional fractionation, 1.8 Gy = 1.8 X 10^5 millirem is significantly higher than any of the X-ray exposures found on EPA’s radiation exposure diagram.

This diagram shows that something as simple as a chest X-ray is only 4 millirem, while a common mammogram is 30 millirem. Other examples of radiation exposure included full body
CT scans (1,000 millirem), transatlantic airplane flights (2.5 millirem) and drinking water radiological limits determined by EPA (4 millirem) (28). Americans on average receive 620 millirem per year, which has been shown to cause no significant harm to humans (28). However as this average dose increases due to therapeutic radiation there is an increasing risk of harmful effects.

Figure 1: Diagram of annual radiation doses from different sources. Compares medical exposure to background and cosmic sources. (Radiation Doses in Perspective 2013 [cited 2013 September 29]. Available from: http://www.epa.gov/radiation/understand/perspective.html).

A 2012 study reviews advances and future directions of cancer and radiation therapy (29). Radiotherapy is an important treatment option for cancer with approximately 50% of all cancer
patients receiving radiation therapy during their illness (29). Today a typical radiation therapy for cancer is 1.5-3 Gy daily for several weeks. This means that over a period of weeks a patient could be exposed to anywhere from 75-150 Gy (29). Although radiotherapy kills cancer cells and helps with tumor suppression, many researchers and clinicians are beginning to ask more complicated questions. How can we optimize the effectiveness of radiation therapy in combinations with other modalities of treatment? Would it be possible to lower radiation therapy effects to normal tissues? There is an ongoing interest in how tissue reacts to radiation and how that information can be used to improve therapeutic doses.

### 2.4 Current Cancer Therapies

Breast cancer is the most common cancer type and the second leading cause of cancer-related death in women, while lung cancer has the highest percentage of cancer-related death in both females and males (Figure 2) (1, 16). Table 1 displays common doses and fractions used for various cancer types. The typical total doses range from 45 ~ 70 Gy which falls well above the EPA common exposures (Figure 1). The 1991 review article looking at tissue tolerances to therapeutic irradiation does not mention breast cancer specifically but states that most partial heart tissue radiation injury was caused by postoperative breast cancer treatment.
In the late 1990s there appeared to be greater development of accurate radiation doses for breast tissue (30). Today, a common total radiation dose for breast cancer is 50 Gy (16). Breast tissue is a complex tissue because it is at an interface in the body, comes in contact with both muscle and skin, and can change significantly depending on the age of the patient. It was originally assumed that the breast was 50% adipose tissue and 50% glandular tissue based on a single 1977 study (31). This concept changed in 1981 when a group of researchers found two major flaws in this approach. The 1977 study was a single study using limited breast samples without morphological verifications, and the term glandular is indefinite and does not distinguish between different tissues (31). After testing 67 different breast tissues from 40 women between the ages of 48 and 90, they found that “glandular” tissue which is actually composed of epithelium and stroma is only on average 17.02% of breast tissue (31). This is important because
this particular type of tissue is at carcinogenic risk from mammography and the actual risk was significantly higher since it was based on 50% composition by volume when in actuality the tissue composition was around 17%.

Previous studies have shown that radiation helps post mastectomy patients but has the adverse effect of skin toxicity (32). This means one of the most important factors for post mastectomy therapeutic radiation is skin dose. This is challenging because of the shape of the chest wall and its limited thickness (32). Many studies have begun to look at differences in breast cancer parameters such as mastectomy or lumpectomy, age, gender, stage or patients who do or do not receive reconstruction (32-38). The German Society of Radiooncology (DEGRO) established an expert panel to provide practical guidelines for breast cancer radiotherapy (39-41).

Most radiation delivered to the heart is background from radiation therapies to other organs. When tumors are located near the heart it is important that doses to tumors are not high enough to cause risk to the heart. Average doses to the heart should not exceed 26 Gy (16). Other considerations when looking at lung cancer include spinal cord tolerance doses (<46 Gy) and esophagus doses (<73 Gy) (16). It is difficult to create a radiation therapy for lung cancer that isolates lungs. Clinicians must take into account the volume and tissue composition of each organ they are irradiating not just the targeted tumor.
**Table I: Doses for Tissues and Organs (16, 25)**

<table>
<thead>
<tr>
<th>Organ</th>
<th>Single Dose (Gy)</th>
<th>Multiple Dose Totals (Gy)</th>
<th>1991 Multiple Dose Totals (Gy)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain</td>
<td>15-25</td>
<td>60-70</td>
<td>45</td>
</tr>
<tr>
<td>Lens</td>
<td>2-10</td>
<td>6-12</td>
<td>10</td>
</tr>
<tr>
<td>Skin</td>
<td>15-20</td>
<td>50-60</td>
<td>50</td>
</tr>
<tr>
<td>Spinal cord</td>
<td>8-10</td>
<td>45-50</td>
<td>47</td>
</tr>
<tr>
<td>VCTS</td>
<td>10-20</td>
<td>50-60</td>
<td>-</td>
</tr>
<tr>
<td>Mucosa</td>
<td>5-20</td>
<td>65-77</td>
<td>-</td>
</tr>
<tr>
<td>Peripheral nerve</td>
<td>15-20</td>
<td>65-77</td>
<td>50 (optic nerve)</td>
</tr>
<tr>
<td>Muscle</td>
<td>&gt;30</td>
<td>&gt; 70</td>
<td>-</td>
</tr>
<tr>
<td>Bone–cartilage</td>
<td>&gt;30</td>
<td>&gt; 70</td>
<td>52</td>
</tr>
<tr>
<td>Thyroid</td>
<td>-</td>
<td>30-40</td>
<td>-</td>
</tr>
<tr>
<td>Optic pathways</td>
<td>8-10</td>
<td>55-60</td>
<td>50</td>
</tr>
<tr>
<td>Breast</td>
<td>1.8 - 3</td>
<td>46-50</td>
<td>-</td>
</tr>
<tr>
<td>Lung</td>
<td>1.8 - 2</td>
<td>&lt; 46 (spinal cord spared)</td>
<td>17.5</td>
</tr>
</tbody>
</table>


Since 1991, fractionations have become more common, making doses available for more types of tissues and organs. Comparing the multiple doses with the 1991 doses (Table I) we can see that the doses have become ranges. Both of these fractionations are based off of the TD 5/5 but one can see that from 1991 to the present, ranges have been developed allowing for differences in patients. Fractionations have also increased from 1991, which could be a result of
better x-ray sources and technology, more detailed research, higher availability and understanding of tissue and more adequate medications for radiation therapy patients.

### 2.5 Radiation of Human Tissue

In order to develop more accurate tolerance doses, researchers need to understand the effects that radiation can have on human tissue. There are currently few studies on the effects of tolerance dose on tissues. Instead doses are derived empirically from past radiation therapies and are created using limited data and clinical observations (8).

The most radiosensitive sites are red bone marrow, gonads and the lens (11), while sites such as the peripheral nerve are fairly radio-resistant (9). These observations are based mostly on the cellular response, but do not always take into account the influence of tissue type, cell migration, and regeneration properties of some tissues (11). Research shows that even doses as small a 1 cGy, can cause altered expression of key regulatory genes (11). Research also shows that radiation effects may not become apparent for days, weeks, months and even years (8, 9, 11).

Cellular response is important to radiation risk. For example, patients with a genetic defect called Louis-Bar Syndrome, which prevents DNA repair causes not only a greater risk of cancer but also increased susceptibility to radiation (8). How does a clinician create a treatment plan for a patient with Louis-Bar Syndrome without fully understanding not only cellular response but tissue and biological response also? A recent review article attempts to tackle current research on tissue reactions to ionizing radiation (11). The article concludes that tissue reaction results from radiation death of target cells as well as functional radiation effects causing impairment of intracellular metabolism, retardation of mitotic activity, impairment of cell-cell interactions, damage to proteins, lipids, carbohydrates and other complex molecules (11). Although a literature review could go into detail on each type of tissue reaction to radiation, it is important to
note that although research is addressing tissue reactions, a working radiation model has yet to be standardized.

X-ray radiation effects on cells have thoroughly considered, but few studies look at the effects on the extracellular matrix, ECM, (a mixture of collagen, elastin fibers, hyaluronan and proteoglycans) (42). A study looking at radiation effects on ECM investigated how mechanical and rheological properties change after exposure to X-ray irradiation (42). This study used bovine pericardium tissue, elastin, and rooster comb hyaluronan to mimic the different ECM parts. The tissues were irradiated at doses of 10, 25 and 50 Gy for the collagen and elastin and 10, 25, 50, 75 and 100 Gy for hyaluronan. These doses are much lower than fractionations (Table I) used for radiation therapy. An initial increase in elastic modulus was found for collagen that may have been due to radiation-induced cross-links, but the final modulus decreased possibly due to the radiation-induced chain scission. For elastin, radiation weakened the tissue, most likely due to chain breakage. For hyaluronan, radiation damaged long polymer chains through free radical action. All of these findings encourage more research of tissues under radiation. If these basic ECM components are breaking down under radiation, what is happening when patients undergo months of radiotherapy, or have recurrence and must undergo multiple treatments throughout their lives?

Since there is limited research on tissue reactions from radiation, an important question would be why? Adequate models do not exist to test tissue-based therapies. In 2012, a research group from the Southwest Medical Center at the University of Texas created a model to test human tissue response (43). They applied different x-ray doses (30.4 Gy, 41.5 Gy, 52.6 Gy, 65.5 Gy, and 76.5 Gy) to the skin of athymic rats then analyzed the resultant wounds. They then look at the various levels of radiation damage up to 65 or 100 days later, which permitted the study of tissue radiation over time. This was a trial study and therefore some results lack significant
samples sizes. After 65 days all 41.5 Gy wounds healed significantly, but ulcerations still covered 5.8% - 47% of the irradiated tissue (43). They found that after 100 days the only dose level that completely healed was the 30.5 Gy dose. Although this model study was an early trial, it suggests that better models need to be created in order to better understand radiotherapy.

Since there is no working model it is difficult to tell what is happening within tissue. Some programs have begun to visually analyze changes in tissue structure (Figure 3) using image characterization. A program called ROSIRIS, started in 2009 by an institute in France, is dedicated to “better understand the mechanisms giving rise to secondary effects in radiotherapy. ROSIRIS is a multidisciplinary programme combining expertise in radiopathology, radiotherapy and physical dosimetry.”

![Figure 3: A comparison of healthy tissue (left) and the same area after 15 weeks of irradiation and a total dose of 27 Gy. (ROSIRIS Programme 2012. Available from: http://www.irsn.fr/en/research/research-organisation/Research-programmes/Rosiris/Pages/ROSIRIS-programme.aspx. © IRSN (HDR of Agnès François)) Used under fair use, 2013](image)

This program has been able to show an obvious effect of radiation on both cancerous tissue (44) and healthy tissue. Looking at the blood vessel (Figure 3) there is a decrease in size of the vessel due to radiation. There is also an increase in the arterial wall thickness due to the radiation doses. It is difficult to determine exactly what is happening to the healthy tissue but
there is a visual change in the healthy tissue around the artery. The radiation has a dramatic effect on the healthy tissue as shown in Figure 3; researchers need to continue to understand what is happening to healthy and cancerous tissue after radiation to better adapt therapeutic radiation treatments.

2.5.1 Radiation Aging

Radiation aging is not a term often used to refer to the human body. It is mostly used in references to polymers (40). Many types of radiation, including x-rays, possess sufficient energy to penetrate polymers and human tissues, which is why they are used as radiotherapy. When x-rays penetrate a material a reaction called ionization occurs, hence the term ionizing radiation. In this process radiation removes an orbital electron from an atom and converts the atom into a positively charged ion (45). As a result of this atom being removed, a covalent bond associated with that atom breaks and the atoms rearrange themselves. This breaking leads to either scission or crosslinking at the site the ionizing radiation penetrates (45).

When a polymeric material is used in a nuclear setting, it can be exposed to high doses of radiation at elevated temperatures. Polymer degradation in air is dominated by oxidation reactions, which is the main mechanism of radiation aging (46). Another important factor in polymer degradation is the exposure temperature. Many polymers show increased degradation when radiation aging is followed by thermal aging (46). From a human tissue perspective, there is adequate oxygen within the human body to cause radiation induced oxidation reactions. Human body temperature (37°C) is higher than accepted room temperature (25°C), which could cause different aging effects than tissues tested at room temperature.

One article looking at water-aging of gelatin-glucose film after exposure to gamma radiation is the closest study to human tissue radiation aging (47). Mixtures of gelatin-glucose or
gelatin-urea, decreased the effect of water aging and were more stable than gelatin itself. Gamma radiation stabilized both gelatin-glucose and gelatin-urea samples. They proposed mixtures of gelatin as potential biomaterials, however the radiation dose used in this study is significantly higher than any tolerance dose for therapeutic radiation.

Many articles investigate irradiation degradation as away to increase the mechanical properties for biomedical applications, which use high radiation doses (2500 Gy – 10,000) and do not take into account the human body environment (47, 48). In these cases, radiation aging is not comparable to radiation aging of human tissue (Table II).

2.6 Radiation of Polymeric Materials

Researchers are interested in irradiating polymers for reasons ranging from nuclear material containment to inducing crosslinking. One thing these research studies have in common is that they generally require high radiation doses and thermal exposure (Table II). A study designed to predict the radiation degradation of elastomeric materials looked at elastomeric seals under gamma radiation at temperatures between 80 °C and 250 °C (49). Another study seeking to improve Teflon for nuclear environments, looked at doses between 2 kGy and 600 kGy at temperatures up to 675 K (50). Studies that looked at inducing material crosslinking used significantly higher gamma-radiation doses than needed in therapeutic radiation: 500 kGy for Teflon (50), 20 kGy for poly(ethylene-alt-tetrafluoroethylene) (51), 100 kGy for Nafion® (52), 25 kGy for polyvinyl pyrrolidone/polyethylene glycol hydrogels (53), and various other doses depending on the polymer being evaluated.
### Table II: Compilation of Various Materials Studied Under Radiation

<table>
<thead>
<tr>
<th>Material</th>
<th>Radiation Source</th>
<th>Dose</th>
<th>Temp. (°C)</th>
<th>Response</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gelatin</td>
<td>Gamma</td>
<td>2.5-25 Mrad</td>
<td>≤77K</td>
<td>Decarboxylation and deamination occur</td>
<td>(14)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15 KGY</td>
<td>25</td>
<td>Cleavage of C-N bonds, forming radicals (I) and (II)</td>
<td>(54)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15, 25, 50, 100, 150, 200 KGY</td>
<td>25</td>
<td>Low levels: uptakes all water. Higher levels increases mechanical properties and crosslinking</td>
<td>(47)</td>
</tr>
<tr>
<td></td>
<td>Gamma &amp; electron beam</td>
<td>7 kGY/H and 11 kGY/s. Totals of 5, 10, 20 and 50 KGY</td>
<td>40</td>
<td>Reduction in viscosity, no difference in chemical reaction between gamma and electron beam</td>
<td>(55)</td>
</tr>
<tr>
<td>Gelatin-Urea, Gelatin-Glucose</td>
<td>Gamma</td>
<td>250, 500, 1000, 2500 Krad</td>
<td>-</td>
<td>Water uptake and swelling reduced for 250 (G-G) and 500 (G-U). Greater stability</td>
<td>(15)</td>
</tr>
<tr>
<td>PCL</td>
<td>Gamma</td>
<td>0-500 kGY</td>
<td>30-55</td>
<td>Improved low heat stability and processability</td>
<td>(56)</td>
</tr>
<tr>
<td>PCL</td>
<td>Gamma</td>
<td>26 Mrad</td>
<td></td>
<td>Gel formation, above this dose scission occurs. 2-5 Mrad tensile elongation increase</td>
<td>(15)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25 kGY (sterilization)</td>
<td></td>
<td>Decreased rate of degradation, affected microstructure</td>
<td>(57)</td>
</tr>
<tr>
<td>PU</td>
<td>Gamma</td>
<td>27 KGY (sterilization)</td>
<td>25</td>
<td>Found larger quantities of carcinogens from gamma sterilization than autoclave</td>
<td>(58)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2.5 Mrad (sterilization)</td>
<td></td>
<td>Crosslinking, linear relationship between irradiation and amount of radicals found</td>
<td>(59)</td>
</tr>
<tr>
<td>PU Biodegradable</td>
<td>Gamma</td>
<td>25 kGY (sterilization)</td>
<td></td>
<td>Two steps of degradation: hard segment then soft segment</td>
<td>(60)</td>
</tr>
<tr>
<td>Poly-acrylamide, poly(vinyl alcohol), polystyrene</td>
<td>Gamma</td>
<td>0 -3 Mrad</td>
<td>-</td>
<td>Gas evolution linear up to 3 Mrad</td>
<td>(62)</td>
</tr>
<tr>
<td>Polypropylene</td>
<td>Gamma</td>
<td>Increasing doses of 25 kGY</td>
<td>25</td>
<td>Stabilization methods for irradiated polypropylene</td>
<td>(63)</td>
</tr>
<tr>
<td>Poly (ester urethane)</td>
<td>Gamma</td>
<td>0-400 kGY, 0.2 Gy/s</td>
<td></td>
<td>Crosslinking at C-C double bonds; chain scission occurs at absences of these bonds</td>
<td>(64)</td>
</tr>
<tr>
<td>Teflon</td>
<td>Gamma</td>
<td>3 kGY/h</td>
<td>340</td>
<td>Crosslinking</td>
<td>(12)</td>
</tr>
<tr>
<td>Thermoset Plastic</td>
<td>Gamma</td>
<td>10 MGy and 1MGy</td>
<td>air</td>
<td>Chain scission, no change in flexural strength</td>
<td>(65)</td>
</tr>
<tr>
<td>LactoSorb®</td>
<td>X-ray</td>
<td>2 Gy doses, total 80 Gy</td>
<td></td>
<td>Material withstands x-ray exposure and can be used for temporary internal fixation</td>
<td>(18)</td>
</tr>
</tbody>
</table>
Another application that causes polymers to be affected by radiation is the process of sterilization. The World Health Organization (WHO) sets standards for the sterilization of drug products and medical devices using ionizing radiation at 25 kGy (66). This means many products that use this type of sterilization must withstand a radiation high dose. These doses may also cause materials to crosslink and degrade while becoming sterilized so it is important to determine the stability of polymers undergoing sterilization (63, 67, 68).

In order to develop a biomaterial that could be a tissue replacement, a thorough understanding of radiation effects at exposures equivalent to therapeutic doses needs to be conducted. There are limited studies applying therapeutic level doses. LactoSorb® a polymer that is used as a bioabsorbable implant, has been studied at radiation doses of 2 Gy, five days a week for up to eight weeks (18). The study observed effects of radiation over eight weeks to determine whether the material would withstand the exposure and found that the material was stable and be used for “temporary internal fixation” when a patient needed radiation in the same region. Although this material is not used for applications such as implants for cancer survivors, it does show one of the only studies using therapeutic radiation doses, including body temperature.

For the materials of interest in this review, gelatin, polycaprolactone and polyurethane limited data on the effects of therapeutic radiation over time is available.

2.6.1 Irradiation of Gelatin

Gelatin irradiation has been partially studied due to the abundance of collagen within the body. Collagen is the most abundant body protein and is a component of skin, tendon, cartilage, bone and connective tissues (19, 54). Gelatin is derived from collagen of either pig or cattle skin and can be considered a human tissue equivalent for many biomaterial studies. Gelatin is also
easily available in large amounts, nontoxic to cells, and biodegradable (69).

Gelatin is often used as a gelling agent in gel dosimeters (70-73). It is currently the most common gelling agent for polymer dosimeters and therefore has been researched under multiple types of ionizing radiation, including X-ray (73). Although gelatin is used as a gelling agent it has been shown to consume long-lived radicals, reducing the polymerization rate (71, 72) and also increases or decreases the sensitivity of the polymers to radiation doses by changing the gelatin weight percentage (70, 72, 74). Since these dosimeters are used in all types of diagnostic screenings they are often studied using lower radiation doses. These doses range from 30 cGy to 400 cGy (72) to 20 Gy (71) but take into account not only the change in gelatin but the change in the polymers used for the dosimeters themselves (polymethacrylic acid, polyacrylamide and variations).

Effects of ionizing radiation on gelatin have been questioned for years in tissue applications. First there was a debate due to contradictory results; either radiation induces degradation or it causes crosslinking (19). One of the first studies, in 1961, by a graduate student, L. Prusak, tried to determine the effects of high-energy radiation on pigskin gelatin (14). Prusak looked at doses between 2.5-25 megarads (25,000 Gy to 250,000 Gy) and found that decarboxylation and deamination occurred in irradiated gelatin in proportion to the absorbed dose of radiation. Prusak also found that lower radiation doses did not severely damage low molecular weight gelatin solutions. He heated gelatin samples to 77 kelvin and found that the increase in temperature had not altered the free radical structure. He concluded that the increase in molecular weight was attributed to crosslinking through covalent and weak hydrogen bonds.

Researchers now have more accurate ways of analyzing radiation effects in gelatin. A recent study using gamma radiation at a dose of 15 Kgy/hour in air at room temperature found that irradiation resulted in formation of free radicals (75). This is similar to studies done with
radiation aging that were previously discussed (9, 10). They conclude that free radicals cause C-N bond cleavage causing gelatin to degrade (75). However, studies also showed gelatin’s ability to crosslink under high doses of radiation >30 KGy (19, 54) and many chemists argue that crosslinking and degradation are not mutually exclusive (12, 14, 19). One problem these studies have in common is that radiation doses are all well above levels of therapeutic radiation. Although many studies were conducted before the updated ICRP standards, there is very limited recent data on therapeutic radiation on gelatin. Since there is a need to better understand effects on human tissue under radiation, it is important that research also provides further understanding of gelatin under therapeutic radiation doses and in the human body environment.

2.6.2 Irradiation of Polycaprolactone

Polycaprolactone (PCL) is a biomaterial that is currently being researched for use as a bioresorbable scaffold for tissue engineering (76, 77). It has already been approved for a number of medical and drug delivery devices (76), which means it may already come in contact with therapeutic radiation. Since PCL is a bioresorbable polymer, it is meant to breakdown in the body over a period of time without causing harmful effects. It also needs to maintain its structure while delivering the drug to the correct target in the body or support a tissue long enough for the body to begin healing itself. In these cases, radiation could increase or decrease the time it takes for this material to degrade, causing possible risks to the patient with PCL scaffolds.

One study has shown that PCL can be photochemically altered, using electron beam or gamma sources, to degrade or crosslink at a controlled rate (78). Using gamma and electron beam radiation low doses (5 Mrad: 50,000 Gy) chain scission will be dominant. Wavelength measurements in their studies were not reported which makes it difficult to compare these results to other X-ray sources, however they concluded that the extent of scission and crosslinking were
adjustable depending on irradiation conditions. This means that when they altered oxygen concentration or light intensity they observed different effects. One condition this study did not consider was temperature dependence.

Yoshii et al. focused only on crosslinking PCL through gamma irradiation to improve heat resistance (56). PCL was irradiated at three different temperature ranges; solid state (33-55°C), molten state (>60°C) and a supercooled state (melted, cooled, then irradiated at 45-55°C) (56). The solid state is of special interest since it represents a temperature range that could include human body temperature, 37 °C.

One study evaluating the irradiation of PCL using high gamma doses (5-60 Mrad: 50,000 – 600,000 Gy), looked into the critical radiation doses of PCL (15), but these doses as in the previously described articles are much higher than any therapeutic dose and cannot be used to predict effects of therapeutic radiation on PCL.

Gamma radiation has also been used to investigate the sterilization of PCL scaffolds that may be used in biomedical applications. Since the standard sterilization dose set by WHO is 25 kGy (66), such studies use 25 kGy or higher doses to evaluate degradation, mechanical properties and cell viability of PCL scaffolds (57, 79-82). However X-ray radiation is not used to sterilize medical devices so many of these articles cannot be directly related to x-ray irradiation.

Just as with gelatin, there is a limited amount of data and research on the effects of irradiation PCL at therapeutic levels. This material, which is currently used for medical devices/drug delivery, should already be thoroughly studied for acute and chronic radiation effects using X-ray radiation at therapeutic levels. Many patients could be at risk if their medical devices begin to degrade at a different rate than estimated due to therapeutic radiation.
2.6.3 Irradiation of Polyurethane

Polyurethanes (PU) are biomaterials often studied for use in fabricating 3D tissue scaffolding (83, 84). PU is of interest from a radiation perspective because it can be designed to biodegrade or not biodegrade *in vivo*. This means if medical implants become available using PU, devices could remain in a patient’s body for an extended period of time, increasing the chance for exposure to ionizing radiation.

Polyurethane studies characterizing gamma radiation exposure have been formed due to the need to sterilize PU medical devices (58, 59, 61, 85). Each of these studies uses a radiation dose at or above 25 kGy (WHO Standard) to study the effects of radiation. Two of these studies looked at the difference between chain-extended and nonchain-extended polyurethanes (59, 85). These studies found that chain-extended PU shows a decrease in molecular weight with an increase in irradiation, while nonchain-extended polyurethanes show an increase in molecular weight with increase in irradiation. One article concludes that cross-linking and degradation occur predominantly within the soft segments (nonchain-extended) of polyurethanes (85); the other agrees while arguing that degradation only occurs in the chain-extended polyurethanes (59). Overall, they both agree that the molecular weight of nonchain-extended polymer makes a difference in crosslinking and degradation.

Another study evaluated biodegradable polyurethanes for use in medical applications and tried to determine whether gamma radiation sterilization is applicable for biodegradable polyurethanes. This was the first study to look at gamma radiation of biodegradable polyurethanes (61). They found that these polyurethanes undergo significant degradation from the sterilization process and that another form of sterilization needs to be used for biodegradable polyurethane (61).

With only a few sterilization studies examining PU, there is a need for study at the
therapeutic radiation doses. Since sterilization uses gamma radiation and not X-ray these studies need to be expanded to include X-ray radiation exposure. As PU materials may be in a medical environment there are always chances that it may come into contact with X-ray radiation.

2.7 Summary

All three of these materials, gelatin, polycaprolactone and polyurethane are being used and investigated for various medical and clinical applications, either as implant/drug delivery systems within the body or as medical devices such as dosimeters and tubing. With the increasing exposure to X-ray radiation during radiotherapy or common diagnostic examinations it is important that the effects on these materials are thoroughly understood.

This research aims to investigate material responses to X-ray radiation. The three materials discussed above, gelatin, PCL, and PU were investigated using low doses of radiation (50 Gy total), commonly found in cancer treatment. This research fills a gap in current therapeutic radiation research.
3 Materials and Methods

3.1 Scaffold Fabrication

3.1.1 Gelatin

Gelatin from bovine skin was purchased from Sigma Aldrich. A 10 wt. % solution of gelatin in deionized water (diH$_2$O) was allowed to bloom (gelatin powder absorbing water) at room temperature for 30 minutes. The mixture was then heated and stirred until fully melted; 100 μL of 0.25 wt. % glutaraldehyde solution was added to crosslink the gelatin. The solution was then cast quickly into a small petri dish and stored at 4 °C overnight. The resultant film was then cut into desired scaffold shape and washed in a 100 mM glycine solution at 37 °C for 1 hour (removing unreacted glutaraldehyde). Samples were washed in excess diH$_2$O at 37 °C for 1 hour, three separate times. Samples were covered in a small volume of diH$_2$O, frozen at -80 °C overnight, lyophilized for 2-3 days and stored in a desiccator until use.

3.1.2 Polycaprolactone (PCL)

Polycaprolactone (inherent viscosity: 1.15 dl g$^{-1}$) was purchased from LACTEL biodegradable polymers (Birmingham, AL). A 10 wt. % mixture of PCL in 1,4-dioxane was heated at 37 °C until fully dissolved. Sodium chloride (grain size: 150-315 μm) was added for a 10 % v/w ratio to create a porous structure. The mixture was then pipetted into a 1mL cryovial and centrifuged for 5 minutes at 3,000 rpm. Cryovials were then placed in liquid nitrogen, for ~30 minutes. Once fully frozen, samples were immediately transferred to the lyophilizer and lyophilized for 2-3 days. Samples were removed from the cryovials and salt leached for 2 days in fresh diH$_2$O, changed twice a day. Samples were then dried at room temperature and placed in a desiccator until use.
3.1.3 Polyurethane

Dr. Matt Becker, The University of Akron, OH, kindly provided the polyurethane. The material is currently being patented and little characterization is currently known. A 15 wt. % solution of polyurethane in dimethylformamide was stirred until fully dissolved. Sodium chloride (grain size: 150-315 μm) was added for a 10% v/w ratio. The mixture was then poured into diH₂O to form 3D scaffolds then cut to the desired shape. Scaffolds were then salt leached in diH₂O at 37 °C in a vacuum oven until the diH₂O fully evaporated (~24 h). Samples were then frozen in water at -80 °C overnight, lyophilized for 2-3 days and stored in a desiccator until use.

3.2 Radiation Source, Exposure and Sample Collection

Radiation exposure was conducted at the Lewis-Gale Regional Cancer Center located in Salem, Virginia. A Varian Clinac 2100 C/D with 6MV X-rays was used to irradiate each sample. Six samples of each scaffold type were exposed to 25 doses of 2 Gy each, over a three-month period (50 Gy total). The samples were irradiated ~2 times a week. Samples were stored in phosphate buffered solution (PBS), pH 7.4, at room temperature throughout exposure and storage. During every other exposure, ~5 mL PBS was collected for analysis from the well plate, in a 15 mL conical tube. Once 5 mL was removed from each plates, PBS was added to replace the removed PBS. A non-irradiated control was used for each scaffold (PCL, PU and gelatin). A control of gelatin samples stored in PBS for 3-months at room temperature using the same PBS collection technique was used for the gelatin samples.

3.3 Environmental Scanning Electron Microscopy, ESEM

A FEI Quanta 600 FEG, Environmental scanning electron microscope was used to determine visual differences in irradiated and non-irradiated samples. One sample of each scaffold type was imaged (Irradiated gelatin, PCL, and PU, non-irradiated gelatin, PCL and PU, and 3 month
gelatin). Each sample was frozen in liquid nitrogen and then cut using a razor to create a cross-section. Both surface and cross-sectioned samples were imaged to determine differences. The ESEM used also included an energy dispersion spectrometer (EDS) to determine surface chemistry if needed.

3.4 **High Performance Liquid Chromatography, HPLC**

High performance liquid chromatography was used to detect the breakdown and dissolution of scaffolds. The PBS collected throughout the radiation process was the source of solution for analysis. All samples were analyzed using size exclusion chromatography and ultra violet detection on an Agilent Technologies 1110 Series HPLC system. Geraldine Magnin-Bissel, at the Virginia-Maryland Regional College of Veterinary Medicine, analyzed the samples.

Due to time constraints and large samples sizes, HPLC results were only run on the PBS collected from the irradiated samples. In the future will need to be run on the non-irradiated samples as well to be certain that all solvents were removed before testing.

3.4.1 **Gelatin**

A 10,000 ppm standard gelatin solution was made by dissolving 50 mg of gelatin in 50 mL of water. A Waters Corporation: Protein-Pak, 300 x 7.8 mm column was used with an aqueous mobile phase at a flow rate of 1 mL/min with an injection volume of 100 μL. Gelatin detection was preformed at 220 nm. A calibration curve was created using the linear portion of a concentration (0.5 – 10 ppm) versus peak area graph.

3.4.2 **Polycaprolactone**

HPLC was used to analyze the release of 1,4-dioxane and 6-hydroxyhexanoic acid from irradiated solutions. 1,4-Dioxane was measured by gas chromatography mass spectrometry (GC-
MS). The concentrated standard was 10,000 ppm solution of 1,4-dioxane in water. Solutions were filtered through a Millipore Amicon Ultracel 3K centrifugal filter at 4,000 rpm for 15 minutes. The column used was a Zebron WaxPlus with a length of 30 m, a diameter of 0.25 mm and a film thickness of 0.25 μm. Helium was used as a carrier gas at a flow rate of 1 mL/min. An electron impact (EI) source was used for mass spectrometry. Single ion monitoring at m/z 88 was employed to detect and quantify 1,4-dioxane. The detection limit for 1,4-dioxane is 1 ppm with a signal over noise ratio of 1:5.

A 6-hydroxyhexanoic acid aqueous standard solution (10,000 ppm) was used. Aliquots were extracted using a Waters Corporation solid phase extraction cartridge. The column used was an Agilent Technologies, HP-5MS, with a length of 30 m, a diameter of 0.25 mm and a film thickness of 0.25 μm. Helium was the carrier gas at a flow rate of 1 mL/min. An electron impact (EI) source was used as the impact source. Single ion monitoring was employed at an ion abundance of m/z 261 to measure silylated 6-hydroxyhexanoic acid. The standard peak used was m/z 131 as a reference for 6-hydroxyhexanoic acid. The detection limit for these samples was 0.2 ppm.

3.4.3 Polyurethane

HPLC was used to analyze the release of N, N-Dimethylformamide (DMF) into irradiated solutions. Standard solutions of 0.1, 0.5, 1, 5, and 10 ppm in DMF in water were prepared. The column used for 1,4-dioxane samples was used for polyurethane. Helium was used as a carrier gas at a flow rate of 1.2 mL/min. An electron impact (EI) source is used to analyze DMF and the abundance of the ion is recorded at m/z 73 for DMF. The detection limit for DMF is 0.10 ppm with a signal to noise ratio of 1:5.

Due to the limited knowledge of the PU (patent pending) degradation products where not
discussed but may be available in the future.

3.5 Differential Scanning Calorimetry, DSC

DSC was used to indicate whether there was a change in glass transition temperature, crystallization temperature, or enthalpy due to irradiation. A Perkin Elmer Diamond DSC equipped with an Intracooler 2P for subambient operation was used. Each sample was held at 30 °C for 2 min then heated from 30 °C to 300 °C at 10 °C/min, held at 300 °C for 1 min then cooled from 300 °C to -50 °C at 10 °C/min, held at -50 °C for 1 min and finally heated back up from -50 °C to 300 °C at 10 °C/min. Screening runs using a sample heating trajectory of 20 °C/min were conducted before each analysis. One sample of each irradiated and non-irradiated scaffolds were analyzed.

3.6 Trinitrobenzene Sulfonic Acid Assay (TNBSA)

TNBSA assay was preformed on gelatin scaffolds. This assay determines the number of free amino groups within a protein. For the assay, ~11 mg of dry scaffold was placed in a 50 mL centrifuge tube, using gelatin powder as a control (for irradiated, non irradiated and 3 month samples). One milliliter of 4% aq. NaHCO₃ and 1 mL of 0.5% aq. trinitrobenzene sulfonic acid were added to each tube. Centrifuge tubes were stirred in a water bath at 37 °C for four hours. Three milliliters of 6 M HCl was then added to hydrolyze the reaction mixture. Centrifuge tubes were then autoclaved at 120 °C and 15-17 psi for one hour to allow the gelatin to hydrolyze and dissolve. Once autoclaved the tubes were removed and diluted with 20 mL of diH₂O. Using a 96 well plate, solution absorbance was measured at a wavelength of 350 nm using a BioTek Multi-Mode Microplate Reader (86). A sample size of three was used in order to preform statistical analysis.
3.7 **Bicinchoninic Acid (BCA) Protein Assay**

A BCA protein assay kit purchased from Pierce Protein Biology Products was used to estimate the amount of soluble protein present after each radiation exposure. A standard procedure was used with the kit at a 5-2000 μg/mL range. Samples were read at an absorbance of 562 nm using a BioTek Multi-Mode Microplate Reader. A sample size of three, using the irradiated and 3 month PBS, were analyzed to preform statistical analysis.

3.8 **Statistical Analysis**

Statistical analysis was preformed on all tests that had a sample size of at least three. Origin data analysis and graphing software was used to create all graphs and statistical analysis in this thesis. A p-value < 0.05 was used in all t-tests and ANOVA testing was preformed to determine significant statistical differences.
4 Results

4.1 Changes in Physical Response

The three scaffold types were evaluated for their physical response to ionizing radiation by visual observation, degree of swelling, and microscopic analysis of microstructure. Gelatin samples displayed physical swelling and changes in color (Figure 4). Swelling was confirmed both after irradiation and from soaking in PBS for three months (Table III). However, irradiated samples (Figure 4 B) displayed significantly greater ($t < 0.05$) swelling compared to the 3 month samples (Figure 4 C). A one-way ANOVA and Tukey comparison was performed to determine that the 1 hour samples were statistically different than irradiated samples but not 3 month samples. Initial visual observations of irradiated and non-irradiated PCL and PU revealed little change in these samples.

Figure 4: Gelatin scaffolds under an optical microscope. A) Non-irradiated gelatin after 1 hour of rehydrating in PBS. B) Irradiated gelatin after 25 doses of radiation in PBS. C) Non-irradiated gelatin after 3 months of dissolution in PBS. There was a visual increase in size between A and B, and a change in color between all three of the samples.
Table III: Comparison of Swelling Behavior in Irradiated and Non-Irradiated Samples (* t < 0.05 for 1 hour compared to irradiated)

<table>
<thead>
<tr>
<th></th>
<th>1 hour</th>
<th>Irradiated</th>
<th>3 Months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Swelling (% increase)</td>
<td>44.08 ± 4.5*</td>
<td>66.4 ± 6.6</td>
<td>52.97 ± 2.4</td>
</tr>
</tbody>
</table>

ESEM was used to investigate any microstructure changes in the three study materials after irradiation (Figure 5). The non-irradiated gelatin sample (Figure 5 A) displayed a distinct interconnected pore structure, that became distorted after irradiation (Figure 5 B). Some of this distortion may be attributed to soaking in PBS for the 3 month duration of the experiment as observed in Figure 5 C. Pore size and shape appear altered after PBS soaking and irradiation. The 3-month samples display a smaller pore size than that of irradiated samples. From cross-sections, it seems that pore sizes decrease in size A > B > C, however, pore size quantification was not preformed for all scaffolds. An unexpected observation was the presence of salt precipitates on surfaces and inside pores of irradiated gelatin samples, as confirmed by energy dispersion spectrometry (EDS) (data in Appendix A). Very little change in the pore size, shape and interconnectivity was observed in polycaprolactone samples due to irradiation (Figure 5 D,E). For the polyurethane samples, the pore sizes and shape were found to be irregular in both irradiated and non-irradiated samples possibly due to difficulties in scaffold fabrication.
Figure 5: Representative ESEM cross-sectional micrographs of Gelatin (A-C), Polycaprolactone (D, E) and Polyurethane (F, G). Non-irradiated images are shown on the left (A, D, F) and irradiated images on the right (B, E, G). Figure C shows gelatin after 3 months of sitting in PBS.

### 4.2 Chemical Changes in Gelatin

Visual gelatin color change observed above was probed further through a TNBSA assay to determine if the extent of cross-linking had been altered. In addition, a BCA assay was performed to measure the amount of protein released into saline over time. TNBSA measures the percentage of free ε-amine groups in the three different gelatin samples as an indicator of the extent of cross-linking present. While an increase in the presence of ε-amine groups was observed for the irradiated samples compared to the non-irradiated; the soaked samples displayed the greatest increase (Table IV).
Table IV: TNBSA Showing the Percentage of Free Amine Groups found in Gelatin Scaffolds

<table>
<thead>
<tr>
<th>TNBSA</th>
<th>Non-Irradiated</th>
<th>Irradiated</th>
<th>3 Month</th>
</tr>
</thead>
<tbody>
<tr>
<td>% Free Amine</td>
<td>13.48 ± 0.14</td>
<td>22.33 ± 2.4</td>
<td>32.85 ± 3.22</td>
</tr>
</tbody>
</table>

A One-Way ANOVA statistical analysis of results was performed (n=3). It was found that p = 0.01, confirming these averages are significantly different. A Tukey mean comparison test revealed that the irradiated and 3 month samples were both significantly different that the non-irradiated samples, p < 0.05. However, a t-test was run to compare the irradiated and 3 month samples finding p > 0.05, concluding that the irradiated and 3 month sample averages are not statistically significantly different.

BCA data compares the release of protein from irradiation exposure and from three months of soaking in PBS (Figure 6). There was a significant increase in the release of protein by weeks 10 and 11 of the irradiated samples. A two sample t-test between weeks 10 and 11, found that both weeks were significantly different than week 9 (p = 4.68 X 10^-4). The 3-month non-irradiated soaked samples have a stabilized concentration over the 11 weeks of sampling. Due to the sampling technique used, the irradiated samples display variable protein concentrations during weeks 1 though 8, as only 1 mL of 5mL PBS was removed for each collection time. Since PBS was left in each well after collection, the remaining PBS may have affected the succeeding collection concentrations.
There was a significant release of protein in the three-month samples after 3 weeks, as compared to 9 weeks in the irradiated samples. * and ** indicate that both week 10 and week 11 are significantly different from week 9 (p = 4.68 X 10^-4, n=3).

4.1 Chemical Analysis of the Collected Saline

HPLC was utilized to further analyze degradation of all three scaffolds. For gelatin, release of gelatin monomer and glutaraldehyde were calculated (Figure 7). For polycaprolactone, release of 1,4-dioxane as well as the 6-hydroxyhexanoic acid were calculated. For polyurethane, release of dimethylformamide (DMF) was calculated.

4.1.1 Gelatin

Gelatin fragments were found to be present in the collected saline (Figure 7) in a similar trend to the BCA analysis (Figure 6). Together, these data sets reveal that irradiated samples
have a significant increase in the gelatin degradation starting at week 10, which may indicate a maximum threshold of irradiation. This may also indicate gelatins normal bulk degradation behavior. No glutaraldehyde was detected during this same time frame.

Figure 7: UV detection of gelatin fragments throughout radiation process. A larger increase in gelatin fragments was measured beginning at week 10. (n=1)

A calibration curve was created using HPLC methods for the glutaraldehyde analysis (detection limit at 0.05 ppm). No detectable glutaraldehyde was released in any of the gelatin samples.

4.1.2 Polycaprolactone

The release profile of PCL was analyzed by the presence of two different chemicals: 14-dioxane (solvent) and 6-hydroxyhexanoic acid (degradation monomer). Neither chemical was found in the GC-MS analyses. Surprisingly, a large amount of glycerol was discovered (Figure 8).
There was a peak in glycerol concentration at week 5. The concentration of glycerol begins to decrease after week 5 and was at its lowest concentration at the end of radiation dosing. A good representation of glycerol release throughout the testing period is shown in Figure 8. Due to the large number of samples only selected samples were used to calculate glycerol concentrations.

4.1.3 Polyurethane

Polyurethane samples were analyzed for the release of DMF using GC-MS. Results show that there was no DMF released in any of the samples.

4.2 Differential Scanning Calorimetry (DSC)

Changes in melting profiles and crystallinity of non-irradiated and irradiated samples were examined by DSC. Changes in crystallinity were calculated by:
\[ \Phi = \frac{\Delta H}{\Delta H_f} \quad (87) \]

Where \( \Phi \) represents the fractional crystallinity (which multiplied by 100 gives the percentage), the heat of fusion \( \Delta H \) is the area under the curve calculated through DSC graphs, and \( \Delta H_f \) is the heat of fusion of the same polymer at 100% crystallinity. The 1st melt scan was used to estimate the changes in crystallinity for both PU and PCL. The 1st melt was chosen to more accurately see changes in the results. The 2nd melt and crystallization peaks may show skewed results due to material deformation from heating and melting the polymers during DSC scans.

4.2.1 Gelatin

The gelatin samples showed no difference between non-irradiated and irradiated samples in DSC scans.

4.2.2 Polycaprolactone

Irradiation of PCL caused a decrease in enthalpy, \( \Delta H \) (heat of fusion) in both the melt region and crystallization regions of the DSC profiles (Figure 9). The decrease in \( \Delta H \) between non-irradiated and irradiated PCL revealed there was less crystalline order in the irradiated polymers leading to decreased crystallinity (Table V). PCL has a more complex temperature profile than the other scaffolds displaying two different melting areas as well as a crystalline region. The \( \Delta H \) values were calculated from the area of the DSC curves. This means that the decrease in \( \Delta H \) also represents a decrease in the area from non-irradiated to irradiated. The peak temperature for non-irradiated and irradiated 1st melt was 58.9 °C and 58.2 °C, respectively.
Changes in crystallinity (Table V) of PCL and PU were calculated using the 1st melt, $\Delta H$ and the equation previously shown. A purely crystalline sample of PCL has been reported as $\Delta H = 139.5$ J/g (88). This value was used to calculate the crystallinity of the PCL scaffolds. A purely crystalline samples of PU has been reported as $\Delta H = 136$ J/g (89) and was used to calculate the crystallinity of the PU scaffolds.

<table>
<thead>
<tr>
<th></th>
<th>Non-irradiated</th>
<th>Irradiated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polycaprolactone</td>
<td>53 %</td>
<td>4.9 %</td>
</tr>
<tr>
<td>Polyurethane</td>
<td>103.3 %</td>
<td>18.2 %</td>
</tr>
</tbody>
</table>

Table V: Change in Crystallinity
4.2.3 Polyurethane

Polyurethane samples displayed a large difference in DSC scans between non-irradiated and irradiated samples as well (Figure 10). The DSC scans show a decrease in area (in correspondence with $\Delta H$) and that peak temperature remained similar for the non-irradiated and irradiated samples (96.44 °C and 91.37 °C respectively). The irradiated samples have a decrease in $\Delta H$ similar to the decrease in the PCL (Figure 9). The 1st melt peak of PU was observed indicating that the amorphous PU, after being heated past it’s glass transition temperature, was more randomly ordered prior to the 2nd melt. Therefore only the 1st melting peak was used. Since PU is already an amorphous material the DSC observed changes in long and short-range order. Irradiated samples show ~5x less order (crystallinity) than non-irradiated samples (Table V).

![Figure 10: A comparison of non-irradiated and irradiated polyurethane scaffolds using differential scanning calorimetry. A large decrease in $\Delta H$ was seen after samples had been irradiated. (n=1)](image)
Both PCL and PU exhibit decreases in $\Delta H$ after being irradiated. PU has a much larger decrease in order than the decrease seen in PCLs crystallinity, 85.1 % and 48.1 % respectively.
5 Discussion

5.1 Gelatin

Physical changes due to irradiation were expected in gelatin since previous research has shown radiation causes damage to normal tissue. However, since gelatin was used as a human tissue equivalent for this study, it was surprising that the effects were so profound.

Gelatin samples displayed the greatest physical changes due to irradiation compared to PCL or PU. Changes in color and swelling were noted, and obvious changes in microstructure of irradiated gelatin were seen in the ESEM images, but not found in either PCL or PU. This indicated that irradiated gelatin samples may be crosslinking and degrading at the same time. Due to the fact that the crosslinking agent (glutaraldehyde) causes gelatin to turn orange once crosslinked, it was expected that the change in color of the irradiated samples from orange to clear indicated uncrosslinking of gelatin. The degradation profiles (TNBSA, BCA, HPLC) confirm that the gelatin was degrading, as large amounts of gelatin were released as well as increased free amine groups. The degradation of gelatin may also be leading to the material swelling, as the bonds break and the material is allowed to expand. This was confirmed by the increase in free amine groups that was observed in both 3 month samples and irradiated samples. It is important to note that irradiated samples have a lower percentage of free amine groups than 3 month samples, suggesting the irradiation was causing the scaffold to maintain more of its bonds. This may be due to an increase in crosslinking from x-ray radiation or it may be a result of the salt precipitates (see also Appendix A).

A phenomenon only occurring in irradiated gelatin samples was the formation of salt precipitates (Figure 12, Figure 13). These salt precipitates may contribute to the stability of gelatin scaffolds during radiation due to a salt shielding (90). Recently discovered in
*Halobacterium salinarum*, intracellular salts provided cellular protection and detoxification, as well as a repair mechanism for oxidative damage due to irradiation. Therefore salt precipitates, once formed, could have protected gelatin samples in this study from a portion of radiation damage and degradation. This article provides evidence that chloride salts protect by scavenging hydroxyl radicals via electron transfer, which then produces chloride radicals (90). These salt radicals are much less reactive and decrease the oxidative damage (90). One question that remains is how these salt precipitates formed on the gelatin samples. This is a topic that may need to be investigated further.

BCA and HPLC analysis show similar degradation profiles for irradiated solutions. Peak degradation occurs weeks 10 and 11, after fairly uniform degradation rates. Due to the testing methods, a uniform profile seen in the early weeks may not be representative; however, the significant increase in weeks 10 and 11 cannot be explained by testing anomalies. If these tests are repeated, it will be important to remove all of the PBS from each sample thus allowing each new collection to be unaffected by previously irradiated PBS.

This sudden increased release of gelatin monomer was only seen in irradiated samples and not 3 month samples suggesting the increase must be due to irradiation. One possible reason for the increase could be that gelatin monomer has a maximum threshold for radiation after 10 weeks (~48 Gy). Examining the HPLC results, no glutaraldehyde was released in any of the irradiated samples. This many indicate that the degradation in the material was not occurring from degradation of the glutaraldehyde bonds. Gelatin’s degradation occurs primarily from hydrolysis of peptide bonds (91). These bonds are located within the backbone and side chains of the gelatin material but are also found in the glutaraldehyde crosslinking. The degradation in the material could be a result of one bond of glutaraldehyde breaking (Lewis Acid bond) and not all of them, allowing for the glutaraldehyde to still be partially crosslinked in the gelatin (Figure
This may be why there was only a partial change in color in the irradiated samples.

Figure 11: Crosslinking mechanism of a protein (in this case gelatin) and glutaraldehyde (92).

Gelatin has also been show to be most stable at a pH between 5-6 and temperatures below ~50 °C (91). As the gelatin is irradiated it may be exposed to increased temperatures from the X-ray as well as changes in pH, which may alter degradation. Further investigation is needed to pinpoint the mechanism of degradation and better analyze affects X-ray may cause in the system.

The irradiated and non-irradiated gelatin exhibited no difference in DSC profiles. These results are in conflict with the HPLC/BCA data. Bonds within the gelatin are breaking (HPLC/BCA) indicating that there should have been some difference in the peak temperature or heat of fusion between irradiated and non-irradiated gelatin. Since there was a change in structure of the gelatin as well as possible backbone breakage the material should be less ordered showing a decrease in enthalpy (ΔH) in DSC scans. It is possible that changes in gelatin were not noticeable in the range of the DSC used. The DSC used to analyze all samples in this study
was unable to analyze at temperatures below 30 °C. Earlier research reveals that the glass transition temperature of gelatin (10 wt. %) can be observed at 37 °C with a gel formation temperature of 22.5 °C (93). In order to quantify this glass transition temperature, S.E.B. Petrie and R. Becker increased the sensitivity of the differential thermal analyzer (DSC) with a microvolt amplifier. It will be important to rerun DSC scans with an alternative DSC able to analyze at lower temperature. The increase in sensitivity that the microvolt amplifier provided in a previous study may be needed to analyze gelatin using DSC.

This research was conducted to understand the effects of radiation on polymeric materials. Gelatin displayed the most noticeable changes due to radiation, which is concerning since gelatin is a mammalian derived tissue and can be considered a human tissue equivalent. Future studies need to be performed to further understand the extent of therapeutic radiation effects on gelatin, in particular the formation of precipitates, the sudden increase in release profile in both the BCA and HPLC results and the reason for the irradiated gelatin’s structural stability even though it seems to be undergoing degradation.

### 5.2 Polycaprolactone

In this investigation PCL was used as a possible biodegradable material since γ-radiation has been shown in some studies to increase degradation rate of PCL. Looking back at PCL studies (Table II) many studies disagree, and no general conclusion has been made on the effects of radiation on PCL. Under γ-radiation PCL has been known to crosslink and/or degrade, depending on the dose rate and amount. In this investigation it was hypothesized that the material would degrade more than crosslink when PCL was been exposed to therapeutic radiation. It was difficult to conclude either crosslinking or degradation since PCL showed little response to therapeutic x-ray radiation levels. There were no visual changes in the microstructure of PCL.
samples. HPLC was used to determine whether PCL degradation was occurring caused by radiation but PCL monomers were not present in the saline. PCL degrades in two different stages, the 1st stage involves molecular weight decreases without deformation or weight loss, the second stage is when molecular weight begins to decrease and the material breaks into pieces (94). The second stage is when 6-hydroxyhexanoic acid would be released since it is a final degradation product in PCL (95). It is possible that the PCL samples were still in the 1st stages of degradation, which would confirm the absences of 6-hydroxyhexanoic acid in the HPLC results. In order to further analyze the degradation of the PCL molecular weight calculations would need to be done. During HPLC analysis glycerol was detected and was further analyzed since available commercial information did not include glycerol as a possible impurity or fabrication addition. The process used in this investigation to fabricate PCL scaffolds also did not involve the use of glycerol during the fabrication of the scaffolds, so the glycerol was introduced prior to receiving the PCL pellets. Glycerol release may be due to the stretching of the chains shown in the DSC scans. The DSC showed a decrease in $\Delta H$ after PCL scaffolds were irradiated. PCL scaffolds crystallinity decreases 48.1 % after irradiation, creating larger amorphous regions within the scaffolds. This may be a possible mechanism for release of glycerol, if it was an impurity in the PCL. As the material begins to lower in crystallinity, glycerol was released until it release rate peaks at 5 weeks. At 5 weeks most of the glycerol had already been released and therefore the concentration of glycerol began to decrease, as less glycerol was available.

It was not expected for PCL to not respond under therapeutic radiation. Although the PCL seemed to be decreasing in crystallinity, there were very few other noticeable changes. PCL may need a prolonged investigation time to evaluate irradiation effects since it can take 6 months to 2 years for PCL to degrade in vivo and >180 days in vitro depending on molecular weight (94, 96).
5.3 Polyurethane

PU was used as a control in this study. It was not expected to show any change due to irradiation as it was designed to be a non-degradable biomaterial. Previous studies (Table II) have used PU only in sterilization processes and the materials had withstood those high radiation levels (exception: biodegradable PU). This investigation shows that the low doses of therapeutic X-ray radiation were having an effect on PU.

There were small inhomogeneities in the morphology of PU, but this may be due to fabrication approaches. The PU scaffolds had to be cut with a cork borer and uneven cuts may have caused differences in PU cross sections. Smaller PU scaffolds were cut from larger PU scaffolds. It is possible that pore structure throughout the bulk was not uniform, thus when the smaller scaffolds were cut to size, they displayed heterogeneous microstructural areas. Since PU was difficult to dissolve in chloroform, it may not have been uniformly distributed causing larger concentrations of PU to occur in some samples and not others. Overall, this small change in microstructure could be due to the fabrication method rather than the irradiation.

Differences in scaffold fabrication may account for differences in DSC spectra as well, although the scaffolds used for non-irradiated and irradiated samples were from the same larger PU scaffold. If it is assumed that these sampling differences were minimal, DSC results contradict the perception that PU is a non-degradable material. With an 85% decrease in crystallinity it is difficult to correlate such a large change to variances in the fabrication. Since the molecular weight of the PU is unknown, the non-irradiated crystallinity is above 100% meaning that the purely crystalline PU used to calculate the literature value for heat of fusion most likely had a lower molecular weight than the PU used in this investigation.

Very few studies, if any, have investigated the use of X-ray radiation on PU (Table II) and it may be true that PU is not degradable under gamma radiation, but there seems to be some
kind of chain movement decreasing crystallinity during exposure to therapeutic x-ray radiation doses. This decrease in crystallinity may ultimately cause this material to degrade. Due to the limited information provided about the PU monomer it is difficult to conclude whether the changes in PU were purely due to the therapeutic radiation.
6 Conclusion

Therapeutic radiation has been used since the X-ray was invented (1980s) for all types of cancer treatments and medical diagnostics. Although doses have evolved and new techniques have helped to improve therapeutic radiation delivery, there is still a lack of knowledge in the effects on human tissue. This investigation showed that normal doses of therapeutic radiation used in cancer treatments affect biomaterials that are currently already being use in clinical applications.

After therapeutic radiation, gelatin samples show degradation but maintain structural stability. The reason for this stability is not fully known, but may be the results of salt shielding and/or crosslinking. These findings are important because gelatin is considered human tissue equivalent. If therapeutic radiation doses are degrading gelatin, what effects may they be having on human tissue?

Irradiated PCL shows decreases in crystallinity (34%) The decrease in crystallinity may be allowing the release of glycerol in to the PBS solution shown in the HPLC results. This glycerol release is important to note because PCL is already used in some biomedical applications. If this glycerol is released into the human body after being irradiated it could cause negative side effects. PU shows similar decreases in crystallinity (85%) from therapeutic radiation to those observed for PCL but PU also shows changes in pore size. Although it is difficult to say whether the pore size alteration is a result of fabrication or irradiation.

Overall, the most important findings in this investigation were the complete alteration of the gelatin scaffolds, both in the physical and chemical parameters and the unexpected release of glycerol from the PCL scaffolds.
6.1 Future Work

In the future it would be beneficial to further investigate the effect of therapeutic radiation on gelatin. A study into what factors are stabilizing scaffolds could lead to a protective strategy, and such a study could show what types of bonds are actually breaking. It is also important to conduct more analysis into the degradation of gelatin both before and after X-ray radiation. Which could be done using Fourier transform infrared spectroscopy (FTIR) allowing for analysis of all the different side chain and crosslinking mechanisms in gelatin. It would be advantageous to do further mechanical studies to determine gelatin strength before and after irradiation, as well as finding a way to increase the sensitivity of DSC measurements to measure changes after irradiation.

The PCL and PU samples need to be more thoroughly characterized before irradiation to better differentiation between therapeutic radiation affects. It may also be beneficial to do further HPLC analysis on the PU and PCL samples to be sure that all 1,4-dioxane and N, N-dimethylformamide are being removed before irradiation. Another future direction may be to test PCL and PU in PBS for 3 months without irradiation, to determine if the changes in DSC are primarily due to radiation. A better fabrication method for PU is needed to create more uniform scaffolds. This is needed determine whether changes in pore size are due to irradiation rather than physical variables caused by fabrication.

This investigation was meant to collect preliminary data on the effects of therapeutic radiation; however, it revealed significant alteration to a primary tissue surrogate, gelatin, which is worrisome and should be investigated further. It also produced unexpected results for a biodegradable polymer, PCL, which is a candidate material already used in biomedical applications. In order to better analyze HPLC, BCA and TNBSA results, the PBS collection technique needs to be altered. In place of collecting only 1 mL of PBS from each well, results
would be more accurate if all the PBS from each well was collected and then refilled allowing for collected PBS to be unaffected by previous doses.
Appendix A

Energy Dispersion Spectrometry (EDS)

After imaging the gelatin samples in the ESEM (Figure 12), a precipitate was seen forming throughout the irradiated samples only.

Figure 12: ESEM images show salt precipitates on the surfaces (A, C) and within the pores (B) of irradiated gelatin samples. Salt covers all surfaces and is confirmed in the EDS scans.

In order to characterize what these precipitates were, EDS was used to determine the chemical structure. It was found that these precipitates were salt crystals and were not found on the 3-month samples or the non-irradiated samples.
Looking at the spectrums, the Sodium (Na) and Chloride (Cl) peaks are prominent in the irradiated samples (Figure 13 A) and are not seen in the non-irradiated samples (Figure 13 B). The non-irradiated samples only show prominent peaks of carbon, which is also seen as a small peak in the irradiated spectrum. The salt precipitates were not seen in PCL, PU or the three-month gelatin samples.

One possible source of these salt crystals is from the PBS saline. In the gelatin samples that were irradiated, x-ray radiation may have caused the salt in the PBS to somehow bind with the gelatin. These results were unexpected but have not yet been analyzed or investigated further.
Appendix B

Alginate

Alginate was originally used as a natural scaffold for the irradiation study. Due to the rapid dissolution of the material it was excluded from further study. Results from the alginate scaffolds are located in this appendix.

Materials and Methods

Alginic acid sodium salt (65-70% guluronic acid, 25-35% mannnuronic acid), from brown algae (Sigma Aldrich) and calcium chloride (CaCl\textsubscript{2}) (Sigma Aldrich) were used to create the alginate gels used in the studies.

Many different ratios and concentrations of alginic acid to CaCl\textsubscript{2} were analyzed through visual and heat drying techniques to determine the ideal concentrations for testing. The concentration chose was a 4:1 ratio of .25 wt. % alginic acid solutions in deionized (DI) water with 0.05 wt. % CaCl\textsubscript{2} solutions in DI water. Both of these solutions were created using deionized water. Once the solutions were made a double barrel syringe was used to mix the two. Alginate solution immediately crosslinked with CaCl\textsubscript{2} to create gel scaffolds.

The alginate was irradiated in the same process as the other samples to maintain consistent testing methods. Alginate was analyzed using HPLC and MS-GC. Using HPLC calcium release was measured from the alginate samples. Calcium standards were prepared in sodium phosphate buffer 0.1 M pH 7.4. Samples were filtered through an Amicon Ultracel 3K centrifugal filter by Millipore at 4,000 rpm. Atomic Absorption Spectrophotometry instrument was used to measure calcium release; the detection wavelength for calcium is 422.7nm.
Results

These samples completely dissolve within approximately four days of sitting in phosphate buffered saline (PBS). Looking at the high performance liquid chromatography results (Figure 14) there is an immediate high release of concentrations of calcium. This release corresponds with the dissolution of the alginate within the first week.

![Graph](image)

Figure 14: Gas chromatogram and UV spectrum release profile for alginate throughout radiation process. Shows an immediate release and then maintains a constant concentration of calcium after 5 weeks.

A control of alginate in PBS with no radiation at room temperature was used to determine if the dissolution was due to the radiation or the gels themselves. Alginate gels were shown to completely dissolve within four days, independent of radiation exposure.

Summary

In conclusion alginate gels dissolved too quickly to analyze for a soft tissue application. Both irradiated and non-irradiated samples dissolved therefore are not an applicable material for this study. These samples were also found to dry out quickly and need to be placed in PBS.
throughout experimentation. Research did find that alginate is capable of being cross-linked using glutaraldehyde similar to gelatin, which may be an alternative fabrication method for future studies to decrease the rate of dissolution of alginate.
References

17. Radiation Doses in Perspective 2013 [cited 2013 September 29]. Available from:


