Polypyrrole: An Interactive Substrate for Bone Regeneration

by

Nahid Rahman

S.B. Chemical Engineering, M.I.T. (1996)

Submitted to the Department of Materials Science and Engineering
in partial fulfillment of the requirements for the degree of

Master of Science

at the

MASSACHUSETTS INSTITUTE OF TECHNOLOGY

June 1998

Author .....

Department of Materials Science and Engineering
May 8, 1998

Certified by .....
Robert S. Langer
Germeshausen Professor of Chemical and Biomedical Engineering
Thesis Advisor

Certified by .....
Michael F. Rubner
TDK Professor of Materials Science and Engineering
Thesis Reader

Certified by .....
Venkatram (Prasad) Shastri
Post-Doctoral Associate of Chemical Engineering
Thesis Co-Advisor

Accepted by .....
Linn W. Hobbs
John F. Elliott Professor of Materials
Chairman, Departmental Committee on Graduate Students

Polypyrrole: An Interactive Substrate for Bone Regeneration

by

Nahid Rahman

Submitted to the Department of Materials Science and Engineering on May 8, 1998 in partial fulfillment of the requirements for the degree of Master of Science in Materials Science and Engineering

ABSTRACT

Current methods of bone repair rely on autografts (bone from a donor site) and allografts (bone from human cadaver). However, these methods are plagued with disadvantages. There is a clear and urgent need to provide alternatives for regenerating and repairing bone.

Bone is known to be one of the many connective tissues in the body that are responsive to exogenous electrical stimulation. Based on this principle, this thesis explores the potential of using an electrically conducting polymer, polypyrrole, as a substrate for bone regeneration.

Optically transparent thin films of polypyrrole, with a polyanionic dopant, poly(styrenesulfonate), were synthesized electrochemically and characterized by X-Ray Photoelectron Spectroscopy, UV/VIS spectroscopy, Scanning Electron Microscopy and by electrical conductivity measurements. In this study, Bone Marrow Stromal Cells (BMSC), which are the progenitor cells to bone cells (osteoblasts), were used as the *in vitro* model system. Their viability, proliferation and differentiation capabilities were evaluated on polypyrrole, in the absence and presence of electrical stimulation. Results indicate that polypyrrole is ideally suited as a substratum for BMSC growth and differentiation. The application of an electrical stimulus through the polypyrrole substrate was found to induce the differentiation of BMSC towards an osteogenic lineage. Thus, polypyrrole, by virtue of its conductive properties, its *in vitro* biocompatibility and its flexibility in altering surface characteristics, has an exciting potential as a suitable interactive substrate for bone regeneration.

Thesis Supervisor: Robert S. Langer
Title: Germeshausen Professor of Chemical and Biomedical Engineering

Table of Contents

List of Illustrations and Figures

Acknowledgements

First, and foremost, I would like to thank my advisor, Bob Langer, for giving me the opportunity to work with him. I have known Bob for many years, including my time spent at MIT as an undergraduate, and he has never failed to amaze me with his dynamic personality, energy, and constant support and encouragement.

Second, thanks are also due to my invaluable mentor, Prasad Shastri. I am grateful to him for introducing me to the project and also for his continuous guidance and insightful discussions during the full course of the project. I would also like to thank Ivan Martin for his time and effort in teaching me cell culture techniques as well as for discussions on bone biology. Thanks are also due to Professor Michael Rubner for important discussions during the thesis writing process.

I would also like to thank the following individuals for their help during various stages of the study: Libby Shaw for UV/VIS spectroscopy, Mike Frongillo for SEM data, Yuan Lu at the Harvard McKay Lab for use of their XPS facility.

I enjoyed working in the multinational Langer Lab environment and I would also like to thank the following people for being there for me: Amir Nashat, Tommy Thomas, Rebecca Carrier, Alexander Zelikin, and my great office mates, Sachiko Hirosue, Jennifer Elisseeff and Shuichi Ando.

Finally, I would like to express my gratitude to my parents for giving me the opportunity to study in the United States. I am indebted to them for their love and support.

CHAPTER 1: INTRODUCTION

1.1 Objectives

The goal of this thesis has been to develop a system to evaluate the candidacy of polypyrrole as an interactive substrate for bone regeneration. In order to reach this objective, several steps have been completed:

1. A polymer system was developed to act as the conductive substrate for bone cell studies.
2. The attachment, proliferation and viability of bone cells *in vitro* were established in the absence of electrical stimulation.
3. The attachment, viability and differentiation of bone cells *in vitro* were established in the presence of electrical stimulation.
4. A mechanism was proposed for the interactions between the polymer system and the bone cells.

1.2 Outline of thesis

This thesis is organized into eight chapters. A general background on bone regeneration and electrically conducting polymers is presented in Chapter 2. Polymer synthesis and characterization are described in Chapter 3. Chapters 4 and 5 describe the interactions of bone cells and the polymer system in the absence of electrical stimulation. Chapter 6 gives a final evaluation of the polymer system as an interactive substrate for bone cells when an electrical stimulus is applied. The key conclusions of the studies are summarized in Chapter 7 and future recommendations are presented in Chapter 8.

CHAPTER 2: BACKGROUND

2.1 Bone regeneration

Over 1 million surgical procedures in the United States each year involve bone repair [1]. Bone defects can result from diverse causes such as trauma, birth defects and disease pathoses. Current methods rely on an adequate supply of autogenous (from a donor site) and allogeneic (from a human cadaver) bone. However, removal of autogenous bone for the grafting procedure requires surgery at a second site and also involves blood loss, pain and increased morbidity. Furthermore, for allografts, there exists the potential for disease transmission or host rejection. Thus, the search for alternatives to autografts and allografts in bone repair and regeneration remains an important topic in medical research.

A variety of biologically compatible materials have been tested for use in bone repair. The materials include naturally occurring compounds such as tricalcium phosphate or hydroxyapatite porous ceramics [2, 3], and synthetic materials including absorbable lactide and glycolide polymers, [4, 5] and ceramic bioglasses [6]. The existing technology, using biomaterials, though effective in many cases, is still beset with numerous difficulties and disadvantages. Thus, there still exists a critical need for improved methods in treating bone defects.

In 1952, Yasuda discovered an interesting property in bone [7]. He first reported that upon mechanical deformation of bone, electricity was produced, the phenomenon known as the "piezoelectric" effect. He showed that the mechanical loading of bone induced electromagnetic potentials or fields that could alter bone metabolism and

produce an increase in bone mass and/or structure [8]. Further studies undertaken by Fukada, Becker, Bassett and others have suggested that the electrical activity observed in bone is a probable mediator of its repair and adaptive remodeling in response to mechanical loading (Figure 2.1)[9-12]. Furthermore, the authors observed that an exogenous electrical stimulus *alone* could stimulate bone regeneration [13-19]. The early success of these experiments with direct current and electromagnetic induction finally led to widespread clinical treatments of non-union bone fractures. However, the importance of localizing the electrical stimulation still remains a challenge [20]. Therefore, a system whereby one can externally control and regulate the stimulus would be extremely attractive.

The concept of "tissue engineering" comes into play here in the development of such a system. Tissue engineering is a field in which the principles of biology, engineering and materials science are applied to the development of functional substitutes for damaged tissue. Langer and co-workers have been developing biodegradable polymer systems that are being explored as three-dimensional matrices for cell transplantation and tissue regeneration [1]. With this approach in mind, one can envision materials that can exploit the piezoelectrical nature of bone and act as interactive scaffolds for bone repair. An interesting class of synthetic polymers that can take advantage of these properties are the electrically conducting or electroactive polymers. Based on their ability to respond to electrical or electromagnetic stimuli, they can act as an interface between the external and physiological environments of a connective tissue such as bone, which is capable of undergoing repair and regeneration on exposure to the same stimuli [21].

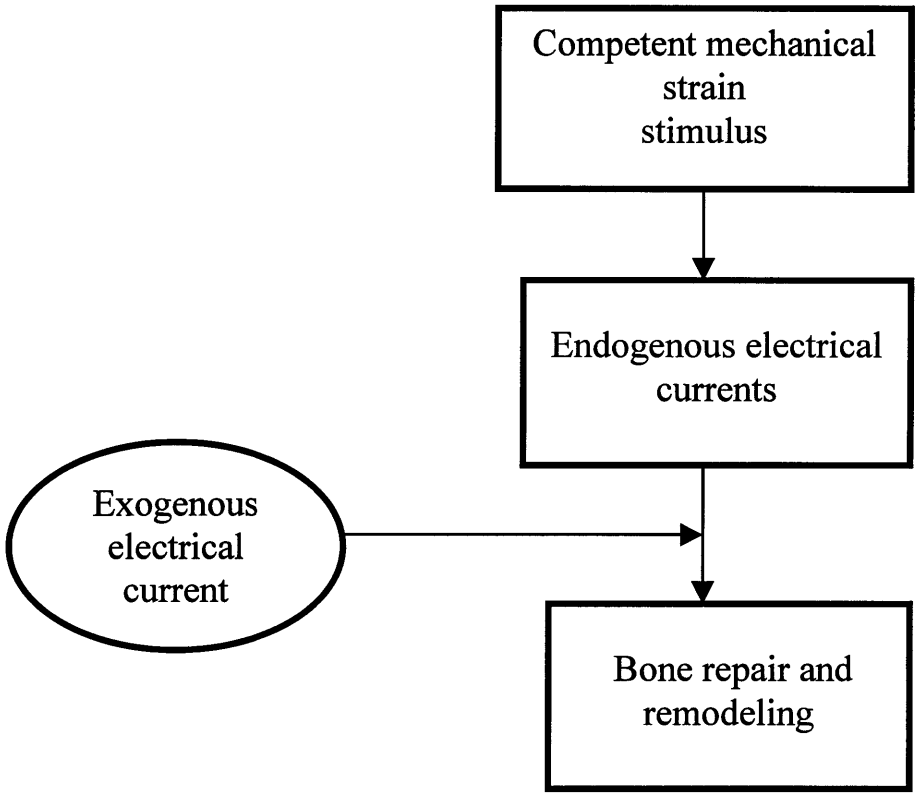


Figure 2.1: "Yasuda's hypothesis". Modified from: Spadaro, A. *Mechanical and Electrical Interactions in Bone Remodeling*. Bioelectromagnetics, 1997. **18**: p. 193-202

An excellent candidate for such an application is polypyrrole, belonging to this class of polymers. The characteristics of this polymer are discussed in the following section.

2.2 Electrically Conducting Polymers

A key property of most polymers, which distinguishes them from metals, is their inability to conduct electricity. However, during the past 25 years, a new class of organic polymers has been devised with a remarkable ability to conduct electrical current. These electrically conducting polymers typically possess a conjugated backbone with a high degree of π-orbital overlap. Through a process known as "doping", the neutral polymer can be oxidized or reduced to become either positively charged (oxidative, p-type) or negatively charged (reductive, n-type). The generation and propagation of charge occurs via polarons or bipolarons along the oxidized polymer backbone. The conductive form of the polymer contains counterions that serve to maintain charge neutrality but do not affect the oxidation level of the polymer. Among the electrically conducting polymers, polypyrrole, polythiophene, poly(p-phenylene) are the most promising, especially for tissue engineering applications.

Polypyrrole, which belongs to the class of aromatic poly(heterocyclics), has been extensively studied. It was first electrochemically synthesized by Diaz and co-workers, [22] and can be synthesized both by chemical and electrochemical means. Through the "doping" process, charge neutrality is maintained by incorporating dopant ions into the polymer backbone. Figure 2.2 shows the mechanism by which the electronic conduction

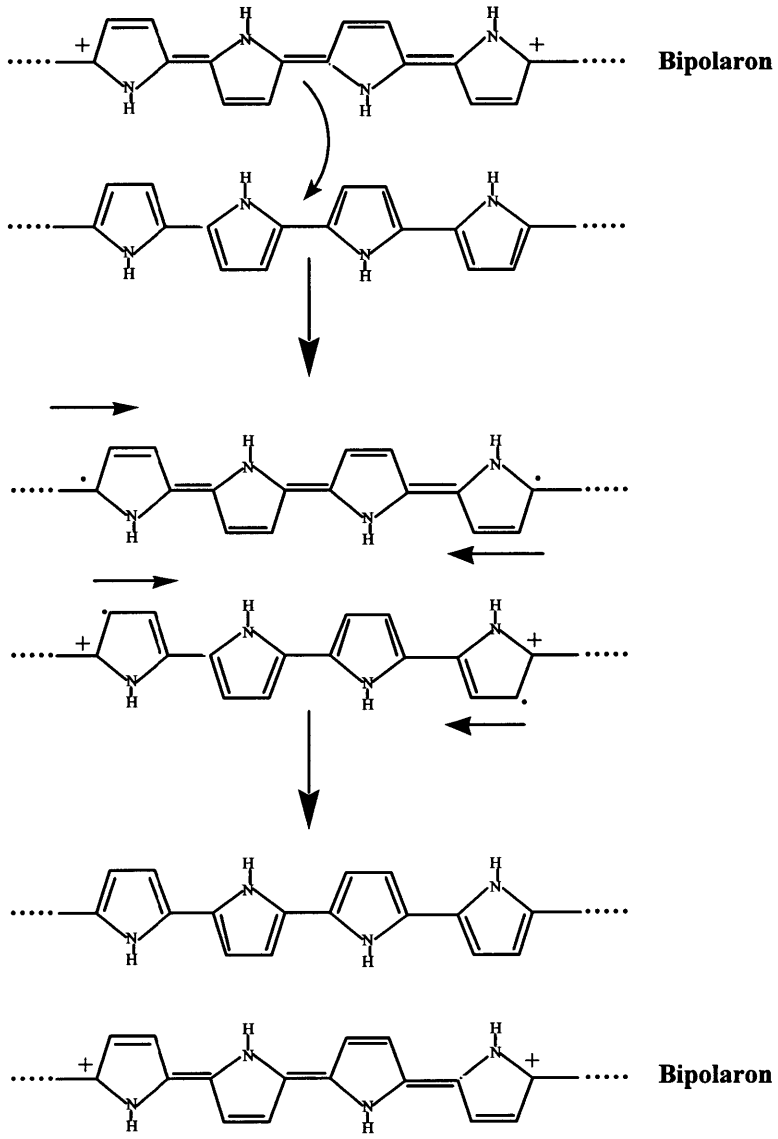


Figure 2.2: Mechanism of electronic conduction in oxidized polypyrrole (PP) via interchain hopping of bipolarons. Reproduced with permission from: Shastri, V.R., *Evaluation of polypyrrole thin films as substratum for mammalian cell culture*. 1995, Rensselaer Polytechnic Institute: Troy, NY.

in polypyrrole occurs. The neutral polymer chain is oxidized with the removal of electrons to form radical cations. The radical ions are delocalized over a portion of the backbone, creating a structural defect known as a polaron, which contains both spin and positive charge. Two polarons can diffuse together and combine spins to form a bond, leaving a bipolaron species. The positive charges on the polymer backbone act as charge carriers for electrical conduction. Conduction can either occur along segments of the conjugated chain, or through charges hopping between chains.

Polypyrrole has been studied extensively due to its chemical and thermal stability, ease of preparation and electroactivity. It has been evaluated for a number of applications, such as amperometric glucose sensors [23], creatinine microbiosensors [24], immobilized enzyme-based analyte detection systems [25] and electrodes to obtain electrochemically controlled dopamine release [26, 27]. Furthermore, its *in vitro* compatibility with mammalian cells has been explored [28, 29]. From these studies, it was shown that both cell-surface interactions and cellular functions on polypyrrole thin films could be controlled by either changing the oxidation state of the polymer or by changing the wettability of the polymer through the use of suitable dopants. Since surface characteristics such as charge density and wettability play a key role in protein-adsorption and cell-biomaterial interactions [30-32], it is desirable to engineer a material that allows flexibility in predicting cellular behavior.

Polypyrrole is a particularly interesting candidate for studying bone repair and regeneration. Since bone is responsive to external electrical or electromagnetic stimulation, it is possible to fabricate an interactive scaffold by using polypyrrole as the

substratum through which one can control the cellular processes involved in bone regeneration. Furthermore, polypyrrole has been evaluated as a candidate for nerve regeneration [33, 34]. It was shown that neurite outgrowth of PC-12 cells, an immortal nervelike cell line derived from rat pheochromocytoma, was significantly enhanced on polypyrrole thin films upon the application of an electrical stimulus. Based on similar underlying principles, polypyrrole can be explored as a possible substratum for bone regeneration.

CHAPTER 3: POLYMER SYNTHESIS AND CHARACTERIZATION

3.1 Introduction

Polypyrrole (PPy) was synthesized electrochemically and characterized by X-Ray Photoelectron Spectroscopy (XPS), UV/VIS spectroscopy and Scanning Electron Microscopy (SEM) and conductivity measurements,

3.2 Experimental

3.2.1 Chemicals and Materials

Pyrrole and sulfonated polystyrene sodium salt (MW 70,000) were obtained from Aldrich (Milwaukee, WI). Activated alumina was purchased from Mallinckrodt (Chesterfield, MO). Indium tin oxide (ITO) conductive borosilicate glass (40 Ω/square, 50 x 25 mm) was obtained from Delta Technologies (Still Water, MN) and used as the electrochemical conductive surface for PP film deposition. Hexane, dichloromethane and methanol were purchased from EM Science (Gibbstown, NJ). A platinum gauze (99.9 % Pt, 52 mesh, woven from 0.1 mm diameter wire) served as the counter electrode and was purchased from Aldrich Chemical Co. An Ag/AgCl electrode was purchased from Fisher Scientific (Pittsburgh, PA) and was used as the reference electrode. Ultrapure water was obtained from a Millipore Milli-Q Reagent Water System (Bedford, MA).

3.2.2 Methods and Procedures

3.2.2.1 Polymer Synthesis

Pyrrole was passed through an activated alumina column, consisting of a standard

9" pasteur pipette plugged with glass wool and packed with activated alumina, until it became colorless. Indium tin oxide substrates were ultrasonically cleaned in hexane, methanol and dichloromethane sequentially for 5 min each.

A three electrode setup was used for the electrochemical synthesis of polypyrrole (PPy): the ITO glass acted as the working electrode, the platinum mesh as the counter electrode and the Ag/AgCl electrode as the reference (Figure 3.1). The electrodeposition solution contained 0.1 M pyrrole and 0.1 M sodium salt of poly(styrenesulfonate) (PSS) and Milli-Q ultra pure water. The sodium salt of poly(styrenesulfonate) served as both the electrolyte and dopant. An EG & G® Princeton Applied Research Potentiostat/Galvanostat Model 253A (Princeton, NJ) was employed as the constant voltage source. PPy films (0.1-0.15 μm) were deposited onto the ITO glass at a constant potential of 0.7 V versus the Ag/AgCl reference electrode. The film thickness was controlled by the passage of charge: a charge of 26.2 mC/cm^2 yields a PPy film of 0.1 μm in thickness [35].

3.2.2.2 X-Ray Photoelectron Spectroscopy (XPS)

Spectra were obtained using a Surface Science Laboratories X-100 spectrometer (Mountain View, CA) employing a monochromatized Al Kα (1486.7 eV) Xray source operated under a source chamber vacuum of ~ 1 $x10^{-9}$ torr. Core level spectra were taken at a take-off angle of 35° (measured with respect to the normal to the sample surface). Photoelectrons were analyzed by a hemispherical multichannel detector in fixed analyzer transmission mode. An electron flood gun (energy 5 eV) was used to compensate for charging during X ray Photoelectron Spectroscopy data acquisition. A nickel mesh in

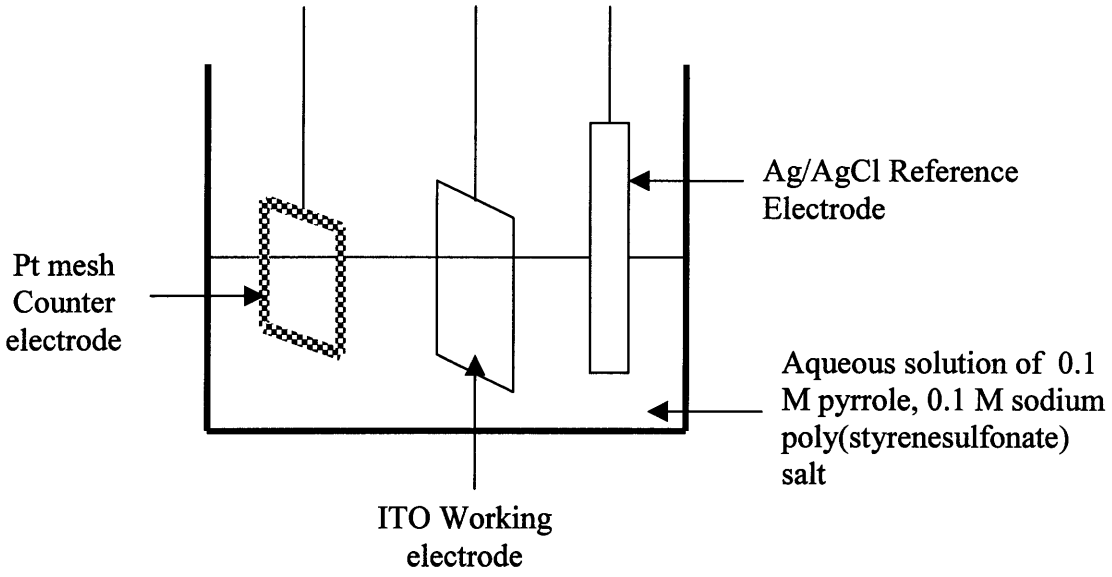


Figure 3.1: Setup for the electrodeposition of PPy thin films onto ITO working electrode.

electrical contact with the spectrometer was placed approximately 1 mm over samples to assist the compensation. For all spectra, the X-ray spot size was 1000 μm^2. Survey spectra were recorded over a binding energy range of 0 to 1000 eV using a pass energy of 300 eV. Surface chemical compositions were determined from peak-area ratios corrected with the appropriate experimentally determined sensitivity factors.

3.2.2.3 UV/VIS spectroscopy

UV/Visible spectroscopic data were obtained using a Cary SE UV-VIS-NIR Spectrophotometer from Varian OSI (Melbourne, Australia). A dual beam system with a scan rate of 600 nm/min was employed.

3.2.2.4 Scanning Electron Microscopy (SEM)

A Joel Scanning Electron Microscope Model 6320 (Akishima, Japan) was used for surface and cross sectional analysis of the PPy thin films. PPy samples were mounted on aluminum stubs using conductive tape to observe the surface. For the cross section of the film, the PPy sample on the ITO glass was cut finely using a diamond cutter. It was mounted in a vice with the interface of interest facing up. Photographs of the images were obtained using a Polaroid instant camera and Polaroid 55 positive negative film.

3.2.2.5 Conductivity

Since the PPy films were grown on ITO (resistance of 40 Ω/sq reported by manufacturer), an estimate of the conductivity was made by measuring the resistance of the films with a Micronta multimeter. The probes of the multimeter were lightly touched

onto the PPy film and the surface resistance was recorded. The surface conductivity, σ, the reciprocal of resistivity, ρ, was calculated from the cross-sectional, area (A) of the film and distance (L) between the multimeter probes.

$$\sigma = \frac{1}{\rho} = \frac{L}{RA} \tag{3.1}$$

3.3 Results and Discussion

The surface properties of the PPy thin films were characterized by XPS. The surface composition of the PPy films at a take off angle of 35° is shown in Table 3.1. The corresponding XPS spectrum is shown in Figure 3.2. The surface composition within a sampling depth of 10-100 Å indicates the presence of Na and S atoms from the sodium salt of poly(styrenesulfonate) (PSS) dopant. The surface N/S ratio was found to be consistent with results from comparable analysis depths [36, 37]. That the surface is rich in negatively charged sulfonate groups is indicated by the presence of Na atoms, which are the associated cations with the pendant sulfonate group. The presence of the Na also indicates that the sulfonate groups are still in the salt form and were not protonated during the electrodeposition process. Furthermore, the almost 1:1 ratio of Na/S (1.57), demonstrates that the source of the Na is not merely a result of adsorbed ions in an electrical double layer, the layer formed at an electrolyte-electrode interface due to the interaction of ions in the electrolyte solution and charges in the electrode.

The presence of the negative sulfonate groups at the PPy/PSS surface is supported by previous work by Prezyna et al.[38]. They suggested the presence of three types of PSS chains in the polypyrrole/PSS films (Figure 3.3). The first (Type 1) includes well entangled chains contained in the bulk of the oxidized PPy film and are used relatively efficiently in doping. Type 2 PSS anions are thought to exist near the film surface, with only a portion of the chains acting as a dopant and the remainder are neutralized by Na cations in the electrolyte. Type 3 PSS chains are only slightly utilized as dopant chains and are loosely held by physical interactions at the film surface. It is the latter two types that have sulfonate moieties available for surface complexation and contribute to changing the nature of the polypyrrole/PSS surface. Thus, it can be concluded that the polypyrrole surface is rich in excess negatively charged sulfonate groups.

The optical properties of Ppy films were determined by UV/VIS spectroscopy (Figure 3.4). The absorption spectrum shows a broad peak at 450 nm, which has been attributed to the oxidized polymer [39]. The broad peak around 800 nm is believed to be due to the presence of bipolarons in the oxidized polymer [40, 41].

SEM was used to analyze the surface and cross section of the PPy thin films. The thickness of the films, which was estimated by the amount of charge passed during the electrodeposition process, was 0.1 μm. This thickness was found to correlate well with the observed thickness of 0.08-0.1 μm, using SEM analysis of the cross section (Figure 3.5). The SEM of the surface of the PPy film is shown in Figure 3.6. At an accelerating

Table 3.1: Surface chemical composition of PP-PSS film of thickness 0.1-0.15 μm as determined by XPS. The "analysis spot area" = 1000 μm^2

Atom	% Composition
O 1s	25.81
Na (Auger)	5.21
N 1s	6.26
C 1s	59.40
S 2p	3.31
N/S	1.89
Na/S	1.57

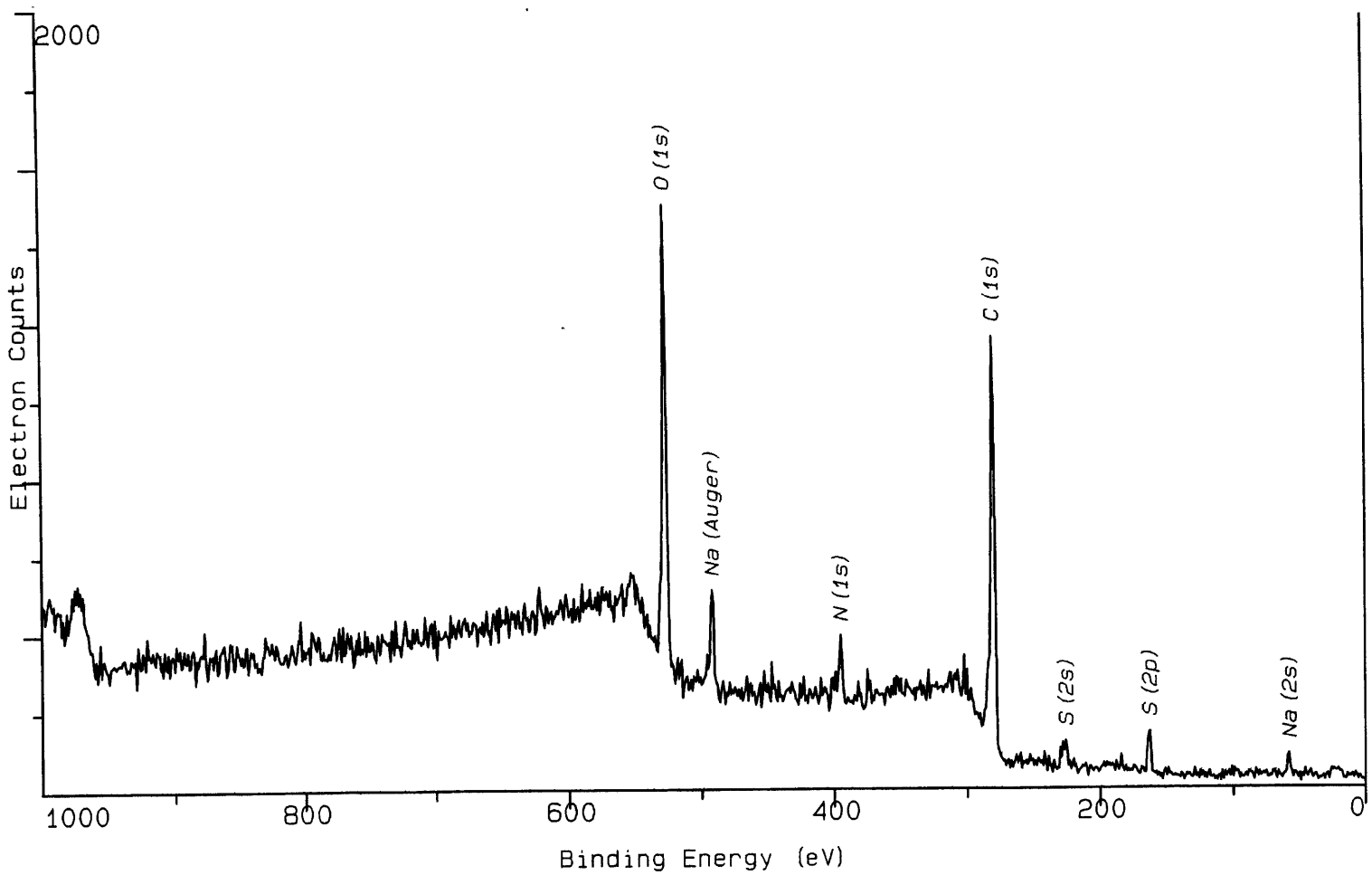
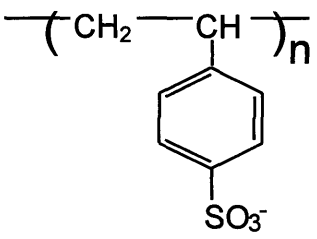


Figure 3.2: XPS spectrum of PP-PSS thin film (0.1 μm thickness) with X-ray spot size of 1000 μm^2 amd electron flood gun energy of 5 eV.

Poly(styrenesulfonate)
PSS



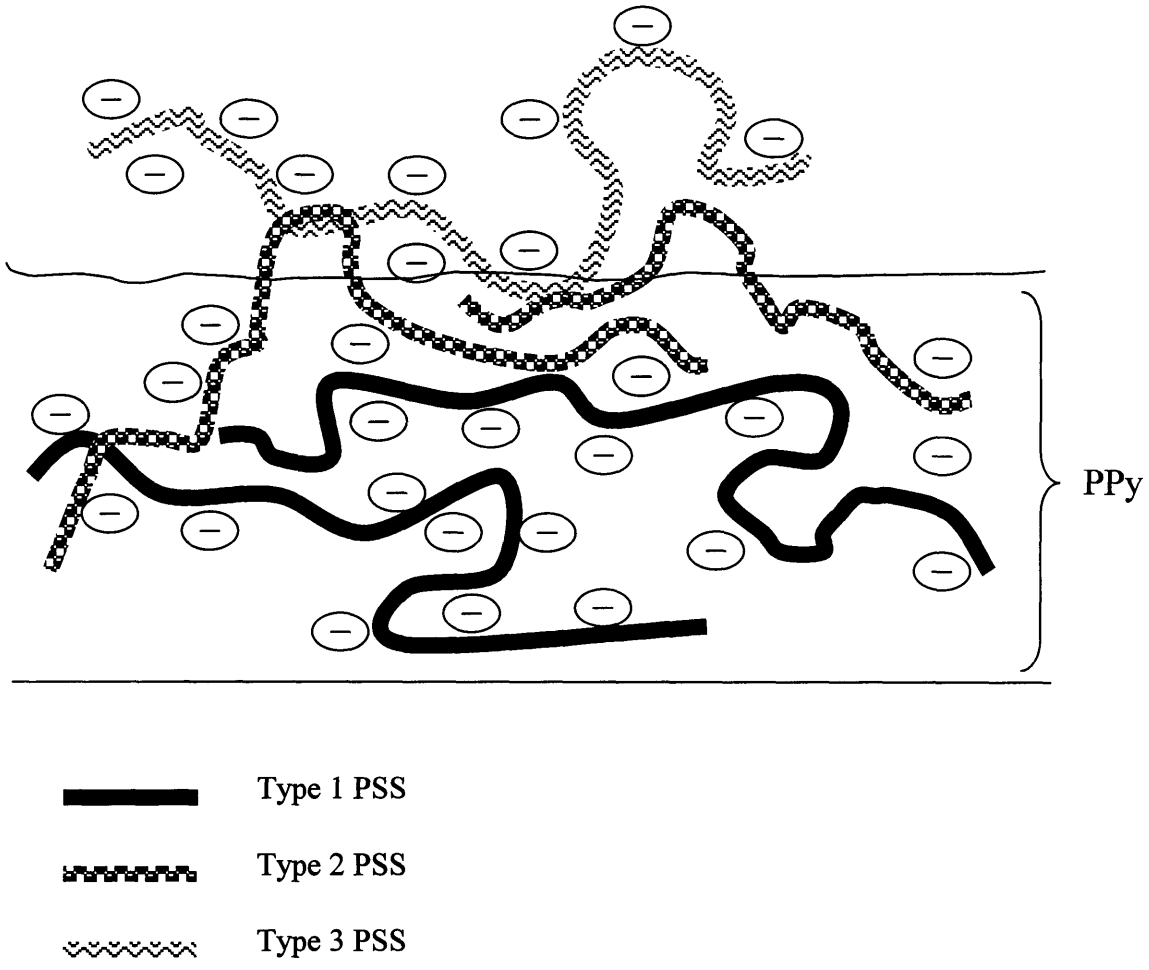


Figure 3.3: Proposed structure of PP-PSS films with three types of PSS chains. Adapted from Prezyna et al. *Interaction of Cationic Polypeptides with Electroactive Polypyrrole/Poly(styrene sulfonate) and Poly(N-methylpyrrole)/Poly(styrenesulfonate) Films.* Macromolecules. 1991. 24: p. 5283-5287

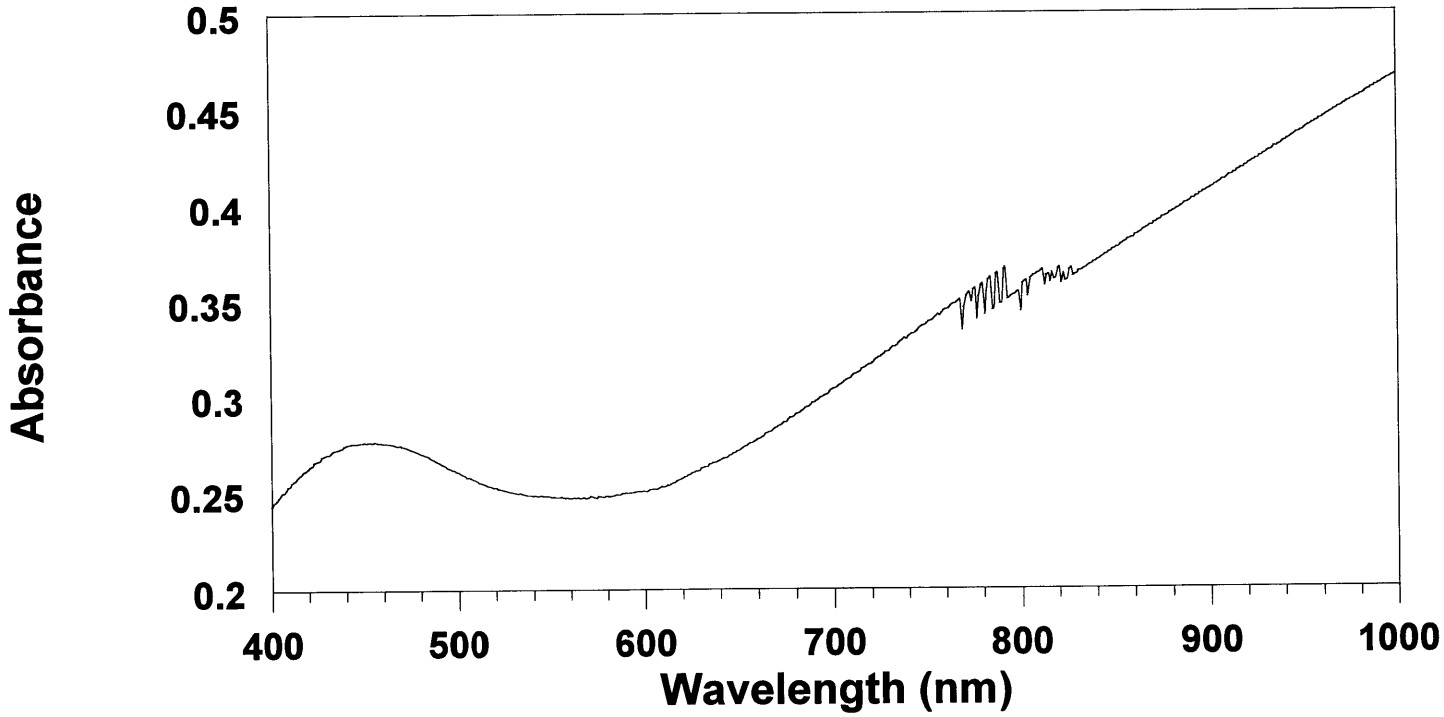


Figure 3.4: UV/VIS absorption spectrum for PP-PSS 0.1 μm thick film.

voltage of 1 kV, the films were seen to have a rough morphology, with numerous nodules. Since a constant potential electrochemical method was used, the growth is by instantaneous nucleation followed by nodular growth on the initially nucleated sites. This leads to the observed rough morphology, which is consistent with previous studies in which polypyrrole was electrochemically synthesized at constant potential in aqueous media [42, 43]. Furthermore, the nodular structure of the PPy/PSS films was also observed by Yang et al. [44]. They investigated the individual conformations of the PSS chains in the films by means of Scanning Tunneling Microscopy (STM). The PSS anions were found to coated on the outside of the helical polypyrrole chains instead of being incorporated fully within the helical structure. This structural arrangement could be a further explanation for the presence of sulfonate moieties at the polypyrrole surface as observed by XPS, and also provide additional evidence for the model proposed by Prezyna (Figure 3.3).

Lastly, the conductivity of the polypyrrole films was evaluated. Based on a rough estimate of the resistance, the conductivity was found to be in the order of 8.5 to12 S/cm. The value confirmed that the polypyrrole was in the oxidized state. However, since we are employing thin films for the purposes of optically transparent substrates for cell culture studies, the conductivity is compromised. Conductivity in the order of 100 S/cm can be obtained with thicker films using the electrochemical synthesis method [45].

3.4 Conclusion

Thin films of polypyrrole were synthesized electrochemically using poly(styrenesulfonate) as the dopant ion. XPS results suggest the presence of negatively

Figure 3.5: Scanning Electron Micrograph of cross section of PPy film.

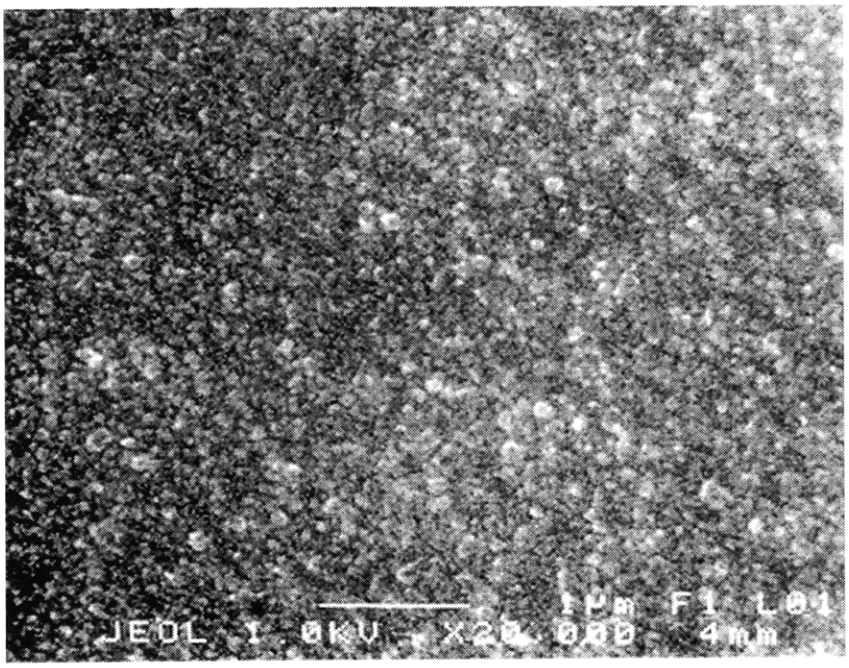


Figure 3.6: Scanning Electron Micrograph of surface of PPy thin film.

charged sulfonate groups at the surface of the films. It is hypothesized that these sulfonate moieties will play a key role in cellular interactions between the surface and cells attached to it. SEM studies confirmed the rough nodular morphology of PPy films consistent with past observations. Conductivity and UV/VIS spectroscopy data established the existence of polypyrrole in its oxidized state. The possibility of using the oxidized polycationic form of polypyrrole with the negative dopant anion, poly(styrenesulfonate), as a substrate for bone regeneration will be discussed in the next chapter.

CHAPTER 4: CELL CULTURE – BASE LINE STUDIES

4.1 Introduction – selection of bone cell culture system

To study the candidacy of polypyrrole as an interactive substrate for bone regeneration *in vitro,* an appropriate cell culture system must be chosen. The key criteria in determining a model system to study were:

- Cells capable of undergoing osteogenic differentiation
- Cells known to respond to electrical fields
- Ease of isolation of cells for *in vitro* and possible future *in vivo* studies
- Cell culture system without contamination by other cell types

Based on these characteristics, Bone Marrow Stromal Cells (BMSC) were chosen as the model system for bone regeneration studies. The following section gives a background and detailed justification for the selection of this particular system.

4.1.1 Bone Marrow Stromal Cells

Adult vertebrates require a continuous supply of osteoblasts (bone-forming cells) for both bone remodeling and regeneration during fracture repair. According to the current hypothesis, there is a reservoir of cells in the marrow stromal system, which is associated with the soft connective tissues of marrow and bone surfaces [46, 47]. These cells have been shown to be pluripotent and are progenitor cells for a number of different cell lineages, including those of bone, cartilage, fat and fibrous tissues [48, 49] (Figure 4.1).

There is substantial evidence showing that bone marrow stromal cells, cultured

both *in vivo* and *in vitro*, can induce bone formation in a variety of animal models [50-53]. The results of these studies are consistent with the hypothesis that part of the renewal of the osteoblast population at bone surfaces involves the recruitment, proliferation and differentiation of these osteoprogenitor cells inhabiting the bone marrow. Thus, bone marrow stromal cells (BMSC) are an appropriate system for the study of osteogenesis and bone regeneration.

As discussed in Chapter 2, bone has been shown to be responsive to exogenous electrical stimulation. Specifically, it has been shown that marrow stromal cells are capable of undergoing osteogenesis upon electrical stimulation [54, 55]. Thus, in order to fulfill our objective of evaluating the potential of polypyrrole, an electrically conducting polymer, as an interactive substrate for bone cells, BMSC satisfy the criterion of being responsive to electrical fields.

In addition, BMSC are easy to isolate and culture *in vitro*. Upon isolating suspensions from the bone marrow cavity, primary cultures of BMSC can easily be separated from hematopoietic cells by virtue of their attachment to tissue culture polystyrene substrate. This ease in isolation is also important to consider for possible future *in vivo* tissue engineering applications. It is not practical to isolate osteoblasts, for example, from a human femur; however, isolation of bone marrow stromal cells can easily be obtained from marrow aspirates from humans and can be expanded *in vitro* [56].

Another issue to consider is the contamination of isolated cells by other cell types. BMSC are easily separated from hematopoietic cells in contrast to cell populations from

osteoblast rich systems such as the calvariae of fetal or neonatal rats, which often contain other cells such as fibroblasts. Thus, BMSC are an attractive system since contamination by other cell types is minimal. A further advantage of BMSC is its comparable activity in both newborn and old [57], whereas osteoblasts obtained from fetal rat calvaria can only be used to model neonatal bone behavior.

Therefore, based on these considerations, BMSC were chosen as the cell culture system of study for interactions with polypyrrole. The culture of BMSC on this substrate is discussed in the following sections.

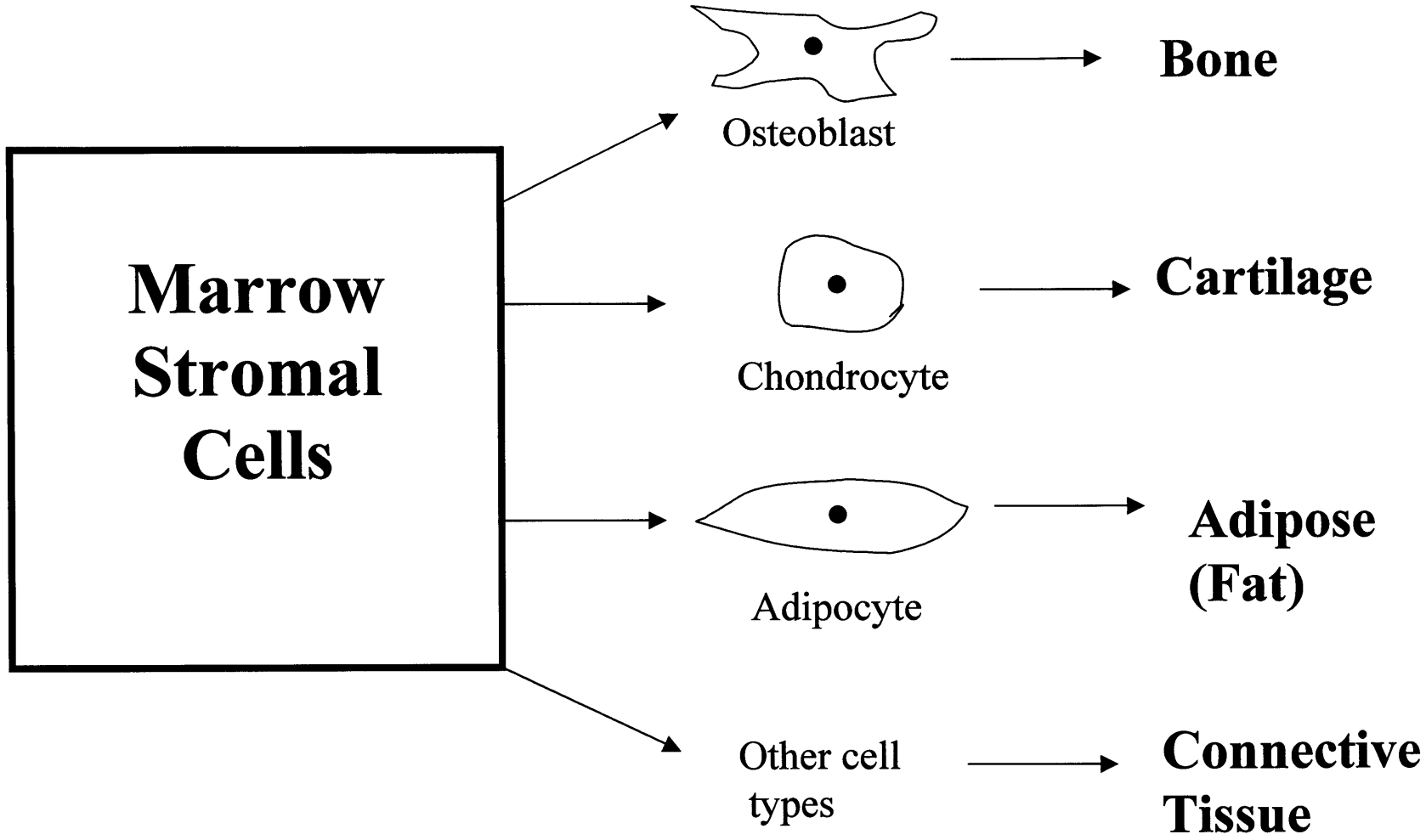


Figure 4.1: Marrow stem cell phenotypes. Marrow stem cells are capable of differentiating through a series of separate and unique lineage transitions into a variety of end-stage phenotypes as shown.

4.2 Experimental

4.2.1 Chemicals and Materials

Dulbeccos's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), Dulbecco's phosphate buffered saline (PBS) without additives, penicillin, streptomycin, non-essential amino acids and trypsin were purchased from Gibco BRL (Grand Island, NY). Recombinant Human fibroblast growth factor-2 was purchased from R & D systems (Minneapolis, MN). Tissue culture polystyrene Petri dishes were purchased from either Falcon (Becton Dickinson & Co., Franklin Lakes, NJ) or Corning (Corning, NY). MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Thiazolyl blue), a mitochondrial stain for cell viability was purchased from Sigma. Alkaline Phosphatase kits were purchased from Sigma.

Cells were viewed using a Diaphot phase-contrast microscope (Nikon, Japan). Images were acquired using a CCD video camera and VM-920 monitor from Hitachi (Tokyo, Japan) and were subsequently digitized using NIH Image software and a Scion LG-3 frame grabber (Frederick, MD).

4.2.2 Methods and Procedures

4.2.2.1 Isolation and culture of Bone Marrow Stromal Cells (BMSC)

The tibia and femur of 2-3 week old bovine calves were obtained from a local abattoir within 4 hours of slaughter. The bones were cleaned of soft tissue and excised in their diaphyseal region. The contents of the bone marrow cavity were aseptically harvested in Dulbecco's Modified Eagle Medium (DMEM) and 0.5 mM ethylenediamine

tetraacetic acid (EDTA). Single cell suspensions were made by repeatedly passing the marrow through needles of different gauges (16 to 20). Cells were centrifuged and were resuspended in DMEM supplemented with 10% fetal bovine serum (FBS), 0.1 mM nonessential aminoacids (NEAA), 100 U/ml penicillin, 100 mg/L streptomycin and 1 ng/ml fibroblast growth factor-2 (FGF-2). Nucleated cells from the bone marrow, consisting of hematopoietic and stromal populations, were counted using a hemocytometer and plated in 100 mm tissue culture polystyrene Petri dishes at $2x10^6$ cells per dish (approximately $25x10^3$ cells/cm^2) in 10 ml of medium. The cells were incubated in a humidified 37 °C/5% CO_2 incubator. BMSC were selected based on their ability to adhere to the Petri dish; non-adherent hematopoietic cells were removed with culture medium during refeeding. The medium was changed after 3 days and twice per week thereafter. When BMSC became near confluent (approximately 2-3 weeks after the primary culture was established), they were detached using 0.25% trypsin/1 mM EDTA and replated in 100 mm dishes at $3x10^5$ cells per dish. When the cell population reached confluency, these Passage 1 cells were trypsinized, and replated as Passage 2 (P2) cells onto polypyrrole (PPy), indium tin oxide (ITO) and tissue culture polystyrene (TCPS). In all experiments, P2 cells were used, as cell attachment decreases with further passaging (Appendix, Figure A.1)

Sterile Plexiglass wells (1 cm x 1.5 cm inner dimensions) were attached to PPy films, ITO substrates or TCPS substrates using autoclaved vacuum grease. Each substrate type was tested in duplicate. All wells and substrates were sterilized by UV irradiation in a laminar flow hood for 24 hours prior to seeding.

BMSC (P2) were seeded into the wells at a density of 3 x 10^4 cells/ml per well and then incubated for 48 hours to permit attachment and spreading. Cell viability assay, morphological analysis and alkaline phosphatase assays carried out after 48 hours are described in the following sections.

4.2.2.2 MTT assay (Cell Viability)

MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Thiazolyl blue) is a water soluble tetrazolium salt, which is converted to an insoluble purple formazan by cleavage of the tetrazolium ring by dehydrogenase enzymes [57]. The cleavage and conversion of the soluble yellow dye to the formazan has been used to develop an assay system for measurement of cell proliferation and cell number. Only active mitochondrial dehydrogenases of living cells will cause the conversion.

After 48 hours, all the wells were evaluated for cell viability on the three substrates: PPy, ITO and TCPS. A stock solution of MTT at 5 mg/ml was prepared and added to serum-free DMEM, without phenol red, at a volume ratio of 1:20. Culture medium was removed from each of the wells and replaced with 0.5 ml of DMEM-MTT medium. After 4 hours of incubation at 37 ^{o}C and 5% CO_2, the medium was removed and the converted dye solubilized by adding 1 ml of acidic isopropanol (6 x 10^{-6} N HCl in absolute isopropanol). Absorbance of the converted dye was measured at a wavelength of 570 nm with background subtraction at 670 nm on a Perkin-Elmer 553 Fast Scan Spectrophotometer. All cell viability values were normalized to those for TCPS for comparison.

4.2.2.3 Morphological Analysis

Thin polypyrrole films permitted the use of light microscopy to study BMSC-biomaterial interactions. Cells were viewed using an inverted phase contrast microscope and under a 10x objective. Images were acquired using a CCD video camera and were subsequently digitized using NIH Image software and a Scion LG-3 image capture board.

4.2.2.4 Alkaline Phosphatase (ALP) Activity

Alkaline phosphatase activity is a marker for the osteoblast phenotype. It is used routinely as an indicator for the degree of differentiation seen in BMSC [58]. Alkaline phosphatase activity was determined as the rate of conversion of p-nitrophenyl phosphate to p-nitrophenol. Each assayed well was washed with PBS and 100 μl of 0.01 % SDS. After 10 minutes, a prewarmed solution (37 °C) of 0.25 ml substrate (Sigma 104-100) and 0.25 ml alkaline buffer solution were added to each of the wells. After a 15 minute incubation period at 37 °C, the contents of each well were added to 5 ml of 0.05 N NaOH to quench the reaction. The absorbance was read at 410 nm on a Perkin-Elmer 553 Fast Scan Spectrophotometer.

4.3 Results and Discussion

BMSC grown on PP films, ITO and TCPS were tested for cell viability (Figure 4.2). BMSC growth was supported on polypyrrole films indicating that polypyrrole is not toxic to them. In contrast, at 48 hours, cell viability was significantly lower on the ITO controls, indicating that the presence of the polypyrrole substrate enhanced cell attachment and proliferation.

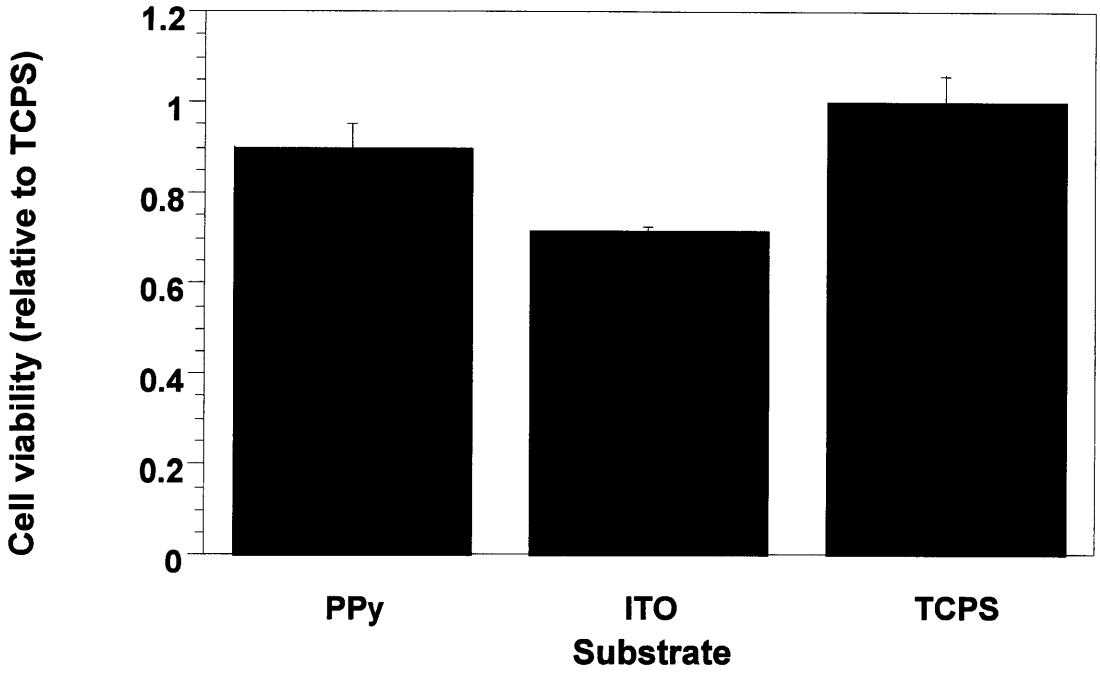


Figure 4.2: Cell viability of BMSC normalized to tissue culture polystyrene. Data is expressed as the mean $\pm$ SD of the mean of three separate experiments. For each experiment, samples were tested in duplicate.

Phase-contrast optical micrographs of BMSC cultured for 48 hours are shown in Figure 4.3. It can be seen that the BMSC attach and spread equally well on all three substrates tested: PPy, ITO and TCPS. The cell morphologies seen on PPy are characteristic of those typically seen in TCPS Petri dishes.

Alkaline phosphatase (ALP) activity, a marker for BMSC differentiation towards the osteoblast phenotype, was found to be significantly higher for cells grown on PPy (Figure 4.4). The activities for each of the substrates are normalized to the corresponding cell numbers obtained from the MTT assay and are represented relative to the TCPS control. It appears that the excess negative charges on the PPy surface, due to the incorporation of the poly(styrenesulfonate) dopant, could be encouraging an increased adsorption of certain matrix proteins that is favorable for the differentiation of the BMSC. The precise mechanism of this observed behavior is still unclear.

It has been shown that the nature of the charged substrate affects the migratory morphology of osteoblasts via the intermediation of specifically adsorbed proteins [59]. For negatively charged substrata, it was shown that the ventral cell membrane was readily visible with only focal areas of close contact with the substratum, in contrast to positively charged substrata where the cell membrane was not distinguishable by transmission electron microscopy. It has been hypothesized that the space between either the osteoblasts or the osteoblast-like cells and negatively charged substrata could allow secretion of extracellular matrix (ECM) directly onto the substratum surface. It is desirable that there be close apposition of bone matrix with the biomaterial, and this is best achieved with ECM laid directly onto the surface. Hence, negatively charged

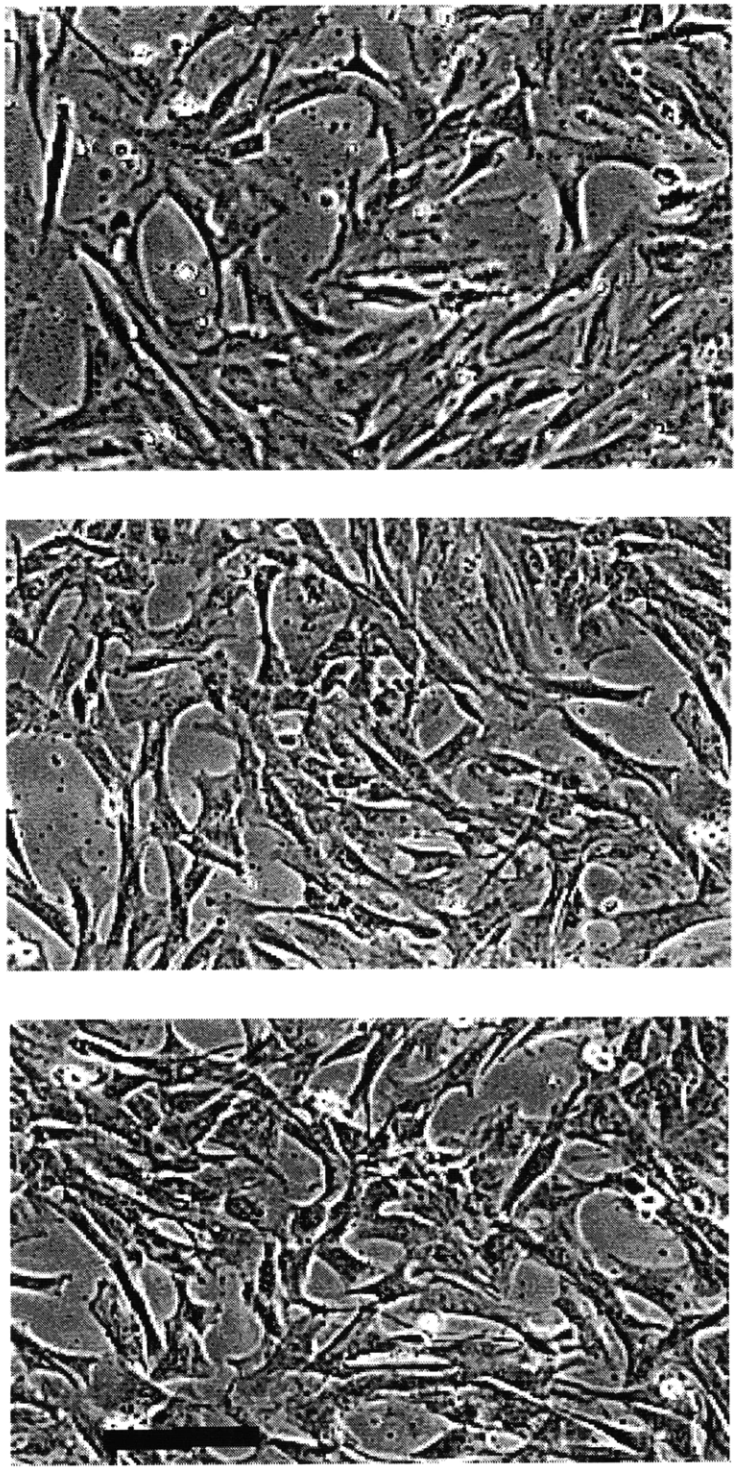


Figure 4.3: BMSC culture on PPy film (TOP); TCPS (MIDDLE); ITO (BOTTOM) after 48 hours incubation. Bar = 100 μm.

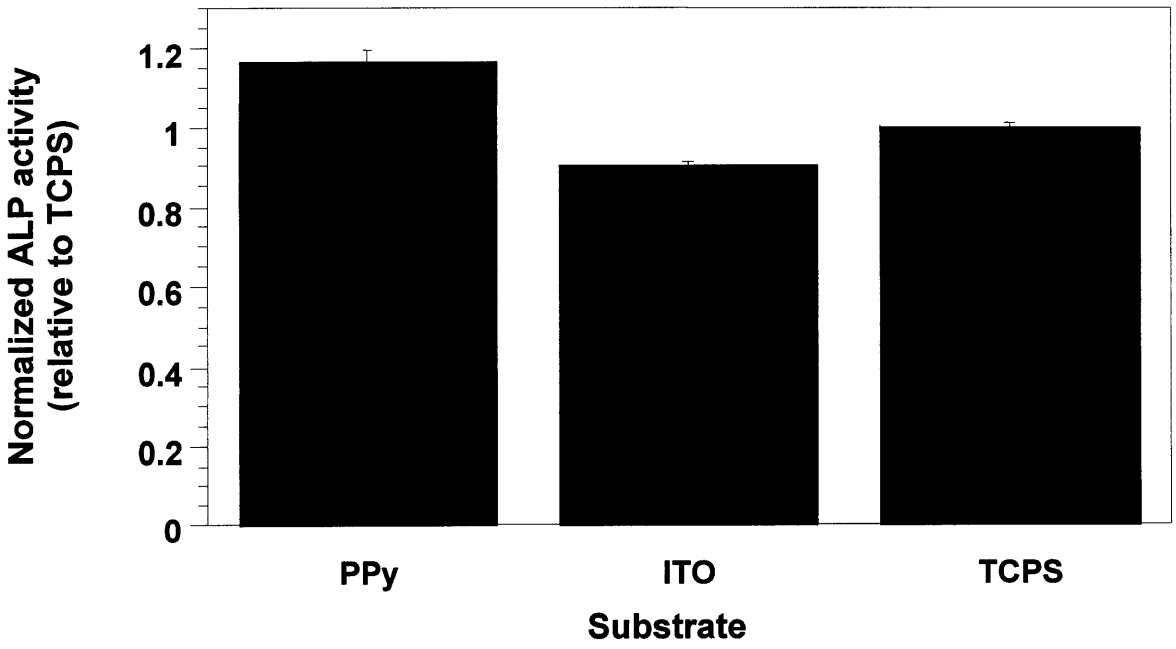


Figure 4.4: Alkaline Phosphatase activity of BMSC normalized on cell number (from MTT assay) relative to tissue culture polystyrene. Data is expressed as the mean $\pm$ SD of the mean of three separate experiments. For each experiment, samples were tested in duplicate.

substrata such as polypyrrole with the dopant ion, PSS, would be ideally suited to fulfill this objective.

Osteogenesis and formation of dense connective tissue has also been demonstrated by employing negatively charged Sephadex beads which were implanted into cranial or mandibular defects in young adult rats [60]. The mechanism by which the charged beads fostered the osteogenic response is not well understood. Our *in vitro* observations with polypyrrole's negatively charged surface interactions with BMSC suggest that the cells may be undergoing differentiation towards the osteoblast phenotype. A similar phenomenon as observed *in vivo* could also be occurring *in vitro* in promoting osteogenesis with negatively charged substrata.

CHAPTER 5: CELL CULTURE - GLUCOCORTICOID STUDIES

5.1 Introduction

It has been shown that stromal cells isolated from the bone marrow can be induced to differentiate along the osteoblast lineage by glucocorticoids. Glucocorticoids are steroid hormones that can regulate gene expression in differentiating cells and in inducing the affinity of the glucocorticoid receptors found in cells [61]. Dexamethasone, a synthetic glucocorticoid, has been shown to induce the osteoblast phenotype and bone formation with stromal cells *in vitro* [51]. Numerous studies with dexamethasone in marrow derived stromal cell culture have shown that an increase in alkaline phosphatase (ALP) activity occurs with the addition of the glucocorticoid [51, 62-68]. The precise role of the steroid in modulating the metabolism and biological activity is not completely understood and is subject to investigation.

The typical "cocktail" of stimulants added to the culture to induce osteogenesis consists of dexamethasone, ascorbic acid and β-glycerophosphate. Ascorbic acid is required for the synthesis of collagen [69, 70] and for osteogenesis *in vitro* [71]. Furthermore, Anderson et al. [72] have demonstrated that ascorbic acid regulates ATPase and ALP activities and protein synthesis in cultures of osteoblast-like cells. The organic phosphate, β-glycerophosphate, has often been used *in vitro* as a potential source of phosphate ions [71, 73, 74].

This section compares the effect of dexamethasone on bone marrow stromal cells grown on polypyrrole to that of tissue culture polystyrene.

5.2 Experimental

5.2.1 Chemicals and Materials

Cell culture reagents are described in 4.2.1. Dexamethasone, β-Glycerophosphate and Ascorbic acid were purchased from Sigma and used as received.

5.2.2 Methods and Procedures

BMSC were isolated as described in 4.2.2.2. P2 Cells were trypsinized and seeded at a density of $5x10^4$ cells/ml in the sterile Plexiglass wells attached to PPy films, ITO or TCPS. After 48 hours, in dexamethasone test cultures, the original medium was removed and replaced by filtered medium (0.22 μm sterile cellulose acetate membrane, Corning, NY) supplemented with 50 μg/ml ascorbic acid, 7 mM β–glycerophosphate and 10^{-8} M dexamethasone. Similarly, fresh medium was fed to control cultures on each of the three substrates at the same time. All samples were incubated for another 48 hours prior to MTT, ALP assays and morphological analysis.

5.3 Results and Discussion

Cell viability of dexamethasone supplemented cultures on PPy and ITO were compared relative to TCPS controls (Figure 5.1). The proliferation of cells was moderately reduced for all cultures in the presence of dexamethasone. The observed behavior is consistent with previous data [63]. Micrographs (Figure 5.2) of the cultures on each of the substrates demonstrate the typical cuboidal shape of the cells on addition of dexamethasone.

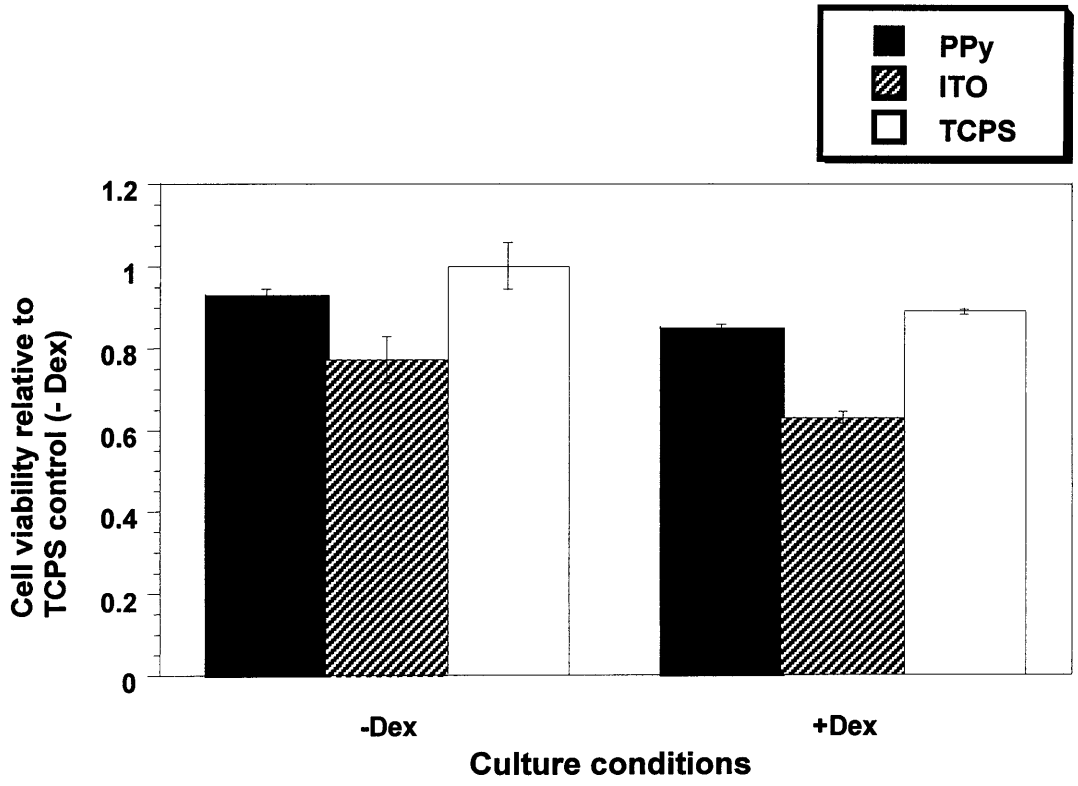


Figure 5.1: Cell viability of BMSC normalized to tissue culture polystyrene control (without dexamethasone). Data is expressed as the mean $\pm$ SD of the mean of three separate experiments. For each experiment, samples were tested in duplicate.

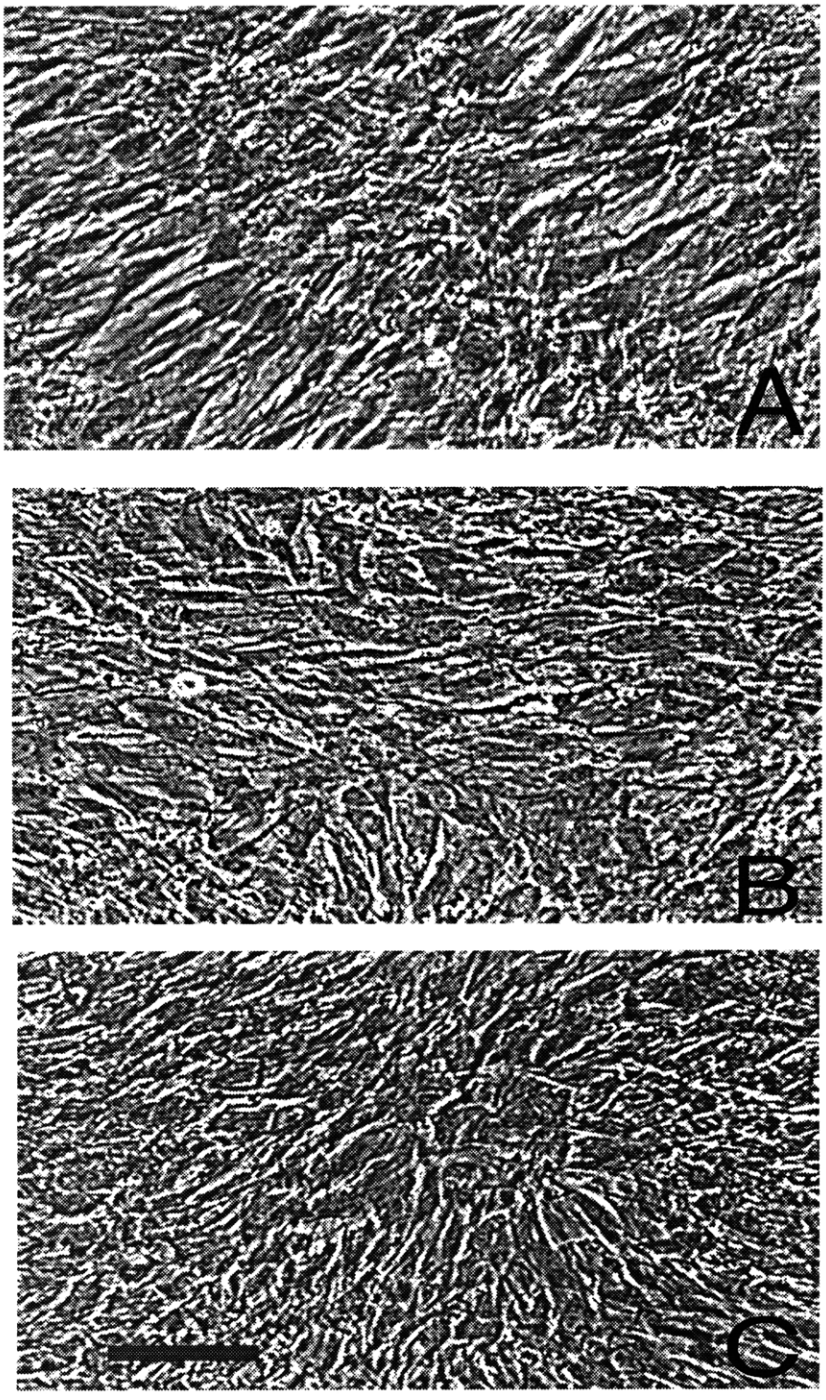


Figure 5.2: Dexamethasone supplemented cultures on A: PPy, B: ITO, C: TCPS, after 48 hours of dex medium. Bar = 100 μm.

ALP activity was stimulated by dexamethasone on all three substrates (Figure 5.3). However, the expression of the enzyme was significantly higher for PPy ($P<0.01$) relative to TCPS controls. Thus, PPy appears to be promoting osteogenesis to the osteoblast phenotype to a greater extent, relative to TCPS, in the presence of the glucocorticoid, dexamethasone. ALP activity is expected to be enhanced with dexamethasone from previous studies with BMSC cultures [62, 63, 65, 67]. However, the enhanced expression on PPy, in contrast to that on TCPS, could again be a result of the interactions between the polypyrrole surface chemistry and adsorption of certain proteins on the surface, which could be triggering the ALP activity via the regulation of the glucocorticoid.

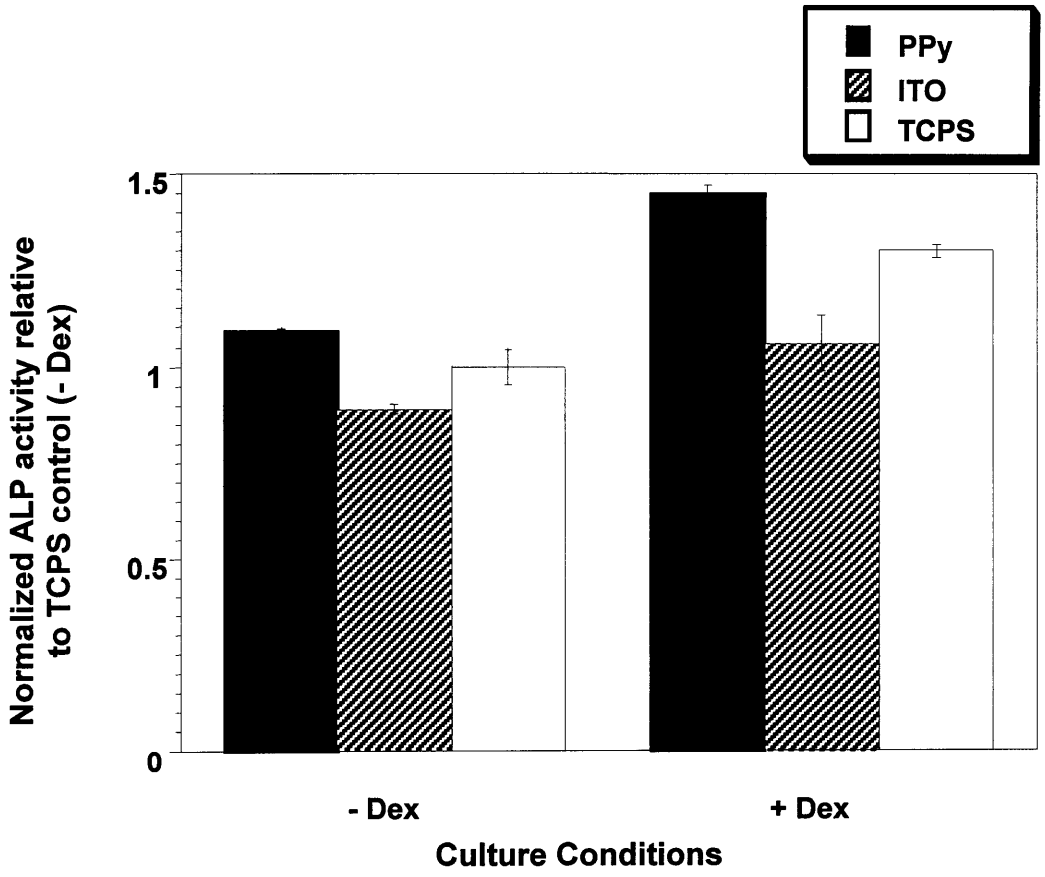


Figure 5.3: Alkaline Phosphatase activity of BMSC normalized on cell number (from MTT assay) relative to tissue culture polystyrene (without dexamethasone). Data is expressed as the mean $\pm$ SE of the mean of three separate experiments. For each experiment, samples were tested in duplicate.

CHAPTER 6: CELL CULTURE – ELECTRICAL STIMULATION STUDIES

6.1 Introduction

This final study evaluates PPy as a medium for the electrical stimulation of BMSC. Numerous researchers have studied the effect of electrical stimulation *in vitro* on bone cells [75-78]. However, there are no reports of bone cells cultured directly on a substrate that was electrically conductive. The use of PPy is thus a novel approach in applying electrical stimulation to bone cells. Through this arrangement, it is hypothesized that electrical coupling between the cells and the electrode will be enhanced since the cells are directly attached to the PPy electrode. Furthermore, the enhanced alkaline phosphatase activity of BMSC from the base line and glucocorticoid stimulation studies (Chapters 4 and 5), have indicated that the nature of the PPy surface, due to the incorporation of the poly(styrenesulfonate) dopant, anion, could be playing a role in the protein adsorption or other unknown cellular processes.

This chapter brings together the conditions of the two previous studies in the presence of electrical stimulation.

6.2 Experimental

6.2.1 Chemicals and Materials

Cell culture reagents as described previously were used. Silver wire and gold wire were purchased from Sigma.

6.2.2 Methods and Procedures

BMSC were isolated as described in 4.2.2.2. Cells were trypsinized and seeded at a density of $5x10^4$ cells/ml in wells attached to each of the three substrates: PPy, ITO and TCPS.

The assembly for electrical stimulation experiments is shown in Figure 6.1. The PPy film or ITO slide served as the anode, a gold (Au) wire placed along the length of the well as the cathode and a silver (Ag) wire acted as a quasi-reference electrode. An EG&G® Princeton Applied Research Potentiostat/Galvanostat Model 263A (Oakridge, TN) was used as the source of constant potential and cells were incubated at 37 °C and 5% CO_2 during the course of the stimulation. All electrically stimulated cells were subjected to a steady potential of 100 mV for 1 hour.

For electrical stimulation (+E) experiments, two conditions were tested for PPy and ITO substrates: presence of dexamethasone (+E, +Dex) and absence of dexamethasone (+E). For dexamethasone supplemented cultures, dexamethasone was added after 48 hours from the initial seeding (methods as described in Section 5.3) and incubated for another 24 hours prior to electrical stimulation. MTT, ALP assays and morphological evaluation (methods as described in Sections 4.2.2.2-4.2.2.4) were performed 24 hours post stimulation. For (+E) cultures, cells were electrically stimulated at the same time point as for dexamethasone cultures (+E, + Dex) and assayed similarly. Control cultures, without electrical stimulation, in the presence (+ Dex) and absence of dexamethasone (Control), were used to evaluate the effect of electrical stimulation for the two conditions. Each substrate type was tested in duplicate for each condition in three

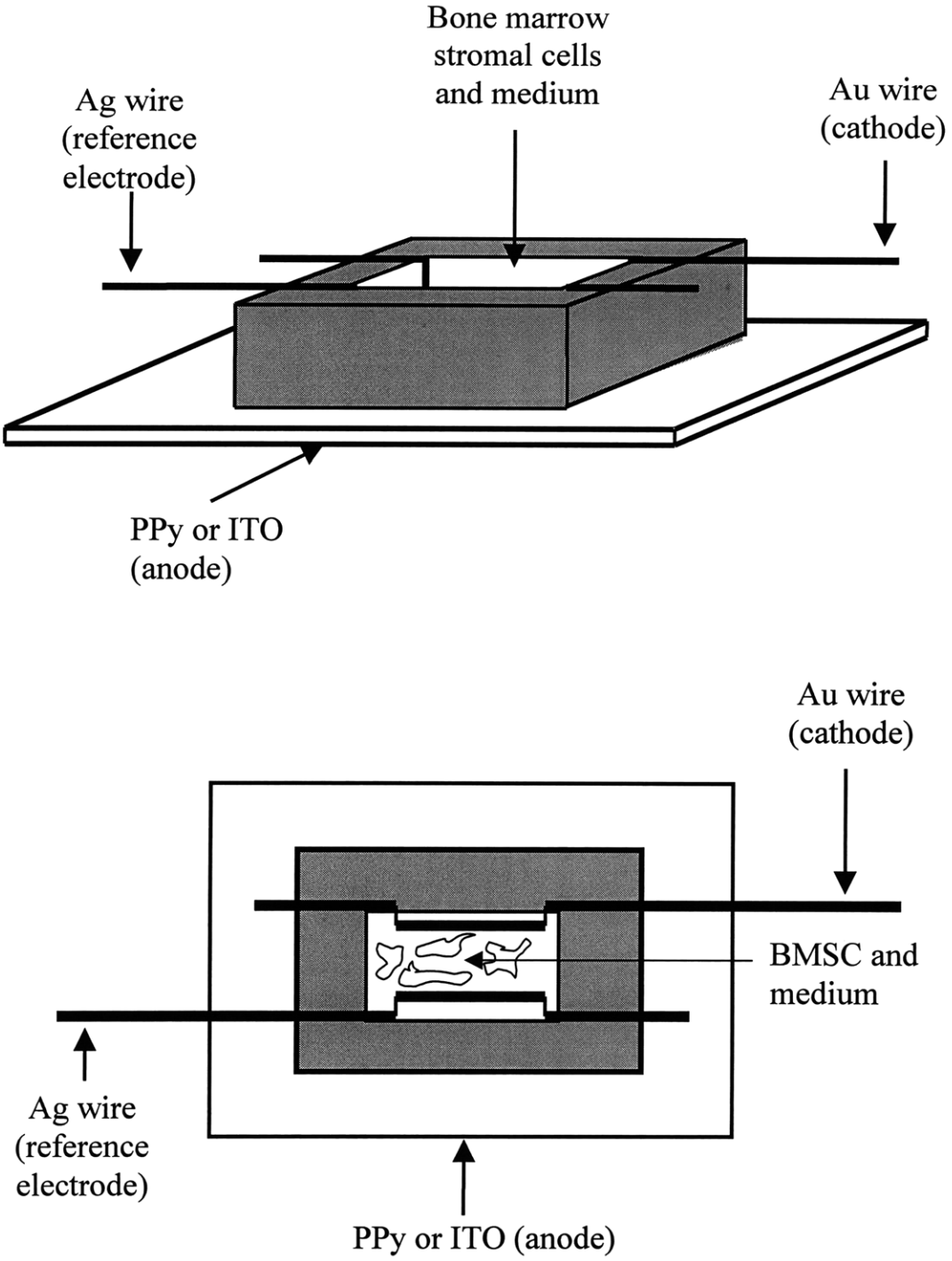


Figure 6.1: Assembly for electrical stimulation experiments.

three separate experiments.

6.3 *Results and Discussion*

Cell viability, normalized to TCPS, was not affected significantly upon electrical stimulation on both the PPy and ITO substrates in the (+E) and dexamethasone supplemented (+E, +Dex) cultures (Figure 6.2). However, relative to PPy, the cell viability of cultures on ITO was significantly lower for two of the four conditions studied: BMSC grown on ITO controls with dexamethasone (+ Dex) only, (consistent with results from Section 5.3), and BMSC grown on ITO with dexamethasone and electrical stimulation (+E, +Dex). The mechanism for the reduced cell number on ITO glass is not understood but supports the hypothesis that the surface of the polypyrrole interacts with charged matrix proteins, both in the presence and absence of electrical stimulation, to support cell growth and attachment. Since the application of the constant potential at 100 mV for 1 hour did not significantly affect cell viability for both dexamethasone and control cultures on polypyrrole, it appears that this stimulus does not initiate cell death during the stimulation and 24-hour post stimulation periods.

Phase contrast micrographs of BMSC cultured on PPy and ITO, in the presence of dexamethasone and exposed to electrical stimulation are shown in Figure 6.3. During this short stimulation period, it is not surprising to see that there was no change in morphology relative to control cultures without the electrical stimulation. The cuboidal shape of the cells as seen in the dexamethasone cultures in Section 5.3, is still maintained upon electrical stimulation.

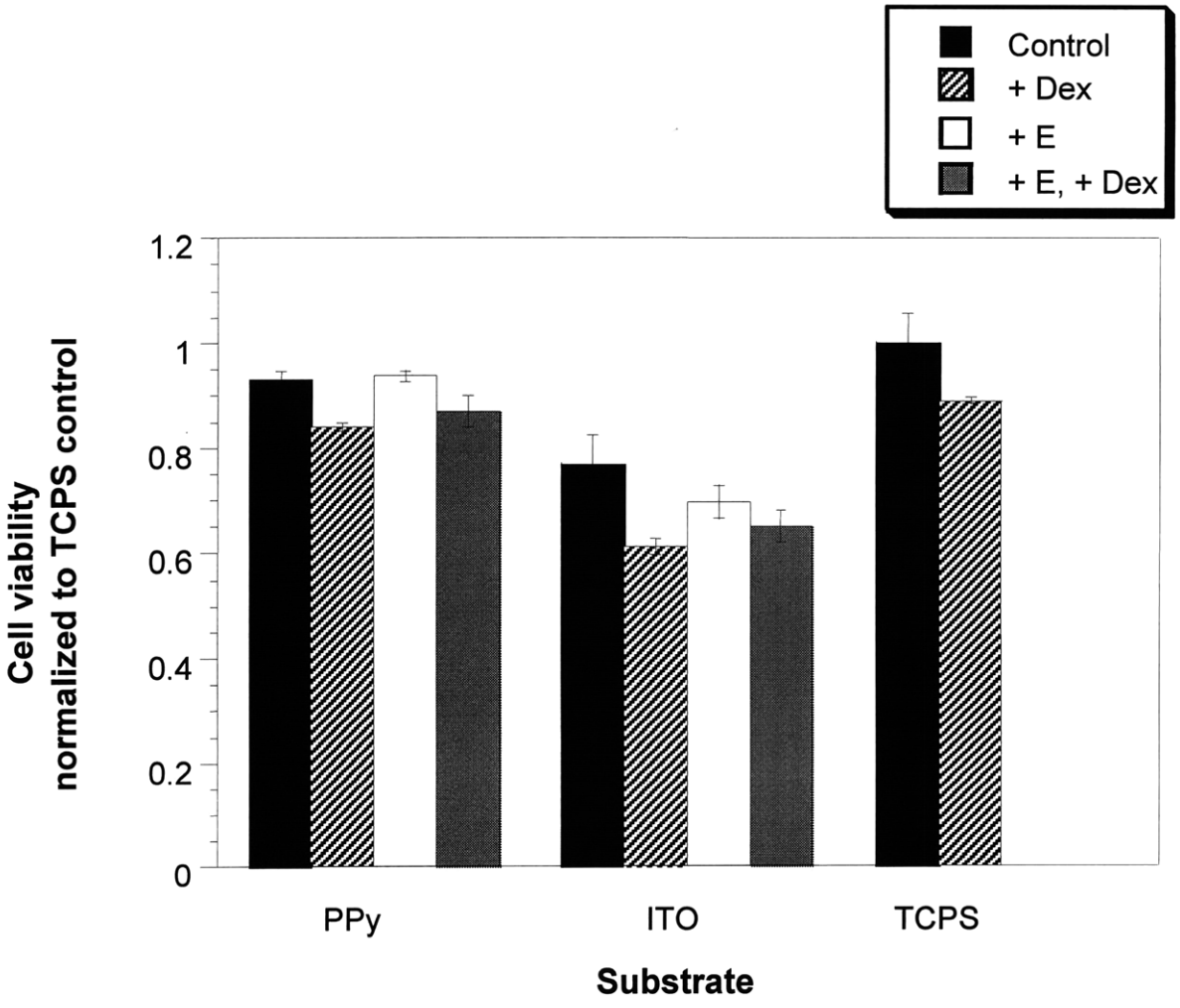


Figure 6.2: Cell viability of BMSC normalized to tissue culture polystyrene control (without dexamethasone). Data is expressed as the mean $\pm$ SD of the mean of three separate experiments. For each experiment, samples were tested in duplicate.

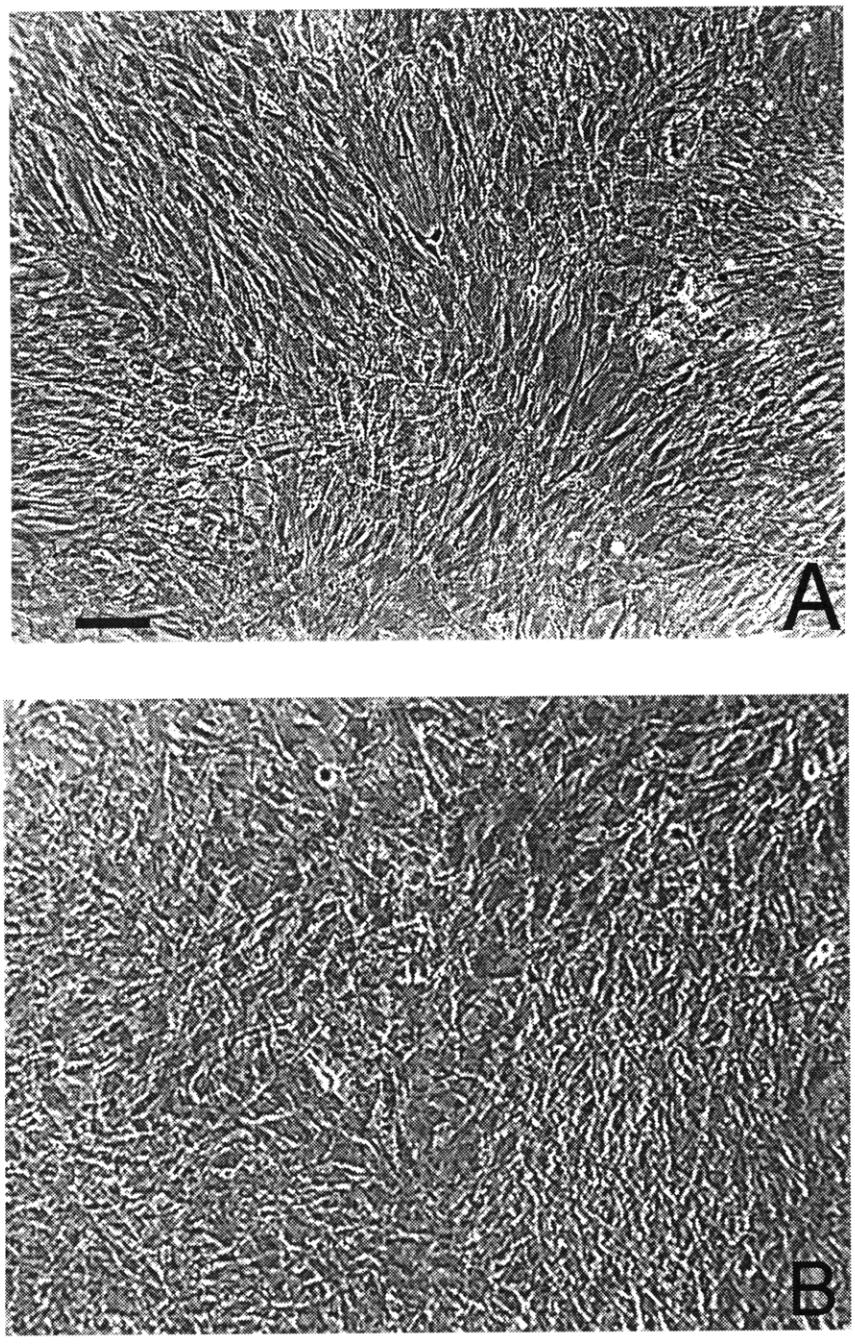


Figure 6.3: BMSC cultures on A: PPy; B: ITO in (+E, +dex) condition, 24 hours after electrical stimulation. Bar = 100 μm.

Although no dramatic changes were observed in the cell morphology of BMSC cultured on PPy in the (+E, +Dex) condition, the ALP activity was found to increase by 40 % ($P < 0.01$) relative to PPy control cultures without electrical stimulation (+Dex) (Figure 6.4). Since the cell viability was relatively unaffected by the application of the stimulus, these observations could be a result of an increase in the number of marrow stromal cells expressing ALP and/or an increase in the amount of ALP expressed per cell. In contrast, ALP activity remained unchanged for electrically stimulated cultures without dexamethasone (+E) versus PPy cultures without both electrical stimulation and dexamethasone (Control). The interaction of the cells with the PPy substrate in the presence of dexamethasone during the electrical stimulus appears to be triggering a mechanism to markedly differentiate the cell along the osteoblastic phenotype. It appears that the presence of the glucocorticoid is essential during the stimulation period to observe this significant increase at the chosen constant potential level of 100 mV for 1 hour.

However, the nature of the surface was also demonstrated to be crucial in determining cellular behavior since ALP activity was significantly increased (+56 %, $P < 0.01$) in PPy cultures in the presence of electrical stimulation and dexamethasone (+E, + Dex) relative to ITO cultures under the same conditions. Even though the ITO substrates are highly conductive (in the order of 800 S/cm as reported by manufacturer), the mere application of a steady potential through the electrode was not capable of inducing osteogenic differentiation of the BMSC. Thus, the interaction of the PPy surface and application of electrical stimulation through the film itself was necessary to obtain the

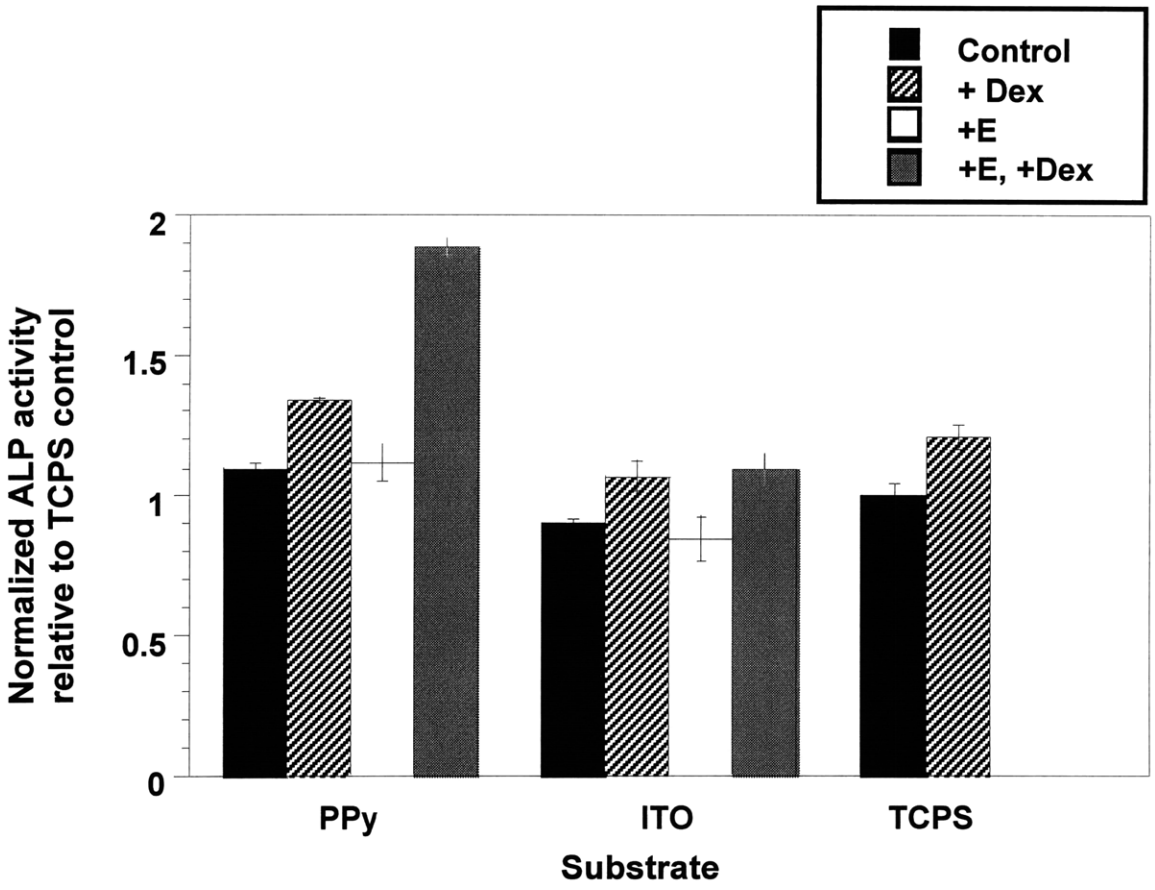


Figure 6.4: Alkaline Phosphatase activity of BMSC normalized on cell number (from MTT assay) relative to tissue culture polystyrene (without dexamethasone). Data is expressed as the mean $\pm$ SD of the mean of three separate experiments. For each experiment, samples were tested in duplicate.

observed increase in ALP activity.

Several hypotheses can be put forth to explain the effect of PPy on BMSC during electrical stimulation. As suggested in the previous two chapters, the excess negatively charged sulfonate groups on the Ppy surface could be playing a role in changing the conformation of certain adsorbed proteins upon the application of electrical stimulation. Furthermore, it appears that there is a coupled effect of dexamethasone and electrical stimulation in enhancing ALP activity on PPy, as the effect was not observed upon application of electrical stimulation (+E) alone. Thus, the interaction of PPy and proteins involved in the dexamethasone regulatory pathway of the alkaline phosphatase gene expression, could also be playing an important role. The mechanism by which dexamethasone induces alkaline phosphatase activity is not fully understood. However, studies have suggested that either a mediator protein is involved in the action of dexamethasone on alkaline phosphatase at a pretranslational level [79] or that the increase in alkaline phosphatase activity in dexamethasone cultures is via the *de novo* synthesis of the ALP enzyme itself [80]. It may be possible that the interaction between an adsorbed protein layer on the polypyrrole surface and the "mediator" protein could be leading to certain conformational changes that result in the elevated activity of ALP.

The role of Ca^{2+} ions in electrical stimulation could also be important in determining the differentiation level of BMSC [76]. Voltage gated calcium channels exist in the membranes of bone marrow cells and osteoblasts in which the ion transport is mediated by electrical stimulation. Ozawa et al. showed that electric fields stimulate the DNA synthesis of MC3T3-E1 (mouse osteoblast-like cells) by a mechanism involving

calcium ions [76]. More recently, it has been shown that capacitively coupled electrical stimulation of these cells induces an elevation in the level of TGF-β, an important growth factor in the formation of bone and cartilage [81]. The mechanism is believed to involve a calcium/calmodulin pathway. Calmodulin is a ubiquitous intracellular calcium-binding protein that interact with a wide range of enzymes. An increase in the concentration of cytosolic Ca^{2+} leads to an activation of calmodulin, which is responsible for many calcium-mediated processes including proliferation and differentiation of cells [81]. Thus, modulation of calcium transport through voltage gated channels, as a result of the electrical stimulation, could be mediating certain cellular differentiation pathways. Furthermore, calcium transport is also affected by dexamethasone [82]. Publicover et al. [82] have shown that low voltage-activated Ca^{2+} channels are induced in rat bone marrow stromal cells by dexamethasone to exhibit osteoblast-like channel modulation. Since the coupling of dexamethasone and electrical stimulation together was necessary to increase the ALP activity in our system, it may be possible that the two calcium transport processes and associated cellular pathways could be interacting to augment the observed osteogenic differentiation.

Lastly, it can also be suggested that the negatively charged polypyrrole surface could be binding the positively charged Ca^{2+} counterions during the electrical stimulation and thus triggering the activity of the alkaline phosphatase enzyme, possibly by means of a transmembrane protein or some other unknown mechanism. Furthermore, if the Ca^{2+} ions are indeed bound to the negatively charged sulfonate groups, the polymer surface could be acting as a localized source of Ca^{2+} ions near the cell surface.

CHAPTER 7: CONCLUSIONS

The following conclusions can be drawn up from this thesis:

1) Thin films of polypyrrole (PPy) synthesized with a polyanionic dopant, poly(styrenesulfonate), support the viability and proliferation of bovine bone marrow stromal cells (BMSC).
2) Upon application of electrical stimulation through the PPy films, BMSC undergo differentiation towards an osteogenic lineage. This result was indicated by an increase in alkaline phosphatase (ALP) activity relative to controls.
3) The presence of a glucocorticoid, dexamethasone, in the culture medium is necessary to observe the increase in ALP activity during the application of electrical stimulation.
4) The nature of the PPy surface, which contains negatively charged sulfonate groups, is hypothesized to be playing a role in cellular interactions to induce the osteogenic diferentiation.
5) By virtue of its conductive properties, its *in vitro* biocompatibility and surface characteristics, polypyrrole is a suitable interactive substrate for enhancing bone cell interactions. The evaluation is a first step toward the ultimate use of polypyrrole as a scaffold for bone regeneration.

CHAPTER 8: SUGGESTIONS FOR FUTURE WORK

1) An understanding of the mechanism of the increased alkaline phosphatase activity of BMSC on electrically stimulated polypyrrole cultures would be very critical in determining how polypyrrole can act as an interactive scaffold for bone regeneration.
2) In this study, the dopant ion chosen was the polyanion, poly(styrenesulfonate). The choice of dopant ion determines characteristics such as wettability and charge density which play a critical role in protein adsorption and cell-substrate interactions. Further work involving polycations as dopants, for example, poly(L-lysine hydrochloride) could be investigated to determine the effect of the type of dopant ions in eliciting the observed BMSC responses [38].
3) We have only investigated the use of polypyrrole as a 2 D substrate for bone regeneration. The fabrication of 3 D scaffolds should be investigated for possible use *in vivo* applications. An alternative approach to electrochemical synthesis that could be employed is the molecular self-assembly method, whereby a layer-by-layer process enables highly conductive forms of polypyrrole to be synthesized [83].
4) Other waveforms for electrical stimulation of BMSC can be investigated. Pulsed Electromagnetic Field (PEMF) stimulation has been employed to stimulate bone cells *in vitro* via a non-invasive method. The effect of using polypyrrole in this configuration could be another option to optimize the observed responses.
5) Finally, *in vivo* studies using an appropriate bone defect system would have to be evaluated for possible future clinical applications.

References

1. Langer, R., Vacanti, J. P., *Tissue Engineering.* Science, 1993. **260**: p. 920-926.
2. Yoshikawa, T.O., H., Dohi, Y., Davies J.E., *Viable bone formation in porous hydroxyapatite: marrow cell-derived in vitro bone on the surface of ceramics.* Biomed Mater Eng, 1997. **7**(1): p. 49-58.
3. Ohgushi, H., Okumara, M., Tamai, S., Shores, E.C., Caplan, A.I., *Marrow cell induced osteogenesis in porous hydroxyapatite and tricalcium phosphate: A comparative histomorphometric study of ectopic bone formation.* J Biomed Mat Res, 1990. **24**: p. 1563-1570.
4. Ishaug, S.L., Crane, G.M., Miller M.J., Yasko, A.W., Yaszemski, M.J., *Bone formation by three-dimensional stromal osteoblast culture in biodegradable polymer scaffolds.* J Biomed Mater Res, 1997. **36**(1): p. 17-28.
5. Ashammakhi, N., Rokkanen, P., *Absorbable polyglycolide devices in trauma and bone surgery.* Biomaterials, 1996. **18**: p. 3-9.
6. Yamamuro, T., *Bone-bonding behavior and clinical use of A-W glass-ceramic*, in *Bone Grafts, Derivatives and Substitutes*, M. Urist, O'Connor, B.T., Burwell, R. G., Editor. 1994, Butterworth-Heinemann: Oxford, U.K.
7. Yasuda, I., *Fundamental problem in the treatment of fracture.* J. Kyoto Med. Soc., 1952. **4**: p. 395-406.
8. Yasuda, I., *Piezoelectricity of living bone.* J Kyoto Pref Univ Med, 1953. **53**: p. 325.

9. Fukada, E., Yasuda, I., *On the piezoelectric effect of bone.* J. Phys. Soc. Japan, 1957. **12**: p. 1158-1162.

10. Becker, R.O., Bassett, C.A. L, Bachman, C. I. I., *The bioelectric factors controlling bone structure*, in *Bone Biodynamics*, R. Bourne, Editor. 1964, Little, Brown and Co.: New York.

11. Bassett, C.A.L., Pawluck, R. I., Becker R. O., *Effects of electrical currents on bone in vivo.* Nature, 1964. **204**: p. 652-654.

12. Bassett, C.A.L., Pawluk, R.J., Becker, R. O., Nature, 1969. **204**: p. 652-654.

13. Lavine, L.S., Lustrin, I., Shamos, M. H., Nature, 1969. **224**: p. 1112-1113.

14. Humbury, H.J., Watson, J., Sivyer, A., Ashley, D. J. B., Nature, 1971. **231**: p. 190-192.

15. Becker, R.O., Spadaro, J. A., Marino, A. A., *Clinical experiences with low intensity direct current stimulation of bone growth.* Clin. Orthop. Rel. Res., 1977. **129**: p. 75-83.

16. Bassett, C.A.L., Pilla, A. A., Pawluk, R. J., *A non-operative salvage of surgically-resistant pseudoarthroses and non-unions by pulsing electromagnetic fields.* Clin. Orthop. Rel. Res., 1977. **124**: p. 128-143.

17. Brighton, C.T., Friedenberg, Z. B., Mitchell, E. I., Booth, R. E., *Treatment of nonunion with constant direct current.* Clin. Orthop. Relat. Res., 1977. **124**: p. 106-123.

18. Watson, J., Downes, E. M., Jap. J. Appl. Phys., 1978. **17**: p. 215-217.

19. Bassett, C.A., L., Pawluk, R. J, Pilla, A. A., Science, 1979. **184**: p. 575-577.

20. Spadaro, J.A., *Mechanical and Electrical Interactions in Bone Remodeling.* Bioelectromagnetics, 1997. **18**: p. 193-202.

21. Shastri, V., Pishko, M., *Biomedical Applications of Electroactive Polymers*, in *Electrical and Optical Polymer Systems*, D.L. Wise, Wnek, G. E., Trantolo, D. J., Cooper, T. M., Gresser, J. D., Editor. 1998, Marcel Dekker: New York. p. 1031-1051.

22. Diaz, A.F., Kanazawa, K. K., Gardini, G.P., J. Chem. Soc. Chem. Commun., 1979: p. 635.

23. Couves, L.D., *Polypyrrole as a potentiometric glucose sensor.* Synth. Met., 1989. **28**: p. C761-C768.

24. Osaka, T., Komaba, S., Amano, A., *Highly Sensitive Microbiosensor for Creatinine Based on the Combination of Inactive Polypyrrole with Polyion Complexes.* Journal of the Electrochemical Society, 1998. **145**(2): p. 406.

25. Guiseppi-Elie, A., Tour, J. M., Allara, D. L., Sheppard, N. F., *Bioactive polypyrrole thin films with conductimetric response to analyte.* Mater. Res. Soc. Symp. Proc., 1996. **413**: p. 439-444.

26. Miller, L.L., Zhou, Q.-X., *Poly(N-methylpyrrolylium) poly(styrenesulfonate). A conductive, electrically switchable cation exchanger that cathodically binds and anodically releases dopamine.* Macromolecules, 1987. **20**(1594-1597).

27. Zhou, Q.-X., Miller, L. L., Valentine, J. R., *Electrochemically controlled binding and release of protonated dimethyldopamine and other cations from poly(N-*

mehtylpyrrole)/polyanion composite redox polymers. J. Electroanal. Chem., 1989. **261**: p. 147-164.

28. Shastri, V.R., *Evaluation of polypyrrole thin films as substratum for mammalian cell culture*. 1995, Rensselaer Polytechnic Institute: Troy, NY.

29. Wong, J.Y., Langer, R., Ingber, D. E., *Electrically conducting polymers can noninvasively control the shape and growth of mammalian cells.* Proc. Natl. Acad. Sci., 1994. **91**: p. 3201-3204.

30. Schakenraad, J.M., Busscher, H. J., Wildevuur, C. R. H., Arends, J., *The influence of substratum free energy growth and spreading of human fibroblasts in the presence and absence of serum proteins.* J. Biomed. Mater. Res., 1986. **20**: p. 773-784.

31. Tamada, Y., Ikada, Y., *Cell attachment to various polymer surfaces*, in *Polymers in Medicine II: Biomedical and Pharmaceutical Applications*, P.G. E. Chiellini, Migliaresi, C., Nicolais, L., Editor. 1986: New York. p. 101-115.

32. van Kooten, T.G., Schakenraad, J. M., van der Mei, H. C., Busscher, H. J., *Influence of substratum wettability on the strength of adhesion of human fibroblasts.* Biomaterials, 1992. **13**: p. 897-904.

33. Shastri, V., Schmidt, C.,, Kim, T.H., Vacanti, J., Langer, R., *Polypyrrole - a potential candidate or stimulated nerve regeneration.* Mater Res Soc Symp Proc, 1996. **414**: p. 113-117.

34. Schmidt, C.E., Shastri, V.R., Vacanti, J. P., Langer, R., *Stimulation of neurite outgrowth using an electrically conducting polymer.* Proc. Natl. Acad. Sci., 1997. **94**: p. 8948-8953.

35. Diaz, A.F., Castillo, J. C., Logan, J. A. and Lee, W. Y., *Electrochemistry of Conducting Polypyrrole Films.* J. Electroanal. Chem., 1981. **129**: p. 115-132.

36. Beelen, E., Riga, J., Verbist, J. J., *Electrochemical doping of polypyrrole: XPS study.* Synthetic Metals, 1991. **41**(Part I): p. 449.

37. Neoh, K.G., Kang, E. T., Tan, K. L., *Limitations of X-Ray Photoelectron Spectroscopy Technique in the Study of Electroactive Polymers.* J. Phys. Chem. B, 1997. **101**: p. 726-731.

38. Prezyna, L.A., Qiu, Y.J., Reynolds, J.R. and Wnek, G.E., *Interaction of cationic polypeptides with electroactive polypyrrole/poly(styrenesulfonate) and poly(N-methyl-pyrrole)/poly(styrenesulfonate) films.* Macromolecules, 1991. **24**: p. 5283-5287.

39. Street, G.B., *Polypyrrole: From powders to plastics*, in *Handbook of Conducting Polymers*, T.A. Skotheim, Editor. 1986: New York. p. 265-290.

40. Scott, J.C., Bredas, J. L., Yakushi, K., Pfluger, P., Street G. B., *The evidence of bipolarons in pyrrole polymers.* Synth. Met., 1984. **9**: p. 165-172.

41. Blackwood, D., Josowicz, M., *Work function and spectroscopic studies of interactions between conducting polymers and organic vapors.* J. Phys. Chem., 1991. **95**(1): p. 493-502.

42. Asavapiriyanont, S., Chandler, G.K., Gunawardena, G. A., Pletcher, D., J. Electroanal. Chem, 1984. **177**: p. 245.

43. Ko, J.M., Rees, H. W., Park, M., Kim, C. Y., *Morphology and Electrochemical Properties of Polypyrrole Films Prepared in Aqueous and Nonaqueous Solvents.* J Electrochem Soc, 1990. **137**(3): p. 905.

44. Yang, R., Naoi, K., Evans, D. F., Smyrl, W. H., Hendrickson, W. A., *Scanning Tunnel Microscopy Study of Electropolymerized Polypyrrole with Polymeric Anion.* Langmuir, 1991. **7**: p. 556-558.

45. Diaz, A.F., Bargon, J., *Electrochemical synthesis of conducting polymers*, in *Handbook of Conducting Polymers*, T.A. Skotheim, Editor. 1986: New York. p. 81-115.

46. Friedenstein, A.J., Potrakova, K. V., Kurolesova, A. I., Frolova, G. I., *Heterotopic transplants of bone marrow: analysis of precursor cell for osteogenic and hemopoietic tissues.* Transplantation, 1968. **6**: p. 230-247.

47. Owen, M., *Lineage of osteogenic cells and their relationship to the stromal system*, in *Bone and Mineral*, W.A. Peck, Editor. 1985, Elsevier: Amsterdam. p. 1-25.

48. Owen, M.E., Friedenstein, A. J., *Stromal stem cells: marrow derived osteogenic precursors.* CIBA Foundation Symposium, 1988. **136**: p. 42-60.

49. Beresford, J.N., *Osteogenic stem cells and the stromal system of bone and marrow.* Clin Orthop, 1989. **240**: p. 270-280.

50. Friedenstein, A.J., Chailakhyan, R.K., Gerasimov, *Bone marrow osteogenic stem cells: In vitro cultivation and transplantation in diffusion chambers.* Cell Tissue Kinet, 1987. **20**: p. 263-272.

51. Maniatopoulos, C., Sodek, J., Melcher, A.H., *Bone formation in vitro by stromal cells obtained from bone marrow of young adult rats.* Cell Tissue Res, 1988. **254**: p. 317-330.

52. Bab, I., Ashton, B. A., Gazit, D., Marx G., Williamson, M.C., Owen M.E., *Kinetics and differentiation of marrow stromal cells in diffusion chambers.* J Cell Sci, 1986. **84**: p. 139-151.

53. Luria, E.A., Owen, M. E., Friedenstein, A. J., Morris, J.F., Kuznetsow, S.A., *Bone formation in organ cultures of bone marrow.* Cell Tissue Res, 1987. **248**: p. 449-454.

54. Friedenberg, Z.B., Brighton, C.T., Michelson, J. D., Bednar, J., Schmidt, R., Brrockmeyer, T., *The Effects of Demineralized Bone Matrix and Direct Current on an "In Vivo" Culture of Bone Marrow Cells.* J Orthop Res, 1989. **7**: p. 22-27.

55. Matsunaga, H., Ijiri, K., Sakou, T., Morimoto, N., *Immunohistochemical study of activating osteoblasts from undifferentiated mesenchymal cells by electrical stimulation.* Bone, 1993. **14**: p. 327-332.

56. Martin, I., Muraglia, A., Campanile, G., Cancedda, R., Quarto, R., *Fibroblast Growth Factor-2 Supports ex Vivo Expansion and Maintenance of Osteogenic Precursors from Human Bone Marrow.* Endocrinology, 1997. **138**(10): p. 4456-4462.

57. Haynesworth, S.E., Goldberg, V. M., Caplan, A. I., *Diminution of the number of mesenchymal stem cells as cause for skeletal aging*, in *Muscoskeletal Soft-Tissue Aging: Impact on Mobility*, J.A. Buckwalter, Goldberg, V. M., Woo, S.L., Editor. 1993: Rosemont, IL.

58. Owen, *Bone Growth at the Cellular Level: A Perspective*, in *Factors and Mechanisms Influencing Bone Growth*, A.D. Dixon, Sarnat, B. G., Editor. 1982, Alan R. Liss, Inc.: New York. p. 19-28.

59. Shelton, R.M., Rasmussen, A.C., Davies, J.E., *Protein adsorption at the interface between charged polymer substrata and migrating osteoblasts.* Biomaterials, 1988. **9**(1): p. 24-9.

60. Krukowski, M., Shively, R.A., Osdoby, P., Eppley, B. L., *Stimulation of craniofacial and intramedullary bone formation by negatively charged beads.* J Oral Maxillofac Surg, 1990. **48**(5): p. 468-475.

61. Cutroneo, K.R., Sterling, K. M., Shull, S., *Steroid hormone regulation of extracellular proteins*, in *Regulation of Matrix Accumulation*, R.P. Mecham, Editor. 1986, Academic Press: New York. p. 119-176.

62. Cheng, S.L., Zhang, S.F., Avioli, L.V., *Expression of bone matrix proteins during dexamethasone-induced mineralization of human bone marrow stromal cells.* J Cell Biochem, 1996. **61**(2): p. 182-193.

63. Locklin, R.M., Williamson, M. C., Beresford, J. N., Triffitt, J.T., Owen, M. E., *In Vitro Effects of Growth Factors and Dexamethasone on Rat Marrow Stromal Cells.* Clin Orthop Rel Res, 1995. **313**: p. 27-35.

64. Shalhoub, V., Conlon, D., Tassinari, M., Quinn, C., Partridge, N., Stein, G. S., Lian, J. B., *Glucocorticoids Promote Development of the Osteoblast Phenotype by Selectively Modulating Expression of Cell Growth and Differentiation Associated Genes.* J Cell Biochem, 1992. **50**: p. 425-440.

65. Malaval, L., Modrowski, D., Gupta, A., Aubine, J. E., *Cellular Expression of Bone-Related Proteins During In Vitro Osteogenesis in Rat Bone Marrow Stromal Cell Cultures.* J Cell Phys, 1994. **158**: p. 555-572.

66. Leboy, P.S., Beresford, J. N., Devline, C., Owen M. E., *Dexamethasone Induction of Osteoblast mRNAs in Rat Marrow Stromal Cultures.* J Cell Physiol, 1991. **146**: p. 370-378.

67. Kasugai, S., Reynaldo, T., Nagata, T., Yao, K-L., Butler, W., Sodek, J., *Expression of Bone Matrix Proteins Associated with Mineralized Tissue Formation by Adult Rat Bone Marrow Cells in Vitro: Inductive Effects of Dexamethasone on the Osteoblastic Phenotype.* J Cell Physiol, 1991. **147**: p. 111-120.

68. Thomson, B.M., Bennett, J., Dean, V., Triffitt, J., Meikle, M. C., Loveridge, N., *Preliminary Characterization of Porcine Bone Marrow Stromal Cells: Skeletogenic Potential, Colony-Forming Activity, and Response to Dexamethasone, Transforming Growth Factor B, and Basic Fibroblast Growth Factor.* J Bone Miner Res, 1993. **8**(10): p. 1173-1183.

69. Kodicek, E., *The effect of ascorbic acid on biosynthesis of components of connective tissue*, in *Structure and Function of Connective and Skeletal Tissue*,

H.R.D. Fitton Jackson S., Patridge, S.M., Tristram, G.R., Editor. 1965: London. p. 307-319.

70. Barnes, M.J., *Function of ascorbic acid in collagen metabolism.* Ann NY Acad Sci, 1975. **258**: p. 264-277.

71. Bellows, C.G., Aubin, J. E., Heersche, J. N. M., Antosz, N. M., *Mineralized bone nodules formed in vitro from enzymatically released rat calvaria cell populations.* Calcif Tissue Int, 1986. **38**: p. 143-154.

72. Anderson, R.E., Kemp, J. W., Jee W. S. S., Woodbury, D. M., *Ion-transporting ATP-ases and matrix mineralization in cultured osteoblast-like cells.* In Vitro, 1984. **20**: p. 837-846.

73. Tenenbaum, H.C., Heersche, J. N. M., *Differentiation of osteoblasts and formation of mineralized bone in vitro.* Calcif Tissue Int, 1982. **34**: p. 76-79.

74. Gehron Robey P., T., J. D., *Human bone cells in vitro.* Calcif Tissue Int, 1985. **37**: p. 453-460.

75. Korenstein, R., Somjen, D., Fischler, H., Binderman, I., *Capacitative Pulsed Electrical Stimulaton of bone cells Induction of Cyclic-AMP changes and DNA synthesis.* Biochim. Biophys. Acta, 1984. **803**: p. 302-307.

76. Ozawa, H., Abe, E., Shibasaki, Y., Fukuhara, T., and Suda T., *Electric Fields Stimulate DNA Synthesis of Mouse Osteoblast-Like Cells (MC3T3-E1) by a Mechanism Involving Calcium Ions.* J. Cell. Physiol., 1989. **138**: p. 477-483.

77. Brighton, C.T., Okereke, E., Pollack, S. R., Clark, C. C., *In vitro bone cell response to a capacitively coupled electrical field. The role of field strength, pulse pattern and duty cycle.* Clin Orthop, 1992. **285**: p. 255-262.

78. Gupta, R., Naranja, R. J., Levit, C. L., Brighton, C. T. *The biochemical pathway of capacitively coupled electric field stimulation of osteoblast-like cells.* in *Proceedings of the 14th Annual Meeting of the Society for Physical Regulation in Biology and Medicine*. 1994. Washington, DC.

79. Green, E., Todd, B. Heath, D., *Mechanism of glucocorticoid regulation of alkaline phosphatase gene expression in osteoblast-like cells.* Eur J Biochem, 1990. **188**(1): p. 147-153.

80. Majeska, R.J., Nair, B.C., Rodan, G. A., *Glucocorticoid regulation of alkaline phosphatase in osteoblastic Osteosarcoma Cell Line ROS 17/2.8.* Endocrinology, 1985. **116**: p. 170-179.

81. Zhuang, H., Wang, W., Seldes, R. M., Tahernia, A. D., Fan, H., Brighton, C.T., *Electrical Stimulation Induces the Level of TGF-β1 mRNA in Osteoblastic Cells by a Mechanism Involving Calcium/Calmodulin Pathway.* 1997. **237**: p. 225-229.

82. Publicover, S.J., Thomas, G. P., El Haj, A. J., *Induction of a Low Voltage-Activated, Fast-Inactivating Ca^{2+} Channel in Cultured Bone Marrow Stromal Cells by Dexamethasone.* Calcif Tiss Int, 1994. **54**: p. 649-661.

83. Fou, A.C., Rubner, M. F., *Molecular-Level Processsing of Conjugated Polymers. 2. Layer-by-layer manipulation of In-Situ Polymerized p-Type Doped Conducting Polymers.* Macromolecules, 1995. **28**(21): p. 7115-7120.

Appendix

Cell viability of bone marrow stromal cells decreases with consecutive passaging. The following graph shows the cell viability from MTT assays for cells passaged from P2 to P5. The values are normalized to the value for tissue culture polystyrene (TCPS) at the first subculture ie. P2. Cells were trypsinized and plated as described in Section 4.2.2.1.

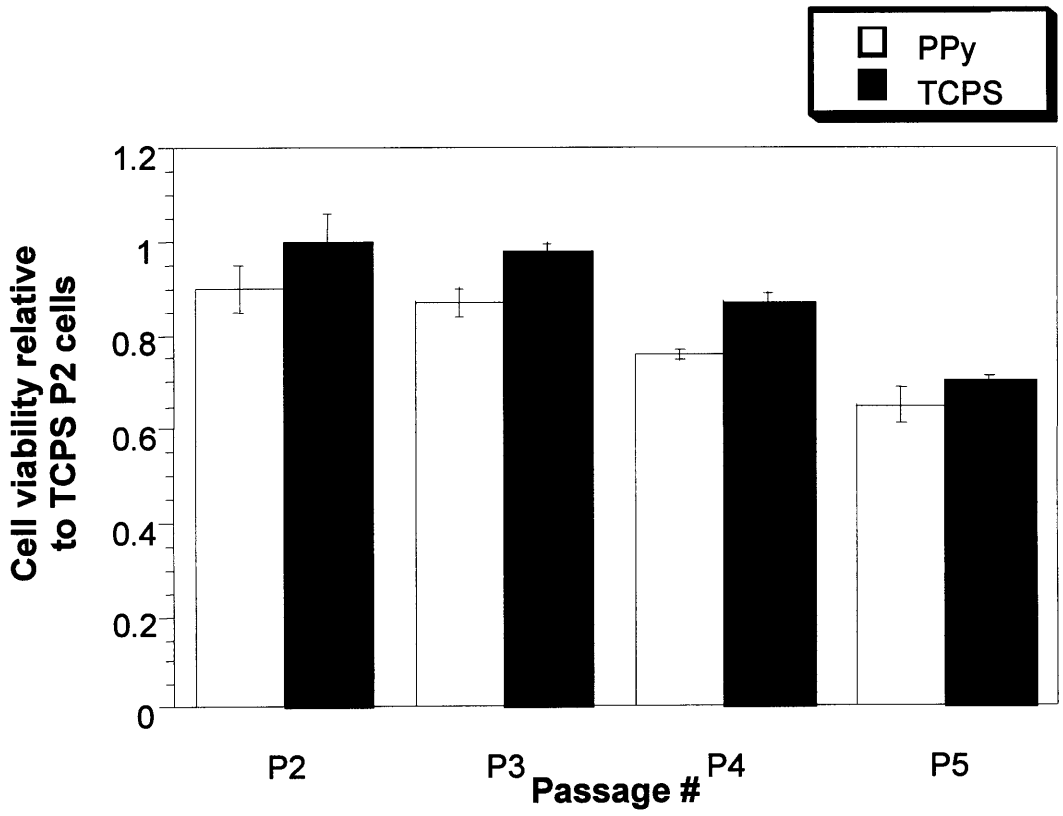


Figure A.1: Effect of passaging on cell viability