ENGINEERED POLYMER IN BONE-TARGETED CELL DELIVERY

Safarova (Yantsen) Yuliya

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ABSTRACT

Osteoporosis is a progressive system skeletal disease associated with the decreased bone mineral density and disrupted microarchitecture of the bone tissue that facilitates fragility and risk of fractures. In osteoporotic conditions the reduction in bone density and strength occurs due to the elevated osteoclastic activity and the diminished number of the osteoblast progenitor cells - mesenchymal stem cells (MSCs). This dissertation is focused on the evaluation of the new approach in cell therapy with membrane engineered MSCs that display covalently-coupled synthetic osteophilic polymers to restore the osteoblast progenitor pool and, at the same time, to inhibit osteoclastic activity in the fracture zones of the osteoporotic bones. The primary active sites of the polymer are bisphosphonate functional groups that target hydroxyapatite molecules (HA) on the bone surface and inhibit osteolysis. N-hydroxysuccinimide (NHS) groups on the other end of the molecule allow the polymer to covalently bind to MSCs' plasma membrane components. The polymer did not affect MSCs proliferation and osteogenic differentiation while inhibiting phagocytic activity of the bone marrow derived macrophages *in vitro*. The therapeutic potential of the polymer-modified MSCs was studied in female rats with the experimentally induced ulna fractures and estrogen-dependent osteoporosis. The osteoporosis was induced by the ovariectomy (OVX). Micro-CT morphometry and histology analysis were used to determine the effect of the injected MSCs on the bone healing. Intravital analysis of the bone density dynamics in the zone of ulna fracture showed a significant increase (27.4% and 21.5%) in BMD at 4 and 24 weeks respectively after the osteotomy of the ulna in the group of animals receiving 4 transplantations (1 million cells, once per week) of the MSC modified with the polymer. The results of the intravital observations were confirmed by post-mortem analysis of the histological slices of the fracture zones.

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(yellow arrows), no signs of dystrophy or degeneration of the bone tissue (red arrow). Calibration=100μm.

LIST OF ABBREVIATIONS

ATRP – atom transfer radical polymerization

BP – bisphosphonate

DMAA - dimethylacrylamide

FBS – fetal bovine serum

GIO – glucocorticoid-induced osteoporosis

HA – hydroxyapatite

IA - intra-arterial

IV – intravenous

MSC – mesenchymal stem cell

NHS – N- hydroxysuccinimide

OI – Osteogenesis Imperfecta

OVX - ovariectomy

PA – Paget's Disease

PTH – parathyroid hormone

WHO – World Health Organization

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Finally, I am grateful to my family, my parents for always believing in me, my husband for always supporting me throughout this academic journey, and to my daughter Sofia for her curiosity to learn this world.

DECLARATION

I declare that the research contained in this thesis, unless otherwise formally indicated within the text, is the original work of the author. The thesis has not been previously submitted to this or any other university for a degree and does not incorporate any material already submitted for a degree.

Signed 

Dated 20-03-2021

CHAPTER 1. INTRODUCTION

1.1 Problem statement

Osteoporosis is a systemic skeletal disease associated with the decreased bone mineral density (BMD), progressive bone loss and deterioration of the bone microarchitecture engendering the elevated risk of fractures. According to WHO, osteoporosis ranks fourth in the world along with cardiovascular, oncological diseases and diabetes mellitus. According to the International Osteoporosis Foundation, 200 million women worldwide are afflicted by osteoporosis, with every third woman and every fifth man suffering from the disease. In Kazakhstan, 12,5% of women and 9,09% of men are affected by osteoporosis. By 2050, the worldwide incidence of most dangerous and “hard-to-heal” hip fracture in men is projected to increase by 310% and 240% in women. This pessimistic prognosis is associated with global aging. According to specialists' forecasts, the prevalence of disability due to the osteoporosis-associated fractures in the World by 2025 will be about 2.6 million cases, and the number of deaths after a hip fracture will reach about 700 thousand per year [1, 2]. Osteoporotic fractures are a serious public health problem, as they cause disability, significant deterioration in the quality of life and mortality. So, only in one European Union each day there are about 1700 fractures, and per year - about 650 thousand cases. The economic losses associated with fracture treatment and rehabilitation in developed countries are a very large expense item in health care. For example, the cost of one case of hip fracture is about 21 000 USD [3].

The decrease in BMD or osteopenia is induced by the upregulated rate of resorption during the bone turnover, age-related decrease in osteoblasts progenitor cells and downregulated calcium absorption. The condition is considered age–related; therefore, the main risk group is elderly people. The most affected group is women in the postmenopausal period. Diminished production of estrogen, the hormone that stimulates bone formation, induces bone resorption and affects calcium homeostasis by increasing calcium absorption from the intestine [1, 4]. According to the

WHO osteoporosis is defined when bone mineral density is more than 2.5 SD below young adult reference.

Osteoporosis could be treated in several ways though most of the treatments are pharmacologic agents that have antiresorptive properties. Bisphosphonates (BP) are the most widely used class of drugs. BPs are pyrophosphates chemical analogues (H_2O_3P-O-PO_3H_2), with substitution of the central hydrolytically labile P-O-P linkage for the hydrolysis-resistant P-C-P bond. They specifically react with the hydroxyapatite group which is found on the site of bone resorption. Bisphosphonates cause the interruption of the osteoclast formation thus disrupting their functional activity and hence improving the formation of the bone tissue. Bisphosphonate-based treatment is widely accepted in clinical practice to manage not only osteoporosis but also other bone-related conditions such as Paget's disease [5, 6].

A new approach in osteoporotic treatment is cell therapy [1, 4]. Besides the increased osteoclastic activity reduction of BMD occurs also due to the diminished number of the osteoblast progenitor cells – mesenchymal stem cells. From this point of view, one of the very attractive approaches for treatment of osteoporotic fractures is MSCs therapy. [7-9]. It has been proposed to infuse autologous MSCs cultured *in vitro* for local bone regeneration. For treatment of osteoporosis an intravenous biotransplant containing from 50 to 500 million of MSCs is administered [10]. Another approach is injection of expanded *in vitro* autologous or allogenic mesenchymal pluripotent stem cells into the bone injury zone to improve the processes of reparative osteogenesis[11-13]. However, the administration of MSCs failed to prevent the ovariectomy-induced bone loss. According to the researchers from University of Davis, they used a novel compound plus a MSC injection and showed the increased rate of bone formation [14]. However, the results of MSCs effectiveness in treating osteoporosis are still contradictory.

1.2 Objective and Structure of the Thesis

Aim: to test the hypothesis that the synthetic engineered polymer is an effective approach for mesenchymal stem cells delivery for bone regeneration in osteoporosis and similar conditions.

Objectives:

1. Characterization of a synthetic osteophilic polymer;
2. Evaluation of the cytotoxicity of the synthetic polymer *in vitro*;
3. Effect of the synthetic polymer on differentiation of the MSCs into osteocytes;
4. Evaluation of the ability of synthetic polymer coated MSCs to stimulate bone regeneration in an animal model of osteoporosis in fracture condition.

In present study mesenchymal stem cells coated with synthetic bisphosphonate-based polymer has been tested in order to promote the regeneration of bone in osteoporosis and similar conditions. The primary active sites of the polymer are bisphosphonate functional groups that target hydroxyapatite molecules (HA) on the bone surface and inhibit osteolysis. N-hydroxysuccinimide (NHS) groups on the other end of the molecule allow the polymer to covalently bind to MSCs' plasma membrane components. Attachment of the polymer to MSCs' plasma membrane allows the cells to bind precisely to the hydroxyapatite component of the bone to localize cellular repair and therapeutic functions to the areas of the affected bone (Figure 1).

The **approach** is unique for several reasons. First of all, the method of synthesis give the ability to control the number of the bisphosphonate groups incorporated into the polymer, and hence the ability to control the strength of binding to the bone. This process also allows controlling the final length of the polymer as this could be a major factor in biological activity of the bisphosphonates. Currently one similar approach could be found in literature. Research group from the University of California Davis are using the bone targeting construct that include alendronate as

bone targeting agent and LLP2A as a cell ligand[14]. However in this Thesis the polymer differs in the cell binding functional group as NHS group was used. **The main novelty of this approach** is to combine a method of cell therapy (MSCs in particular) with the use of a bisphosphonate-based polymer that is responsible for targeted delivery of the cells to the bone and for reduction of the osteoclastic activity and therefore prevention of the bone demineralization.

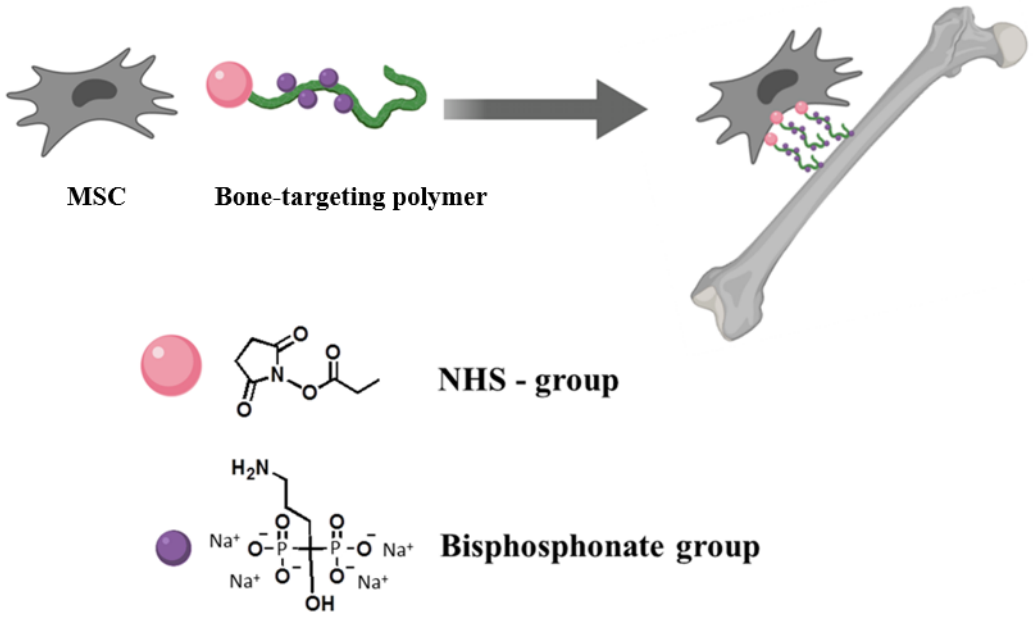

Figure 1. Graphic representation of the synthetic polymer binding to the cells and bone

Current PhD Thesis is separated into 4 Chapters:

Chapter 1 provides the background information on the osteoporotic condition and various traditional treatments as well as describes the hypothesis and overall structure of the Thesis.

Chapter 2 brings the background on the osteoporotic condition and further details on stem cell treatments, cell surface modifications for targeted delivery and animal models used in the preclinical research.

Chapter 3 is focused on the *in vivo* studies of the polymer interaction with the mesenchymal stem cells and bone chips to evaluate the optimal concentrations for binding and its effect on the viability and further differentiation of the MSCs.

Chapter 4 describes the results for *in vivo* studies aimed to assess the therapeutic potential of the proposed approach in ulna fracture regeneration in OVX condition.

1.3 Role of Collaborators

The current dissertation is a multidisciplinary study aimed at developing a novel approach for the enhanced bone regeneration in complex condition of the osteoporotic fracture. This thesis was supported by the Ministry of Education and Science of the Republic of Kazakhstan, which financed the PhD program, project grant № 0118PK01040 MES RK and the Nazarbayev University CRP grant № 091019CRP2113.

The part of the thesis that involves polymer synthesis using ATRP technique was performed in collaboration with the Laboratory of Dr Alan Russell from Carnegie Mellon University and in particular Dr Hironobu Murata as a leading chemist.

All aspects of this thesis have been reviewed by the team of supervisors: Dr Sholpan Askarova, Dr Alan Russell and Prof Gonzalo Hortelano. Several contributions were also received from the colleagues working at the National Laboratory of Astana of Nazarbayev University including Dr Farkhad Olzhayev, Dr Bauyrzhan Umbayev, Dr Andrey Tsoy and Aiym Kaiyrlykyzy

This thesis is based on my work, and it was written by me. Individual contributions are described in detail in the contribution section of the author, where applicable.

1.4 Thesis Outputs

Journal Articles

1. Y.Safarova, B.Umbayev, G.Hortelano, Sh.Askarova. Mesenchymal stem cells modifications for enhanced bone targeting and bone regeneration. Regenerative Medicine, **16 April 2020** (Ahead of print) doi.org/10.2217/rme-2019-0081

2. Y.Safarova (Yantsen), F.Olzhayev , B.Umbayev, A.Tsoy , G. Hortelano, H.Murata, A.Russell and S. Askarova. Mesenchymal stem cells membrane-engineering enhances osteoporotic bone fracture regeneration// Bioengineering, 2020, 7(4):125; https://doi.org/10.3390/bioengineering7040125

3. B.Umbayev, A-R. Masoud, A.Tsoy, D.Alimbetov, Farkhad Olzhayev, Aiym Kaiyrlykyzy, Yuliya Safarova, Terence Davis, Sholpan Askarova. "Elevated levels of the small GTPase Cdc42 induces senescence in male rat mesenchymal stem cells". Biogerontology, Biogerontology, 19(3), 287-301, 2018 DOI 10.1007/s10522-018-9757-5

Local journals

1. Y.Safarova, F.Olzhayev, B.Umbayev, A.Yerkebayeva, A,Karenkina, I.Kotov, A. Russell, and S. Askarova. Perspective approaches for treatment of low-energy injury bone tissue injuries using bioengineering methods and cell therapy. **Science and Healthcare**, 2019 (Vol.21) 5, p. 68-80 (Russian)
2. Olzhayev F.S., Safarova Ju.I., Tsoy A.K., Umbayev B.A., Askarova Sh.N. Cell therapy approach for correction of osteoporosis-associated fractures using adipose-derived mesenchymal stem cells functionalized with osteophilic polymer. **Experimental Biology.** 2018; 4(77):59-72 (Russian)
3. Olzhayev F.S., Safarova (Yantsen) Y.I., Umbayev B.A., Tsoy A.K. and Askarova Sh.N. Animal model of ulna fracture in experimentally induced osteoporosis. Vestnik KazNMU, 2020, №3 :218-223 (Russian)

Patent

- Patent of RK № 34147 “Stimulation of reparative osteogenesis of osteoporotic bone fracture and similar pathologies based on combined application of MSCs and synthetic bone targeted polymer” (2020)

Presentations at the Conference:

- Oral presentation, BioMaH Conference (Biomaterials for Healthcare), Rome, Italy, October, 2016.
- Oral presentation at Astana Biotech 2018 – Astana, Kazakhstan,12-13 June 2018
- Oral presentation at 3d Congress of Orthopedic Traumatologists of the Republic of Kazakhstan and VII Eurasian Congress of Orthopedic Traumatologists – Nur-Sultan, Kazakhstan, September 2019

Awards

- Best oral presentation among young scientist, BioMaH Conference, Rome, Italy, October, 2016.

Abstracts

- Y.Safarova (Yantsen), F.Olzhayev, D.Idrissova, B.Umbayev, H.Murata, R.Koepsel, Z.Zhumadilov, A.Russell and Sh.Askarova. *MSCs functionalized with osteophilic polymer enhance compact bone regeneration in osteoporotic rat model in vivo.* 3rd Annual Stem Cell Congress, Oxford Global, 3-4 November 2016, London, UK. P-2. (Poster)
- Y.Safarova (Yantsen), F.Olzhayev, D.Idrissova, B.Umbayev, H.Murata, R.Koepsel, A.Russell, Sh.Askarova. *MSCs functionalized with osteophilic polymer enhance compact bone regeneration in osteoporotic rat model in vivo. 4th International Scientific Conference on Regenerative Medicine and Healthy Aging*. May 11-12, 2016, Astana, Kazakhstan, p.45

- Y.Safarova (Yantsen), F.Olzhayev, D.Idrissova, B.Umbayev, H.Murata, R.Koepsel, Z.Zhumadilov, A.Russell and Sh.Askarova. *Mesenchymal stem cells coated with synthetic bone-targeted polymer as a new approach for managing of osteoporotic bone fracture regeneration.* 1st biennial conference BioMaH October 17-20, 2016 Rome, Italy. - p.227-230

- Askarova Sh.N., Safarova (Yantsen) Y.I., Olzhayev F.S. Promising approaches for treatment of traumatic bone tissue injuries using bioengineering and cell therapy. Materials of 3d Congress of Orthopedic Traumatologists of the Republic of Kazakhstan and VII Eurasian Congress of Orthopedic Traumatologists. – Nur-Sultan, 2019. – P. 77 – in Russian

CHAPTER 2. LITERATURE REVIEW

2.1 Pathophysiology of osteoporotic conditions

The molecular mechanisms behind the osteoporosis are well defined. Bone metabolism depends on two types of cells. Osteoblasts, or bone-forming cells, generate the bone tissue. Progenitors of the osteoblasts in adult organism are mesenchymal stem cells that reside in the bone-marrow cavity, adipose tissue and synovial fluid and subsequently can be isolated from those tissues. Second type of cells that maintain bone equilibrium are osteoclast, or bone resorbing cells. Osteoclasts derive from the hematopoietic lineage and produce hydrolytic enzymes and metalloproteases (MMP-3 and MMP-6) that digest organic component of the bone matrix[15].

These two populations of cells are in a delicate balance, which can be disturbed both by growth factors that are secreted by bone cells, and by the optimal ratio of calcium and magnesium in the bone tissue. There is a pattern when the magnesium content falls in the blood, balance is restored due to the work of the kidneys by means of less retention of calcium ions, and if the concentration of magnesium in the body increases, the kidneys accordingly remove less calcium from the body. First of all, for this very reason, the body needs magnesium itself and vitamin B6, which helps to keep it in the cell[16].

The activity of osteoclasts and osteoblasts is also subject to the humoral regulation. When the level of the hormone estrogen decreases, the level of osteoclast activity rises above the level of osteoblasts and result in disproportionate destruction of the bone tissue and its depletion that subsequently promote osteoporotic condition.

There are two types of osteoporosis - primary and secondary. Primary osteoporosis further divides onto type I (postmenopausal) or type II (senile). In osteoporosis of the type I, a distinctive feature is the loss of bone mass of the cancellous bones[17]. The cancellous bone has larger surface

area and is located in the bodies of the skeletal vertebrae and at the end of the tubular bone (bones of the wrist, femur), and contribute to the critical fractures in these places. People with this type of osteoporosis are more likely to experience a fracture of the femoral head. Type II osteoporosis is so called senile osteoporosis that is characterized mainly by loss of the cortical bone mass. This type of osteoporosis occurs in older people aged 70-80 years and is associated with gradual age-related decrease in stem cell progenitors. Among the forms of the secondary osteoporosis are also rheumatic diseases, endocrine diseases, pathology of the digestive tract, blood system, kidney disease, as well as many other conditions and genetic abnormalities[18].

2.2 Animal Osteoporotic Models

Three common types of the osteoporosis model in animals are postmenopausal, glucocorticoid and disuse model besides the genetically modified animals[19]. In our research we focused on postmenopausal model to resemble the physiological conditions in osteoporosis type II. The selection of an appropriate animal model should be based on several criteria such as suitable disease analogue and known biological background, reproducibility and repeatability of the model, cost and affordability and ethical concerns[20].

Osteoporotic condition can be modeled in different animals including mice, rats, rabbits, dogs, sheep, pigs and non-human primates. Traditionally all the experiments involving primates meet more strict ethical concerns compared to that of rats or mice. Non-human primates are widely used to evaluate the efficacy of the osteoporotic treatment as large animal model for final phase of preclinical testing[21]. Sheep is used for orthopedic research as they are more compliant and cost-effective in maintenance for long-term studies[20]. Dogs were extensively used in bone related research as they possess considerable basic multicellular unit-based remodeling. Also they are

cheaper than primates and more compliant to work with. Although several authors have shown the decrease in bone density after OVX[22, 23] one of the main limitations is resistance of the canine organism to the estrogen deficiency. Removal of the ovaries does not bear the same effect as in humans and do not lead to significant bone loss associated with the human osteoporotic condition. Mice are usually used for the manipulations with the genome and creating models of the genetic bone disorders such as osteogenesis imperfecta, or introducing the enhanced expression of the various bone proteins such as BCL-2 to promote the osteogenesis[20].

OVX rat model is most widespread and accepted model for the postmenopausal osteoporotic research. One of the main limitations of the rat animal model is the lack of Harversian system compared to human. In humans, cortical porosity is caused by the Harversian remodeling, but in rats most cortical bone loss is in endosteum and bone gain in the periosteum. In this course the larger animals that possess Harversian system seem to be more appropriate for the osteoporosis research. However the accommodation of sheep, dogs and primates are more cost effective, labour-consuming and may not always resemble the process of the postmenopausal osteoporosis in human (eg in dogs)[24]. Taking into account the difficulties with other animal models the lack of harversian remodeling in rat is an limitation that can be overcome.

2.3 Traditional Treatments

Osteoporosis is a part of aging so it cannot be cured but there are several pharmacological strategies that can result in bone strengthening for some time. Traditional treatments used in clinical practice include bisphosphonates, hormonal therapy (hormone replacement therapy with estrogen or recombinant parathyroid hormone), selective estrogen modulators and dietary calcium supplement intake. There are also several non-pharmacological strategies involving the vertebral plastic surgery

such as vertebraplasty and kyphoplasty. Though it only relieves the symptoms of the vertebral compression[25].

Promising management of postmenopausal osteoporosis is hormone replacement therapy such as estrogen or combination of estrogen and progesterone. These drugs have a therapeutic effect only after long-term use for several years and affect humoral regulation of the bone metabolism. The effectiveness of such drugs is pronounced. According to the HOPE (Health, Osteoporosis, Progestin, Estrogen) trial oral treatment with conjugated equine estrogens (CEE) prevented the bone loss from the vertebrae and hip and improved other symptoms associated with the menopause such as lipid and bleeding profiles, endometrial hyperplasia and vasomotor function[26]. The prolonged therapy with CEE overall decreased the risk of fracture in osteoporosis[27]. However the discontinuation of the estrogen treatment leads to the termination of the beneficial effect and the bone loss occurs almost at the same rate as before the treatment, so called "catch up bone loss".[28] Drawbacks include the higher risks of breast cancer development [29].

Selective estrogen modulators (SERMs) are the prospective pharmacological agents that act as estrogen agonist to maintain the bone density. On the other hand in breast tissue SERM act as a estrogen antagonist and prevents the breast cancer development[30]. SERMs decrease the osteoclast activity[31]. It also activates the expression of osteoprotegerin and downregulate the RANKL level facilitating recruitment of the osteoblast progenitor cells and preserving BMD[32].

Other option of the hormone therapy include treatment with parathyroid hormone (PTH) analogues such as teriparatide, the first FDA approved osteoporotic drug, and abaloparatide[33]. The drugs resemble the physiological effect of PTH and enhance the osteogenesis from the osteoblasts. According to the clinical trial of Saag et al, treatment with teriparatide had more pronounced effect than traditional bisphosphonate treatment resulting in improved BMD in vertebrae and hip and decreased vertebral fractures rate[34]. However, teriparatide group had more incidence of

hypercalcemia. Besides the treatment with PTH analogues has been also associated with the risk of osteosarcoma[35].

One of the novel ideas in bone metabolism management is use of the receptor activator of NF-kB ligand (RANKL) inhibitors. RANKL or osteoprotegerin ligand (OPGL) plays a vital role in activation of the osteoclasts that are responsible for bone resorption [36]. Denosumab is a drug that is human monoclonal antibody suppressing RANKL. FREEDOM trial assessed the effectiveness of Denosumab for 36 months showed that risk of the vertebral fractures decreased by 69%, risk of the non-vertebral fractures – by 20% and hip fractures by 40%[37, 38]. Adverse effects of the prolonged use of Denosumab include hypercholesterolemia, musculoskeletal pain and hypocalcemia[33].

2.4 Bisphosphonates

One of the most common strategies in managing osteoporosis is bisphosphonate therapy[39-41]. Bisphosphonates (BP) are the most widely used class of drugs. BPs are chemical analogues of pyrophosphates (H_2O_3P-O-PO_3H_2), with substitution of the central hydrolytically labile P-O-P linkage by the hydrolysis-resistant P-C-P bond. They specifically react with hydroxyapatite group which is found on the site of bone resorption. Bisphosphonates cause the interruption of osteoclast formation thus disrupting their functional activity and hence improving the formation of the bone tissue. Bisphosphonate-based treatment is widely accepted in clinical practice to manage various bone-related conditions including as Paget's disease [39].

Bisphosphonates comprise the whole group of chemical compounds and can be divided into 2 groups according to the N terminal between itself [42]. It is aminobisphosphonates that have a more pronounced effect than "simple" bisphosphonates. Aminobisphosphonates are not absorbed by the osteoclasts and therefore have an additional osteolytic effect. In particular, they inhibit the

mevalonate metabolic pathways by blocking the enzyme farnesyl diphosphate synthetase, which destroys a specific protein. The destruction of the latter leads to the accumulation of abnormalities in osteoclasts and accelerates their apoptosis and leads to an additional decrease in bone resorption. The antiresorptive effect of the various aminobisphosphonates depends precisely on the ability to interfere with this enzymatic pathway[43]. Bisphosphonates are also able to inhibit the osteolytic activity of cancer cells in bone tissue. The ability of the bisphosphonates to suppress pathological bone resorption and stimulate bone formation determines their therapeutic effect in osteoporosis [44, 45]. Treatment with bisphosphonate drugs has come a long way from clinical trials to commercial drugs approved by the FDA for clinical use. Table 1 below shows the drugs available on the market.

Table 1. Bisphosphonates in clinical practice

International name	Commercial name	Manufacturer	Relative activity	Doses in osteoporosis
Simple Bisphosphonates				
Etidronate	Didronel EHDP	Procter & Gamble	1	400 mg daily for 2 weeks every 3 months
Tiludronate	Skelid	Sanofi	10	40 mg per day
Clodronate	Bonefos Syndronate Loron Clodron	Schering	10	—
Amino bisphosphonates				
Pamidronate	Aredia Pamired Pamidronate Pamiredin Pamitor Pamyphos	Novartis	100	30–80 mg every 4 months
Alendronate	Fosamax Osteomax Lindron Rekostin Alendronate-Stoma Fosalen Ostalon Alendros 70	Merk	1000	5–10 mg per day or 70mg every week
Ibandronate	Boniva	Roche	and 1000	2,5 mg per day or

	Bondronat Bondron	Glaxo Smith Kline		150 mg every month
Risedronate	Actonel Actonel Ca^{++}	Aventis and Procter & Gamble	2000	5 mg per day or 35 mg every week
Zoledronic acid	Zometa Aclasta Reclast Zoledronic acid	Novartis	10000	2–4 mg per year

2.5 Stem cell treatments

Besides the increased osteoclastic activity reduction of BMD occurs also due to the diminished number of the osteoblast progenitor cells – mesenchymal stem cells. From this point of view, one of the most attractive approaches for treatment of the osteoporotic fractures is MSCs therapy[7-9].

Transplantation of MSCs is performed either systemically or locally. Systemic administration is represented by the intravenous (IV) and intra-arterial (IA) injection of the cells, while the local administration implicates direct injection of the cells into the region of regeneration. To maintain the oxygen and nutrient supply systemic route is preferred besides this route of transplantation is less invasive and cells can easily penetrate through the vessel walls to the target tissue[46]. Though, according to the previous studies 35% of the administered cells tend to home in lung tissue[47-50], the number of cells residing in lungs reduces dramatically to 2%[51]. Further migration of transplanted MSCs from the lungs are mediated by the inflammation process in the tissue of target [52]. Chemokine CCL21 is expressed in the vessels near the inflammation site and is a driving force that attracts MSCs to the area of inflammation[53]. Therefore, the efficiency of the systemic route of transplantation to regenerate bone tissue could be compromised by the other ongoing chronic inflammation processes. Among other drawback is the tendency of administered MSCs to accumulate in the areas of abnormal cell proliferation such as breast or ovary cancer[54].

There are several approaches to navigate the cells directly to the site of interest. The targeting moiety can be stimulated by an independently administrated component, for example, an injection of parathyroid hormone (PTH). Treatment with PTH along with administration of MSCs promotes cell migration to the area of bone defects and facilitates further differentiation of the cells[55]. Generally, recruitment of the MSCs to the site of fracture is initiated through the stromal cell-derived factor 1 (SDF1)/C-X-C chemokine receptor type 4 (CXCR4) axes. Though, PTH administration alters the mechanism of MSCs recruitment to the amphiregulin pathway where epidermal growth factor (EGF)-like ligand is secreted in the affected area[55, 56].

Alternative way of MSCs administration is a local transplantation directly to the site of bone fracture. One of the significant benefits of the local transplantation is close proximity of the cells to the areas of bone defect. Conversely, the survival of administered cells is uncertain since oxygen and nutrients are not readily available at the sites of injection. In this consequence the degree of cell engraftment largely depends on the delivery system that will place the cells at or force the MSCs to migrate to the site of bone defect.

2.6 Cell surface modifications for targeted delivery

In consistence with studies of Wu et al MSCs express almost 19 receptors on its cell surface. All of them can potentially be exploited for targeting and homing [57]. However these naturally occurring receptors are only effective in direct transplantation of MSCs without prior expansion *in vitro.* However, during expansion of MSCs in laboratory condition most of the receptors on the surface of cells are lost [58]. This produces a whole research area for the biomedical engineering of receptors and ligands in tissue-specific delivery of MSCs (Table 2).

Table 2. Target moiety and corresponding agents for surface modifications of cells

Target moiety	**Agent**	**References**
Hydroxyapatites	Bisphosphonates	[14, 59]
CXC4R	SDF-1	[60]
E-selectins	CD44 glycoform	[61]
P-selectins	SLeX	[62]

Note. CXC4R - C-X-C chemokine receptor type 4; SDF-1 - Stromal cell-derived factor-1, sLeX – sialyl Lewis X.

For instance, MSCs rolling and homing to SDF-1 that is expressed in the bone marrow and ischemic tissue is facilitated by a C-X-C chemokine receptor type 4, CX4CR [63, 64]. In this respect, in a study of Jones *et al* MSCs were incubated with SDF-1 for one hour before transplantation to elevate the expression level of CXCR4 receptor. This approach improved the engraftment of the cells both in wild type and in osteogenesis imperfecta mice (OIM), as well as amended the bone quality and plasticity to fracture, particularly in OIM animals[60].

As mentioned before, MSCs have a strong affinity for the inflamed tissues, and evidence suggests that endothelial-expressed P- and E-selectins are used to selectively attract MSCs to the sites of inflammation.[65]. The selectins are a type 1 transmembrane cell adhesion molecule and regulates the first step of leukocyte recruitment during inflammation. There are a lot of glycoproteins that are physiological ligands for selectins on the surface of MSCs such as P-selectin glycoprotein ligand 1 (PSGL-1), E-selectin ligand 1 (ESL-1), CD34 and CD44 [65]. To improve MSC trafficking to bone, Sackstein et al used a glycan engineering technique. The native CD44 glycoform on the surface of MSCs was changed ex vivo into hematopoietic cell E-selectin/L-selectin ligand. (HCELL). According to the results within the hours upon systemic transplantation of MSCs cells migrated and assembled to the bone marrow [61]. Correspondingly Sarkar et al.

suggested that MSCs could be targeted to the bone marrow using a nanometer-scale polymer framework containing a sialyl Lewis X (SLeX), also known as cluster of differentiation 15s (CD15s) or stage-specific embryonic antigen 1 (SSEA-1). SLeX is a tetrasaccharide carbohydrate that is normally found on the leukocytes surface as an active selectin ligand binding site[66, 67] and facilitates cell rolling and engraftment into the inflamed tissue with strong expression of the P-selectins [62].

In a study of Guan et al researchers created a two-end framework that target the cell surface via a synthetic peptidomimetic ligand (LLP2A) and bisphosphonate (alendronate, Ale) [14]. The construct promoted the migration and differentiation of MSCs down the osteogenic lineage *in vitro.* According to the obtained results the administration of the LLP2A-Ale modified cells augmented the formation of the trabecular bone in a mouse model of osteoporosis (estrogen-dependent model) mainly due to the effective homing and retaining of the cells with the bisphosphonate in the bone [14].

CHAPTER 3. *In vitro* analysis of the polymer interaction with mesenchymal stem cells

3.1 INTRODUCTION

Bone marrow-derived mesenchymal stem cells are promising agents in cell therapy. Most common source of MSCs in clinical practice is iliac rest. MSCs can also be isolated from the proximal and distal tibia, mandibular, proximal and distal femur, vertebral bone and humerus [68]. However, the bone marrow form the posterior iliac crest proved to be the most efficient in terms of the resulting number of MSCs [69]. Like all other stem cells, MSCs possess self-renewal capacity and ability to differentiate towards the terminal cell type. MSCs are the precursors of adipocytes, chondrocytes and osteocytes and consequently can differentiate into the corresponding cell types. The ability of MSC differentiate towards the osteogenic lineage makes them an attractive approach in orthopedics applications.

However, to obtain the clinically relevant number of cells that would be sufficient for transplantation in therapeutic purposes mesenchymal stem cells should be increased *in vitro* conditions. During the *in vitro* expansion almost all surface receptors that are responsible for the homing of MSCs are lost[58] . To add bone moiety, the cells could be engineered in several ways. First, the cells can be genetically modified to express certain receptors, for example the BMP-2[70-73], BMP-4[74, 75], BMP-6[76, 77], BMP-7[78], BMP-9[79-81]. The DNA genetic manipulation of the cells following the subsequent transplantation bears the risk of the expression of the desired receptor through the whole life and may lead to the unforeseen consequences.

Another approach in bone-targeted delivery of cells is the modification of the cell surface by adding the ligand that will drag the cell towards the corresponding receptor. This area of the surface engineering is relatively new and holds a great potential in future as it does not involve the long term effect. As for today a lot of targeting agents are used in the bone research, including SDF-1 [60], CD44 glycoform[61], sLeX[62] and bisphosphonates[14, 59].

In this regard the idea of creating the construct that might navigate the cells towards the tissue of interest was attractive. Using the ARTRP synthesis an osteophilic polymer was synthesized and contained in its structure several functional groups. The N-hydroxysuccinyl group covalently binds to the proteins on the surface of the cell membrane. Another functional group is represented by the bisphosphonate – alendronate. The alendronate possess the affinity towards the hydroxyapatite on the bone surface and thus is navigating the cells conjugated to the polymer to the bone tissue. In this Chapter of the Thesis *in vitro* interaction of the polymer with the cells and bone chips was analyzed. The effect of the polymer on the subsequent cell proliferation and differentiation of the cells down the osteogenic lineage was also evaluated to confirm its potency in further regeneration use.

3.2 MATERIALS AND METHODS

3.2.1 Polymer Synthesis

Initiator synthesis

The Dean-Stark apparatus was used to reflux a mixture of 4-aminobutanoic acid (20.6 g, 200 mmol), benzyl alcohol (100 mL, 965 mmol), and p-toluenesulfonic acid monohydrate (45.6 g, 240 mmol) in toluene (200 mL) for 5 hours at 160^0C. After allowing the mixture to cool to room temperature, 100 mL diethyl ether was added, and the precipitated crude compound was filtered out. Recrystallization of benzyl ester intermediate from ethanol and diethyl ether yielded 62.6 g (88 %), mp 106-108°C. At 0^oC, a mixture of the intermediate (11 g, 30 mmol) and triethylamine (10 mL, 70 mmol) in CH_2Cl_2 (150 mL) was slowly applied to a solution of 2-chloropropionyl chloride (3.2 mL, 33 mmol) in CH_2Cl_2 (10 mL). The mixture was washed by stirring for 2 hours at room temperature with 100mL of water 2 times, 3 times with 100mL of aqueous saturated $NaHCO_3$, 3 times with 100mL of 5% HCl aq. and again with 100mL of water 2 times. To extract the solvent, the organic phase was dried with $MgSO_4$ and then evaporated. The benzyl ester of N-2-chloropropionyl-4-aminobutanoic acid was dried in vacuo, yielding 6.8 g. (80%).

*Synthesis of Block 1***(F1)**

A polymerization tube was filled with DMAA (5.1 mL (50 mmol) for sample 1, 20.4 mL (200 mmol) for sample 2, or 25.5 mL (250 mmol) for sample 3), fluorescein O-methacrylate (400 mg, 1.0 mmol), N-2-chloropropionyl-4-aminobutanoic acid benzyl ester (283 mg, 1.0 mmol), IPA. After charging the solution with argon for 30 minutes, Me6TREN (460 mg, 2.0 mmol) and Cu(I)Cl (200 mg, 2.0 mmol) in 10 mL of water were added under argon flow. For 18 hours, the polymerization was carried out at room temperature. The resulting mixture was dialyzed in deionized water for 2 days using a Mwco 1,000 dialysis tube (Spectra/Por®, Spectrum Laboratories Inc., CA), and then lyophilized..^{1}H NMR (300 MHz, C*D*Cl_3) data analysis is shown on the Figure 2.

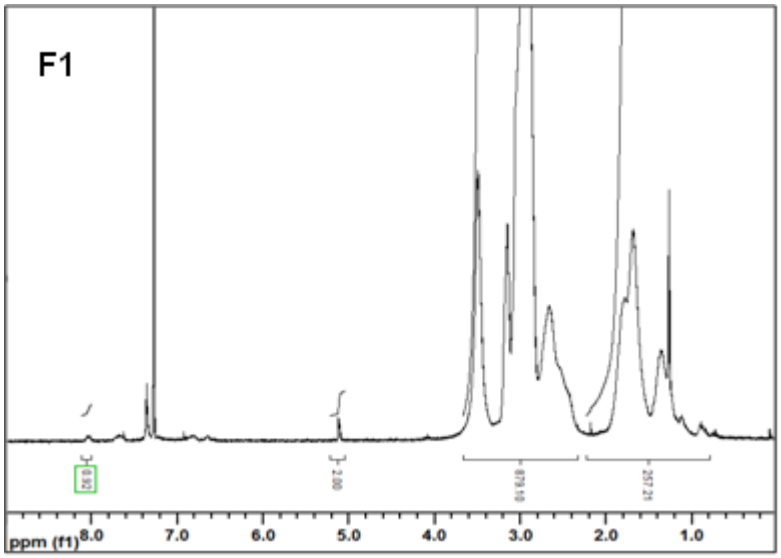

Figure 2. Synthesis of Block 1 **(F1)**

Synthesis of Block 2 **(F2)**

In a polymerization tube, DMAA (103 mL, 1.0 mmol), NHS monomer (142 mg, 0.5 mmol), polymer F1 (700 mg, 0.1 mmol of Cl end group), IPA (12.5 mL), and acetonitrile (12.5 mL) were added. After charging the solution with argon for 30 minutes, an argon-charged solution of Me6TREN (46 mg, 0.2 mmol) and Cu(I)Cl (20 mg, 0.2 mmol) in IPA (10 mL) was applied under argon flow. The mixture was transferred through silica gel after being heated at 40°C for 18 hours to dissolve the copper catalyst. Precipitation with diethyl ether yielded polymer F2. The ether-insoluble portion was filtered out and dried in a vacuum overnight. ^{1}H NMR (300 MHz, C*D*Cl$_3$) data analysis is shown on the Figure 3.

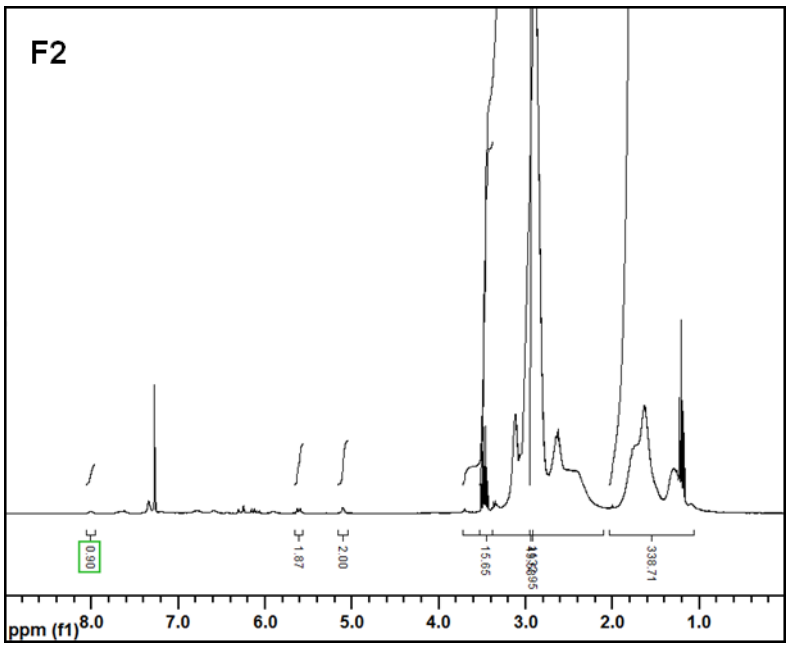

Figure 3. Synthesis of Block 2 **(F2)**

Third block polymer synthesis using aminobisphosphonate - PBPfOH **(F3)**

A sodium alendronate solution (120 mg, 0.37 mmol) in 25 ml of 0.1M sodium phosphate buffer (pH9.0) was combined with a polymer F2 (475 mg) in 5mL of DMSO Overnight, the resulting solution was stirred at room temperature. The solution was treated with 1.0 mL of 1 N NaOH and mixed for 3 hours at room temperature. After dialysis in deionized water with a Mwco 1,000 dialysis tube, polymer F3 was extracted via lyophilization.. ^{1}H NMR (300 MHz, C*D*Cl$_3$) data analysis is shown on the Figure 4.

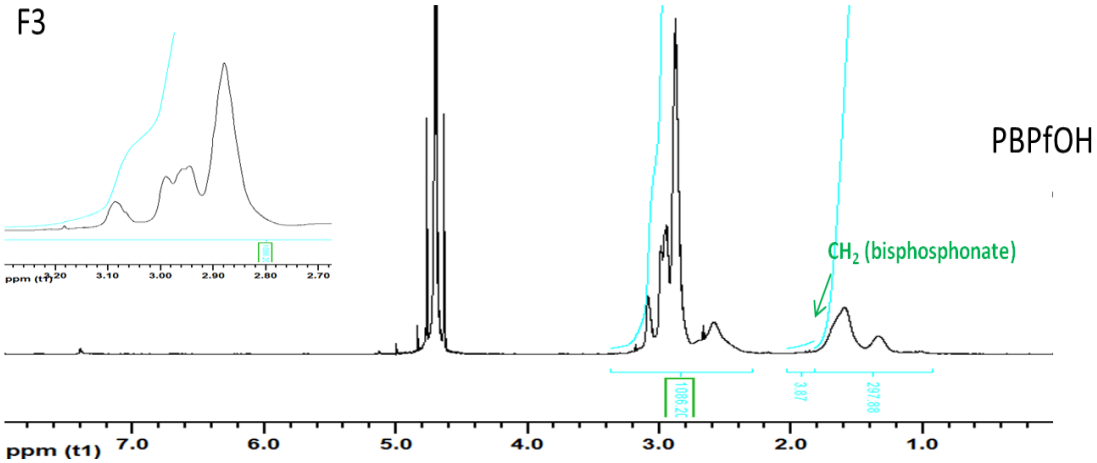

Figure 4. Synthesis of Block 3 **(F3)**

Induction of NHS group – PBPfNHS **(F4)**

In a solution of Polymer F3 (200 mg) in 10 mL of deionized water, EDC•HCl (39 mg, 0.2 mmol) and NHS (24 mg, 0.2 mmol) were placed and stirred at room temperature for 30 minutes. Using a Mwco 1,000 dialysis tube, the polymer F4 was purified and lyophilized.. ^{1}H NMR (300 MHz, C*D*Cl$_3$) data analysis is shown on the Figure 5.

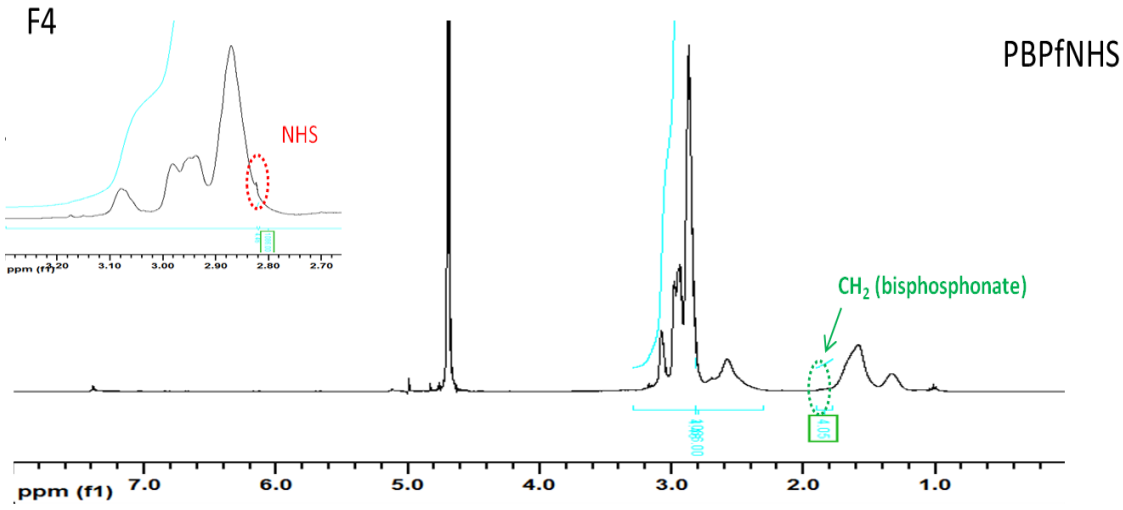

Figure 5. Synthesis of complete polymer *PBPfNHS* **(F4)**

The control polymer was also synthesized. The PAB-f-NHS polymer substitutes amino butanol for alendronate and binds to cells but not to hydroxyapatite or bone.

3.2.2 Isolation and characterization of MSCs

Bone marrow-derived MSCs for the experiments were isolated from the rat necropsy samples according to the published earlier protocol [82]. In brief, femur and tibia bones were obtained in aseptic condition, rinsed in the PBS + 5%Antibiotic solution for 5 minutes, cleaned from all the soft tissue. Further epiphysis were dissected and the marrow cavity was flushed several times

with a heparin and Dulbecco's minimum essential medium (DMEM, Gibco) solution. The obtained cells were filtered with 70micron mesh filter, then placed in 50ml tube and centrifuged at 100xg for 7 min. Centrifuged cell pellets were resuspended in DMEM complete media (DMEM+10%FBS+1%Ab), plated in T25 culture flasks and cultured to the third passage. MSCs nature of the obtained cells was confirmed by the flow cytometry and immunofluorescent staining with CD90, CD105, CD34, CD45 and CD31 and shown on the Figure 6.

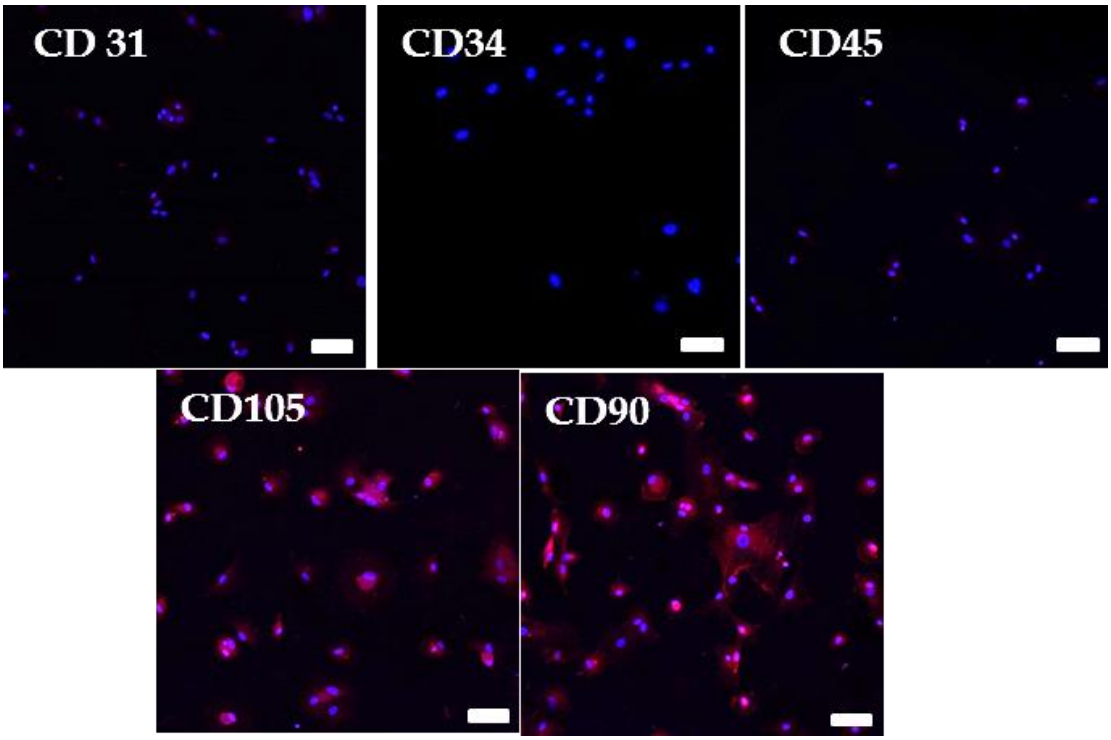

Figure 6. MSCs staining with immunofluorescence for the negative markers CD31, CD34, CD45 and positive markers (purple) CD90 and CD105 with DAPI-stained nuclei (blue). Calibration=50μm.

3.2.3 Functionalizing the MSCs with polymer – optimal condition

MSCs ($1.0\pm1x10^6$/ml) were incubated with the polymer (1 mg/ml in PBS, pH 8.0) for 10 min at 37^0C. Further cells were centrifuged at 300xg for 5 min, washed 3 times with PBS pH 7.4.

3.2.4 Cell Proliferation Assay

Cell proliferation assay was performed using Cell-Titer Glo Luminescent Cell Viability Assay (Promega, USA). This assay counts the number of viable cells in a culture by measuring the amount of ATP present, which is a sign of metabolically live cells. 1 mg/ml of polymer was incubated with MSCs($1.0\pm1x10^6$/ml) for 10 min, rinsed 3 times in PBS, plated on a 96-well plate (Costar, USA) and cultured for 0, 1, 2, 4, 24, 48 and 72 hours at 37°C, 5% CO_2 . At various time points the CellTiter-Glo® Reagent was supplemented to the wells mixed with an orbital shaker for 2 minutes. The plate was incubated at room temperature for 10 minutes for stabilization of the luminescent signal and readings were recorded with a Biotek Hybrid Reader (Biotek, USA).

3.2.5 Effect on the differentiation of MSCs

24-well culture plates were seeded with polymer modified MSCs in complete DMEM media (15% FBS, 1% Pen/Strep) and kept in incubator overnight. Then, the media was replaced with osteogenic media (Invitrogen's StemPro Osteogenesis Differentiation Kit) and cultured for 14 days.. Change of media was performed every 2-3 days. In control group non-modified and modified MSCs were cultured in complete DMEM media. In 14 days alkaline phosphatase was performed according to the manufacturer's protocol (SigmaAldrich, USA).

3.2.6 Polymer ability to inhibit the osteoclast activity –Pitt Assay

To obtain the culture of osteoclasts first the macrophages from the bone marrow were isolated in accordance with the protocol of Tevlin *et al* [83]. In short, bone marrow was harvested from the compact bones of the rat necropsy samples and the acquired cell suspension was separated with gradient cell separation media (Ficoll, SigmaAldrich, USA). Cells were then maintained in macrophage induction media (MEM, 10% FBS, 1% Pen/Strep, 10 ng/ml M-CSF) and in 3 days

media was substituted with osteoclast induction media (MEM, 10% FBS, 1% Pen/Strep, 10 ng/ml M-CSF + 10 ng/ml RANKL). Staining with tartrate resistant acid phosphatase (TRAP, SigmaAldrich) was performed to confirm the osteoclastic profile.

Osteoclast resorption assay was carried out with 24-well plate that had been coated with inorganic bone mimetic compound (Corning, Sigma). Osteoclasts were pleated at density of $2x10^4$ cells per well. The polymer at concentration of 0.5 mg/ml, 1mg/ml and 2 mg/ml was added to the media on the following day. Normal osteoclast induction media was added to the control wells, and as a positive control commercially available alendronate (Londormax, Greece) was added. After 7 days cells were fixed with the 4% paraformaldehyde (PFA). Von Kossa staining was used to differentiate between the resosrbed and unresorbed areas. Images were obtained with Zeiss Microscope and areas were calculated with FIJI software.

3.3 RESULTS AND DISCUSSION

The PBP-f-NHS polymer (MW8 kDa) was synthesized using atom transfer radical polymerization. The advantages of this type of polymerization include the control over the structure and composition i.e. molecular weight and introduction of functional groups the polymer ends. There is also a large diversity for the monomers and initiators that could be used. In current polymer synthesis DMAA molecule served as the backbone and 2 functional groups – N-hydroxysuccinyl and alendronate. The structure of the polymer was confirmed after each step of synthesis with H^1NMR and HPLC. The polymer was stored at $+4^0$C prior to coating with MSCs. The optimal amount of the polymer and optimal conditions were empirically established through the serial trials of the concentrations and incubation time. For the enhanced binding of the polymer to the cell surface PBS was modified to have pH 8.0. The pH was changed to normal 7.4 once the incubation ends and the washing starts. The polymer was visualized through the fluorescent group that was added in the final step of polymerization. The Figure 7A demonstrates the polymer coated MSCs in the bright light field and upon fluorescent exposure. Binding efficiency of the polymer to the surface of cell membrane was measured using the signal from the fluorescent group. Number of cells that exhibit fluorescent signal were calculated versus the total number of cells to produce the percentage of the binding efficiency of 70% straight after the modification. Quantitative results are shown on the Figure 7B. The fluorescent signal remained stable around 50 % from 2 to 4 hours and faded thereafter. In 12 hours no fluorescent signal could be detected not through the microscope neither from the spectrophotometer. The polymer is either internalized or degraded or the fluorescent groups lose its ability to produce the signal.

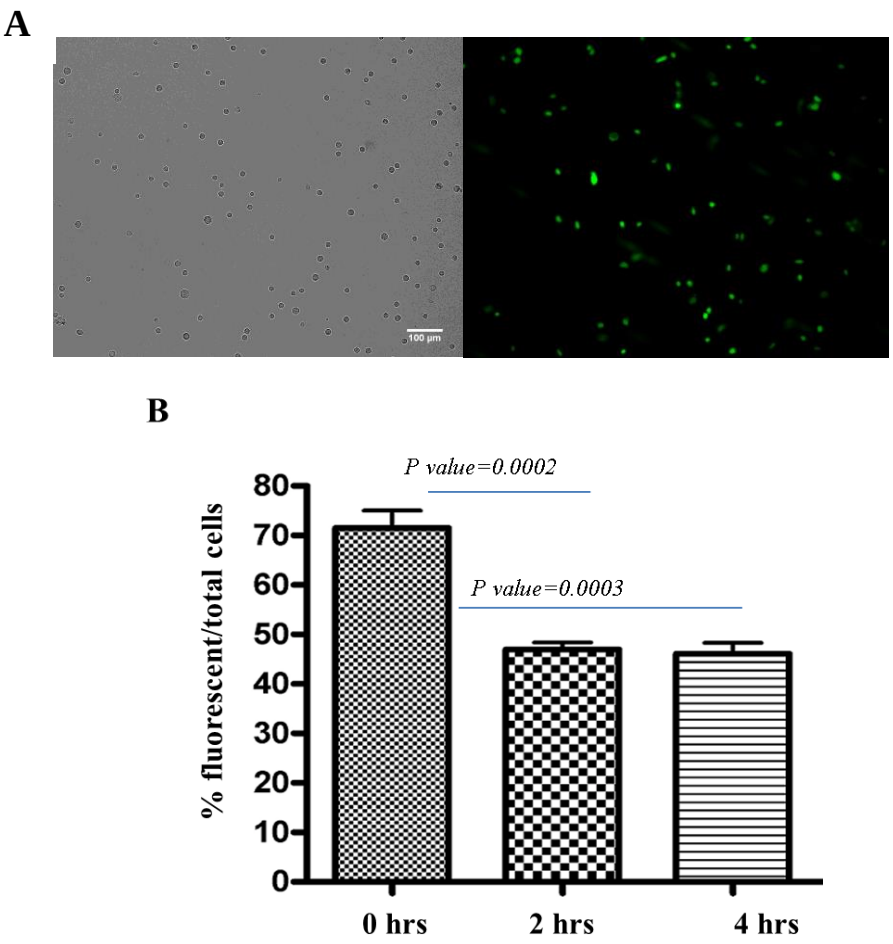

Figure 7. Modification of MSCs with polymer PBP-f-NHS.

A - PBP-f-NHS (1mg/ml) conjugated with MSCs ($1.0\pm1x10^6$/ml); B – Binding efficiency of the PBP-f-NHS (1mg/ml) to MSCs ($1.0\pm1x10^6$/ml).

The bone-targeting potential of the functional alendronate group was evaluated in vitro with the help of the bone chips. An additional polymer (PAP-f-NHS) was synthesized that did not possess the bisphosphonate group in its structure and served as a control. The results shown in Figure 8 demonstrated the statistically significant increase in polymer affinity towards the bone chips. Compared to the control area that was covered with the PBP-f-NHS polymer was almost two times larger.

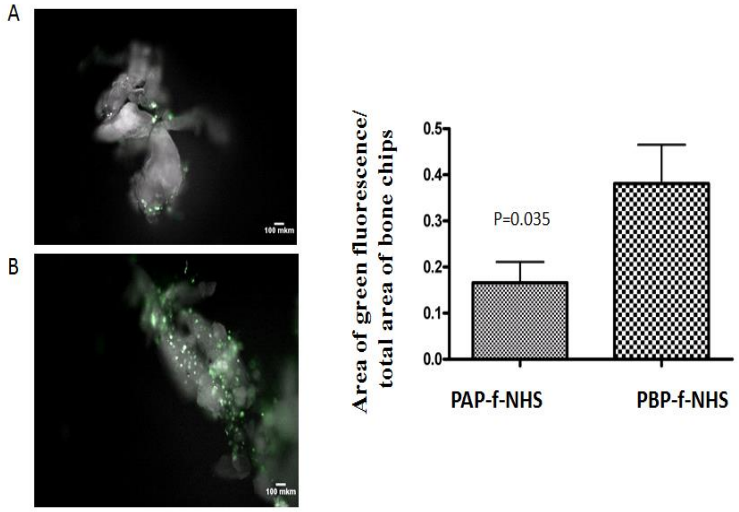

Figure 8. The bone-seeking potential of the PBP-f-NHS polymer shown on the bone chips *in vitro.* A – Bone chips coated with PAP-f-NHS polymer; B – bone chips coated with PBP-f-NHS ; C – quantitative analysis of polymer coated areas.

Since the polymer is chemically synthesized and contains the traces of possible toxic substances some concerns on its biological toxicity arises.

Figure 9A shows the results of the proliferation assay of MSCs modified with polymer and control (non-modified) cells. According to the data no significant difference was observed between the proliferation of the control group and the polymer modified cells. Therefore it was concluded, that concentration of polymer in 1mg/ml has no effect on the cell viability in culture and it ability to proliferate *in vitro* in short-term (4 hours) and long-term (72 hours) perspective. Difference between the groups did not reveal the statistical significance, $p = 0.383$.

To assess the effect of the polymer on the differentiation of the modified cells down the osteogenic lineage alkaline phosphatase staining was performed. Obtained results are shown on the Figure 9B. Alkaline phosphatase is considered to be the early osteogenetic marker[84]. Areas of the elevated activity of alkaline phosphatase are stained dark pink (white arrows). No difference was revealed between the polymer modified and non-treated MSCs cultured in osteogenic media for 14 days in their ability to differentiate into the osteoblasts. Level of ALP expression in those groups

was equally distributed through the whole culture. MSCs cultured in the normal DMEM media did not show any staining and confirmed the experimental results.

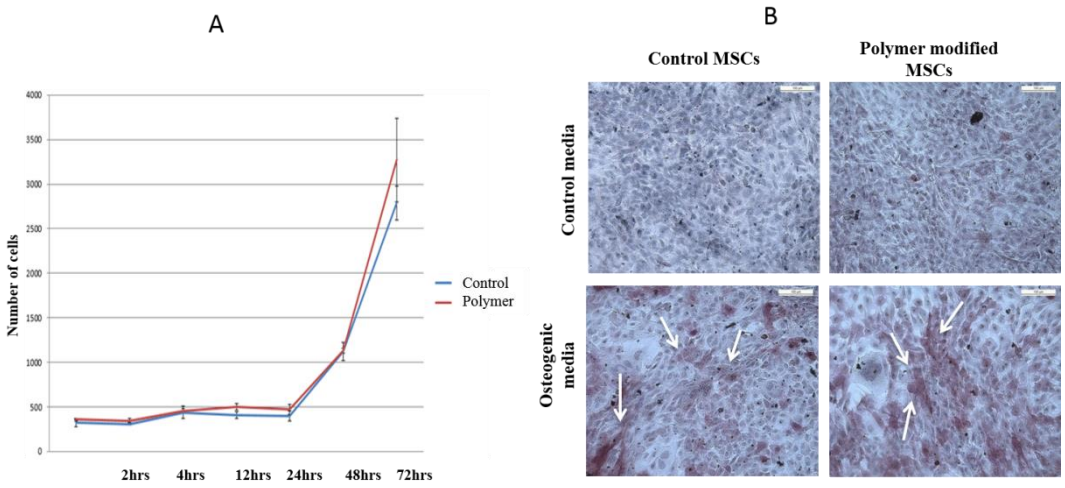

Figure 9. Proliferation assay and osteogenic differentiation of polymer modified and non-modified MSCs. A - Quantitative analysis of cell proliferation assay ($p = 0.383$, one-way ANOVA on Ranks); B - Qualitative analysis of osteogenic differentiation with ALP Assay, 10X

Bone marrow derived macrophage precursors were isolated and differentiated towards the osteoclastic lineage to assess anti-osteoclastic potential of the polymer alone. To identify the differentiated osteoclast tartrate-resistant acid phosphatase staining was performed. The TRAP staining is considered to be the histological marker of the osteoclasts that reveals the activity of the tartrate-resistant acid phosphatase[16]. Besides the TRAP activity another key feature of osteoclasts is a presence of the large number of nuclei (10 to 100). Figure 10A shows the representative images from the microscope where the large number of the nuclei cells can be detected inside the cell and the activity of TRAP can be observed. The shape of the cells is irregular and polygonal and has several processes. As one of the functional features of the osteoclast is bone degradation, lysosomal vesicles containing proteases can be detected.

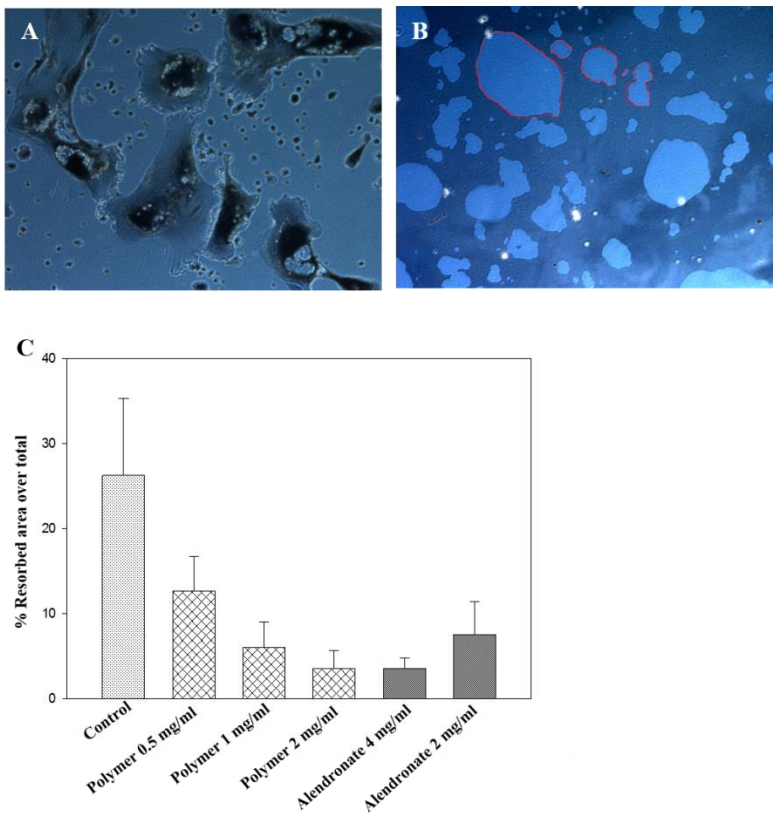

Figure 10. PBP-f-NHS polymer effect on the osteoclastic activity.
A - TRAP staining of the osteoclasts, 20X; B - Pit Assay: light areas – resorbed by osteoclasts, 10X; C - Quantitative analysis of Pit Assay: data is presented as a percentage of resorbed area over the total area ($p \leq 0.001$, one-way ANOVA).

Commercially available plates with inorganic surface that mimics bone were seeded with the osteoclasts and supplied with media that contained different concentrations of the polymer and commercially available alendronate product served as a control (Londromax, Greece). The bone degradation ability of the osteoclasts was measured with Pit Assay. The area of the resorbed and un-resorbed surfaces was calculated with FIJI software (extension of ImageJ). The resorbed areas are indicated as a lighter spots on the Figure 10B. Quantitative analysis is shown on the Figure10C where the percentage of un-resorbed over the total area was calculated. According to the results in a control group with normal media the resorption rate was 26%. The media containing osteophilic polymer in the concentration of 0.5 mg/ml decreased the resorption rate to 13% that is almost 2 times compared to the control. Polymer in concentration of 1mg/ml reduced the phagocytic ability

of osteoclasts almost three times (6%) and had similar effect compared to the standard dose of commercially available alendronate 2mg/ml (7.5%). Percentage of resorption area in a group of polymer concentration of 2mg/ml and alendronate 4mg/ml had the similar results of 3.6%. The obtained data strongly supports the ability of the polymer alone maintain the key feature of the bisphosphonate molecule and suppress the osteoclastic activity.

3.4 SUMMARY

After the intensive *in vivo* studies the osteophillic polymer was found to bind to the cell membrane for at least 4 hours and polymer coated cells retained the affinity towards the bone tissue. The polymer did not have any detrimental effect on the cell viability or its ability to proliferate for 3 days(72 hours). One of the most significant findings was the absence of polymer's effect on further cell differentiation down the osteogenic lineage as it gives a perspective to more efficient regeneration of the bone tissue in pathological condition. There were some concerns of the anti-osteoclastic effect of the bisphosphonate molecules incorporated into the polymer to have same effect as non-bound bisphosphonate drugs. However, the Pit Assay results confirmed that polymer in concentration of 1 mg/ml has the same effect on the osteoclastic activity in vitro as commercially available alendronate (Londromax, Greece).

CHAPTER 4. POLYMER MODIFIED MSCs SHOWING THE REGENERATIVE POTENTIAL I*N VIVO*

4.1 INTRODUCTION

Increased time for the fracture to heal in estrogen-dependent osteoporosis is related to induced osteoclast activity and diminished number of osteoblast precursors - MSCs [25, 85]. Thus, a promising approach is administration of MSCs to manage the bone pathological conditions particularly in age-associated diseases such as osteoporosis [86, 87]. The main problem is to isolate the sufficient amount of autologous MSCs for clinical use. This issue can be resolved through the expansion of acquired cells in laboratory condition *in vitro*. *In vitro* expansion of MSCs, on the other hand, alters the expression profile of the surface receptors and alters their homing potential as opposed to freshly isolated MSCs [88-90]. Furthermore, transplanted MSCs have a proclivity for homing to pathological cell proliferation sites, such as breast cancer [54]. In this regard bone-targeting potential was improved through the membrane engineering with bisphosphonate-based polymer that has high affinity for hydroxyapatite (Chapter 3). A model of estrogen-dependent osteoporosis was developed by ovariectomy (OVX) to determine the fracture-regenerating ability of MSCs modified with bisphosphonate polymer in osteoporosis *in vivo*. Different types of osteoporosis including postmenopausal type I and age-related type II could be recreated in animal model. OVX animal model is well recognized and licensed by FDA since most osteoporosis cases are estrogen-dependent. [91]

A model of the non-union bone fracture was performed by ulnar osteotomy. The period for the recovery of non-union bone fractures in healthy rats is usually 12 weeks [92, 93]. Though fractures take longer time to heal in osteoporotic condition. According to research by Namkung-Matthai et al, osteoporosis impacts fracture healing early on, leading to a 23 percent drop in BMD and decreased bone callus development after 3 weeks[94]. Kubo et al. found that bone recovery was

compromised in the late stages of fracture healing. Radiological and histological tests after 12 weeks showed reduced BMD and diminished callus quantity, which compromised woven bone formation[95].

In this Chapter the regenerative potential of the membrane engineered MSCs was assessed *in vivo* in combination of the two pathological conditions – the fracture on the background of ongoing osteoporosis. The main hypothesis of this part of work is to confirm the homing of the modified cells to the bone tissue and subsequent increase of BMD in the areas of fracture in short and long-term perspective.

4.2 MATERIALS AND METHODS

4.2.1 Ethical Guidelines

All the studies were carried out in compliance with the US Department of Health and Human Services' (HHS) ethical standards, Institutional Review Board (IRB) and approval by the Ethics Committee of Nazarbayev University's Center for Life Sciences (Registration number IORG 0006963).

4.2.2 OVX and Ulna fracture model

To perform the short-term and long-term in vivo experiments 45 female outbred rats were obtained and housed in individually ventilated cages maintaining the temperature at 21±2°C, 55±10% humidity and a 12 h day/night cycle. Water and standard rat food was supplied *ad libitum.* Estrogen-dependent osteoporotic condition was surgically induced in 40 females Wistar rats, 12 weeks old, with average weight 200-300g, by the bilateral ovariectomy (OVX). Sham operations were performed in 5 healthy female rats and served as a control group. 3 rats were excepted from the experiment due to unrelated health conditions. microCT IVIS Spectrum (Caliper, USA) was used to measure the bone density before the OVX procedure and 3 months after. Once the osteoporotic condition was confirmed, the fracture model was developed. Open ulna fracture was performed surgically in ulna shaft region in a proximity of the radiocarpal joint under the isoflurane anesthesia. The post-operative period was followed with oral anesthetic to reduce the animal distress. Following the next day after the fracture, polymer modified MSCs ($1x10^6$ of cells in 200 μl of PBS) were transplanted locally into the area of fracture. To assess the combination effect of the polymer and MSCs also single solutions of polymer alone (1 mg/ml in 200 μl of PBS); and MSCs alone ($1x10^6$ MSCs in 200 μl of PBS) was also injected in separate groups. 200 μl of PBS were administered to the animal in the control group. In 4 weeks and 24 weeks bone density was analyzed with microCT IVIS Spectrum (Caliper, USA).

4.2.3 Survival of MSCs

Luciferase-transfected cells were used to evaluate the survival and homing of the cells at the fracture site. Luciferin was prepared at 15 mg/mL in PBS and sterilized through a 0.2 μm filter for stock solution to be used as a substrate for luciferase expression. Luciferin at concentration 10 μL/g (150 mg/g) of body weight was injected through IP route 10-15 minutes before imaging with μCT machine (IVIS Spectrum CT, Caliper, USA). Images were processed with the Living Image 4.3.2 software (Caliper, USA).

4.2.4 μCT morphometry

A μCT machine (IVIS Spectrum CT; Caliper) was performed with x-ray mode with 150μ voxel size, 440 Al fitter, 50 kV, resolution 425, FOV LxWxH 12x12x13cm. The approximate dose was 52 mGv per scan. The 3-dimensional rebuilding and BMD measurements were executed with the Living Image 4.3.2 software (Caliper). The obtained image was saved in the DICOM format and stored. The BMD was determined as the optical density in the bone volume. Region of interest (ROI) was quantified with 10mm cylindrical volume of interest placed in the area of the fracture.

4.2.5 Histological analysis

Cervical dislocation under isoflurane anesthesia was performed to sacrifice rats *at* 4 and 24 weeks of the experiment. After, the ulna with the fracture was excised and stored in 4% PFA (pH 7.2-7.4). Bone fragments were then decalcified and processed in paraffin. Parafifn blocks were cut into the 7-10 microns sections and stained with hematoxylin and eosin for further microscopic analysis of the fracture healing zone.

4.2.6 Statistical Analysis

Data were processed and presented as mean ± SD. One-way ANOVA and unpaired T-test were used to analyze mean difference between the experimental groups. If data failed the normality test, Mann-Whitney test was performed to assess the differences between the various groups. Values

were considered significantly different at the $p \leq 0.05$ level. Statistical analysis was accomplished with the SigmaPlot 11.0 software. Number of animals per group was calculated using the method based on the law of diminishing terms or so called "resource equation" method [96]. This method can be used in exploratory studies to find the any level of difference between the groups. Value "E" is degree of freedom of analysis of variance (ANOVA) and is calculated by following formula:

E = Total number of animals − Total number of groups

Though, this method is based on ANOVA, it is applicable to all animal experiments. The value of E should lie between 10 and 20. If E is less than 10 then adding more animals will increase the chance of getting more significant result, but if it is more than 20 then adding more animals will not increase the chance of getting significant results. In experiment design the number of groups is 4 (Control, Polymer, MSCs and MSCs with Polymer), so taking into the account value E of 20 (as a measure of more significant results) total number of animals should be at least 16, 4 animals per group correspondingly. Taking into account the complexity of animal handling 1 animal was added to each group to minimize the risk and unexpected death of animal would not affect the significance of the results.

4.3 RESULTS and DISCUSSION

Estrogen-dependent osteoporotic model was experimentally induced in laboratory rats through the OVX procedure[97]. An ulnar fracture model was developed based on the ulnar osteotomy model in rabbit [98]. The model was used in fracture repair studies, and confirmed a reduction in BMD in osteoporosis. In a methodological context, the ulnar fracture is also preferable since no external bone fixing is necessary. The neighboring radius acts as a splint which supports weight bearing. The micro-CT IVIS Spectrum was used to determine bone density one day before OVX and three months after surgery. The differences in the bone density were measured as a ratio of final bone density (3 months after OVX) to the initial pre-operation measurements in each animal density. When the ratio equals to 1, then the bone density remains the same; the ratio below "1" represented reduced BMD. If the BMD declined by 10% or more relative to the original measurements, the osteoporosis induction was deemed successful.

Figure 11 indicates that the bone density of control animals improved significantly (by 8%), which was consistent with the animals' normal physiological maturation. On the contrary the animals that were ovariectomized, however, BMD reduced by 20%, indicating the emergence of osteoporotic condition.

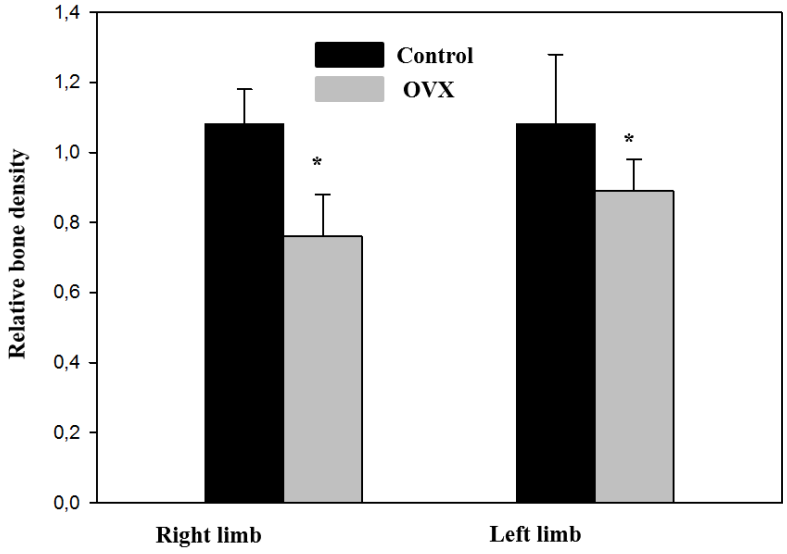

Figure 11. Osteoporotic rat model using OVX. Data are presented as Mean ± SD, *-p≤0.05 in comparison with control (unpaired t- test).

Once the osteoporotic condition was confirmed, open fractures were produced in ulnar regions. The following day the desired treatments with PBS, polymer, non-modified MSCs, and the polymer modified cells were injected into the fracture zones. BMD was measured at the sites of fracture in 2 hours after the surgery and in 4^{th} and 24^{th} weeks. Differences in the bone density were measured as a ratio of final bone density to the initial density (at the time of fracture) in every individual rat. Then, the median values were calculated for each group (Table 3). When the ratio equals to 1, then the bone density remains the same; values >1 indicate augmented bone density and improved bone regeneration. For the positive control group animals with no OVX but with ulna fractures were exploited (n=5). Since biomechanical properties of healthy rats' bones are recovered after 4 weeks [99], the measurements were terminated and the same data as a positive control in subsequent experiments were used.

Table 3. The results of relative bone mineral density measurements in 4 and 24 weeks upon fracturing.

Animal Group	Relative bone density ratio (after fracture/before fracture) Median (IQR 0.25-0.75)	
	4 weeks	**24 weeks**
Negative control (OVX, ulna fracture)	0.914 (IQR 0.671-1.053) (n=9)	0.737(IQR 0.640-1.029) (n=4)
Polymer	0.845 (IQR 0.727-0.994) (n=10) *P=1.00 •P=0.032	0.621(IQR 0.435-0.652) (n=5) *P=0.066 •P=0.008
MSCs	0.976(IQR 0.674-1.141) (n=8) *P=0.597 •P=0.354	0.596(IQR 0.453-1.379) (n=4) *P=0.486 •P=0.268
MSCs+ Polymer	1.274(IQR 1.046-1.421) (n=10) *P=0.003 •P=0.058	1.215(IQR 1.124-1.754) (n=5) *P=0.032 •P=0.095
Positive control (no OVX, ulna fracture)	1.103 (IQR 0.971-1.148) (n=5) *P = 0.046	No data

Note: Differences between experimental groups were analyzed with Mann-Whitney Rank Sum Test: * - compared to a negative control; •- compared to a positive control.

The sites of fractures were imaged under the X-ray mode 2 hours after the fractures and 4 weeks after and are presented in Figure 12.

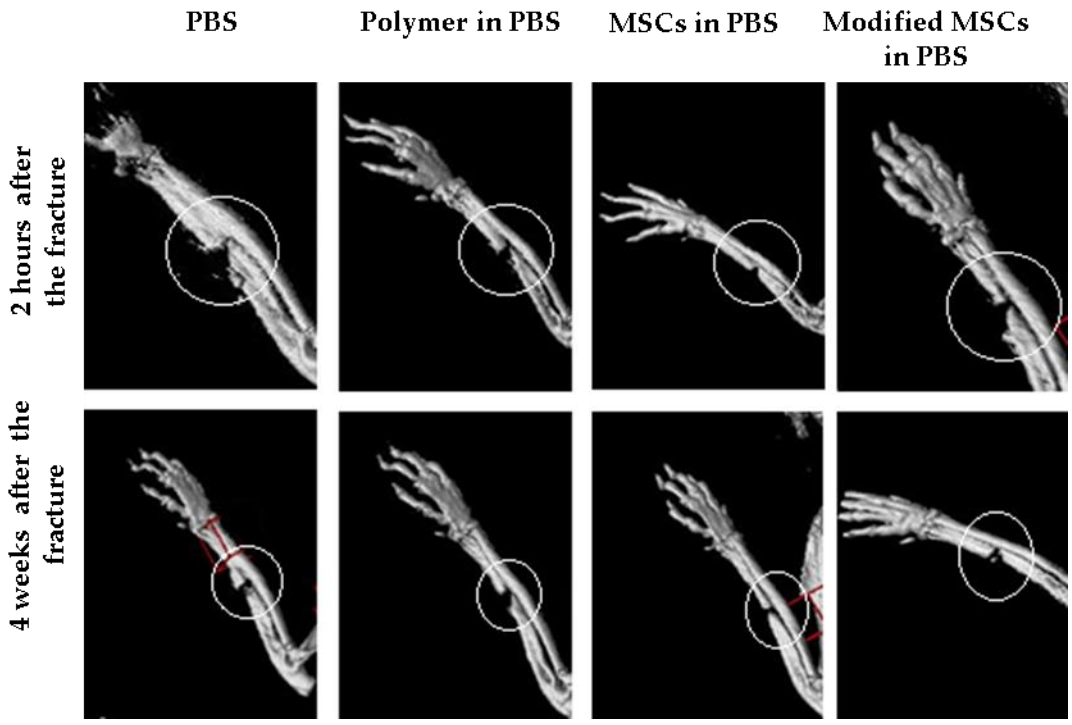

Figure 12. Rat ulna CT images in 2 hours and 4 weeks after the surgically induced fracture (In Vivo Imaging System, Caliper, USA).

Figure 13 shows that 4 weeks after the surgery, bone density decreased slightly in the control group (by 13%), in the groups that obtained only polymer (14%) and only MSCs (6%), but there were no statistically important variations between all three groups. On the contrary in the group of animals that received the membrane-engineered MSCs, bone density improved by 27% ($p \leq 0.001$) as compared to the control group (PBS). 19 animals were sacrificed in 4 weeks, while the remaining 18 were held for further study. In 24 week the measurements of bone mineral density were repeated. In Group 1-3 bone density was continuously declining (20%, 44,1% and 19 % respectively), and only in group 4 statistically significant increase in BMD up to 39% was observed (≤ 0.05).

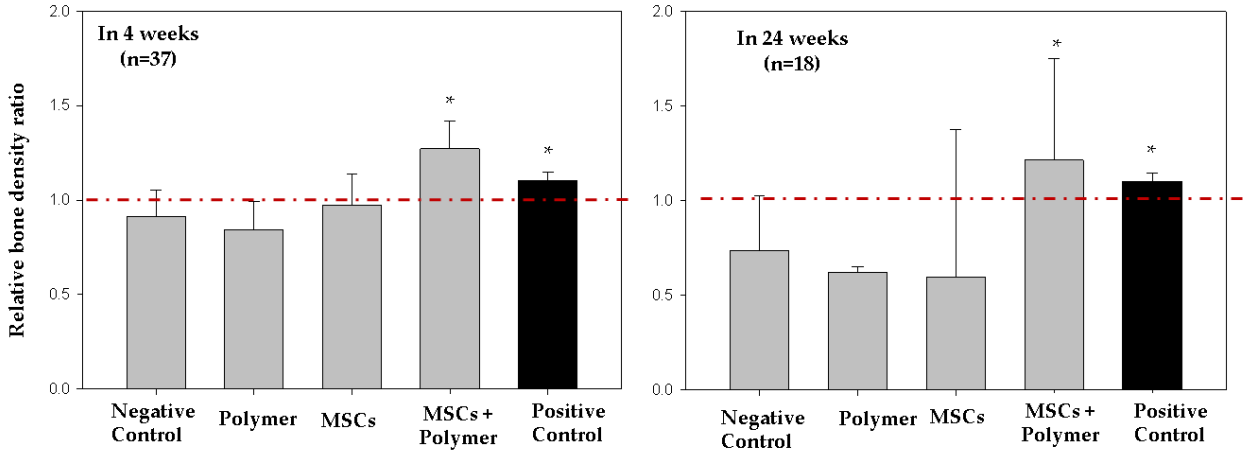

Figure 13. BMD measurements in the ulna fracture region in 4 and 24 weeks after the treatment. Data are presented as Median (IQR 0.25-0.75), *p≤0.05 in comparison with a negative control, • p≤0.05 – in comparison with a positive control (Mann Whitney Rank Sum Test).

The fate of the transplanted MSCs was monitored using the preliminary transfection of cells with Luc-LVT lentiviral particles. Bioluminescent signal was detected *in vivo* with optical imaging system IVIS Spectrum CT (Caliper, USA). Figure 14 demonstrates the results of the bioluminescent analysis. The transplantation of cells was confirmed the following day after administration and the signal was clearly observed in 7 days. However in 14 days upon injection the signal was not detected. One of the possible reasons that could be associated with the survival of MSCs or their migration deep into the bone that affects the ability of the imaging system to perceive the signal.

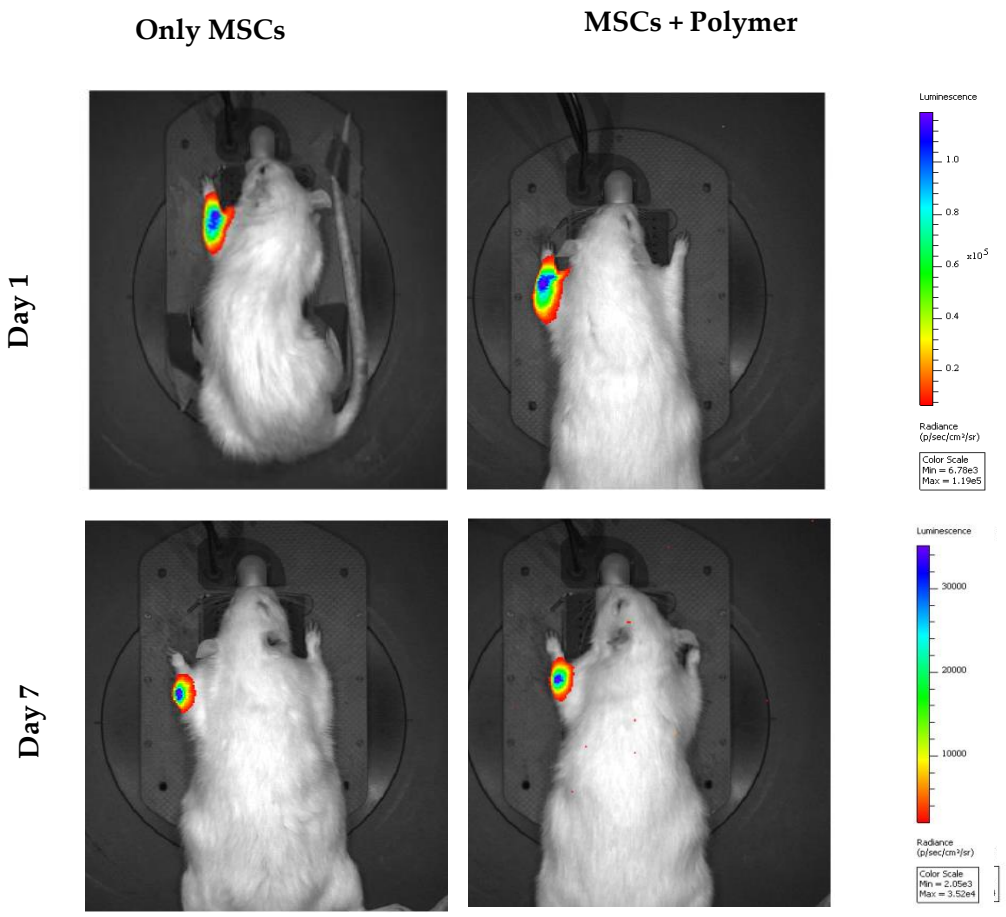

Figure 14. *In vivo* tracking of the transplanted MSCs with LUC-LVT particles in bioluminescent spectrum.

Post mortem histological examination of the ulnar fracture zones confirmed the findings of the micro-CT study and shown on the Figures 15-16. After four weeks of fracturing, the control group showed obvious signs of bone injury including vascular congestions, large lacunae cavities with internal hemorrhage, small porous cavities, and linear and cross-sectional breaks (Figure 15). The presence of small regions of fibrocartilage development and areas of fibrotic adhesions of old bone fragments indicated incomplete regeneration.

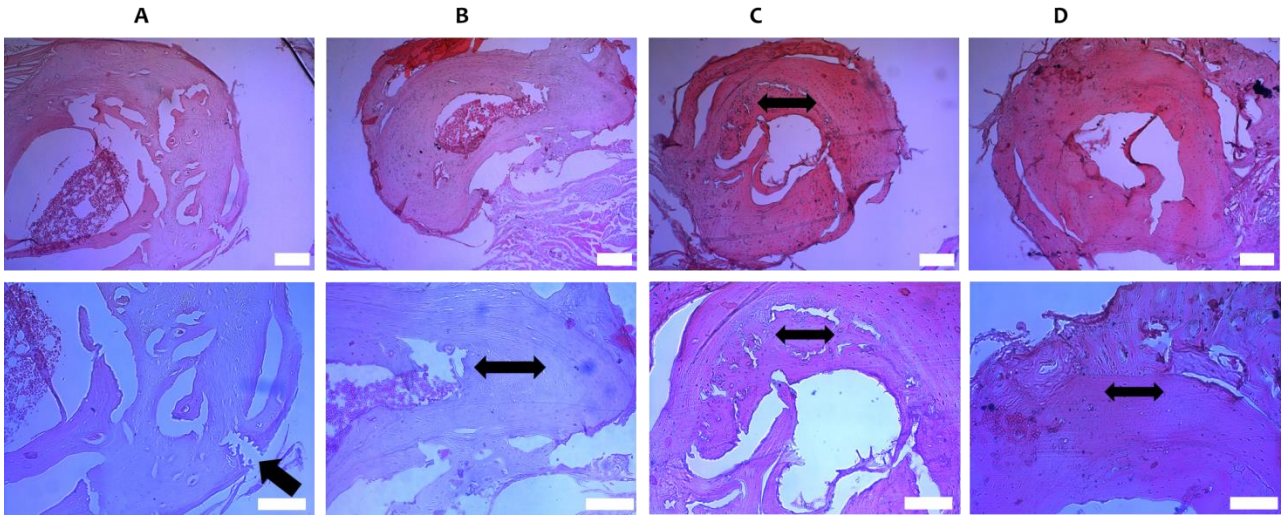

Figure 15. Cross-sectional histological analysis of ulna in 4 weeks after the fracture. Staining with Hematoxylin and Eosin; upper row - 10X, lower row - 20X. A - PBS group, the clear separation of the broken end (an arrow); B - Polymer group, fracture gaps filled with fibrous tissue (two-headed arrow); C - MSC group, signs of early bone regeneration between the fracture gaps (two-headed arrow); D – Polymer modified MSCs group, most pronounced osteogenesis with fracture gaps filled with bone tissue (two-headed arrow). Calibration=100µm.

Linear and cross-sectional breaks, as well as lacunae cavities, were found in the ulna sections of animals that were treated with the polymer solution (Figure 15). Some reactive modifications such as tidemarks parallel to the periosteum and unevenly mineralized tissue with spotted calcification were seen, which suggests the polymer had a minor beneficial effect. The bone tissue of animals injected with unmodified MSCs showed evidence of the aggressive osteogenesis, such as hyperplasia and proliferation of the progenitor cells in the periosteum's cambial layer (Figure 15). Development of early trabeculae consisting of bone spicules with poor calcification was also observed. The group administered with polymer-modified MSCs, on the other hand, showed the most significant signs of osteogenesis. Wide areas of the fibrocartilaginous callus with ossification fusing the two fracture ends were present (Figure 15). Patches of just-woven bone and newly formed trabeculae from the mature osteoblasts were found (Figure 15).

Fracture healing is usually divided into three stages. Fracture hematoma, inflammation, and the development of granulation tissue define the first stage level, which is reactive in nature. Collagen fibers bind the damaged bone ends during the second reparative process, and osteoblasts begin to form spongy bone. At this stage, some spicule will appear. Endochondral ossification turns the fibrocartilaginous callus into hard calcified tissue (woven bone). Bone remodeling is the final stage, after which the bone regains its original form, composition, and mechanical strength.

After four weeks, the group of animals given only bisphosphonate polymer had a similar 13 percent decrease in bone density, which progressed to a drastic 44 percent decrease after 24 weeks. These results, though, are not statistically relevant and should be explored further. Injection of pure MSCs, on the other hand, resulted in a slight decline in bone density after four weeks, but by 24 weeks, the bone density had dropped by the same 20% as the control group. This data suggests that MSCs have a short-term beneficial impact but not a long-term effect. After four weeks, the group that obtained coated MSCs had a substantial statistically significant increase in bone density (27%) in the fracture region. After 24 weeks of monitoring, the treatment with polymer modified MSCs resulted in a sustained rise in bone density of up to 39 percent.

After 4 weeks, histological analysis of the control group showed some signs of early reparative processes, including fibrous union of old bone fragments. The polymer-coated MSC group demonstrated successful osteogenesis with the formation of fibrocartilaginous callus, while groups 2 and 3 showed additional signs of mineralization and the bone spicule formation. While no full fracture healing was found in all of the groups by 24 weeks, group 4 had the most marked development of the woven bone. Surface modification with bisphosphonate groups is thought to have facilitated the bone healing in several ways: first, by enhancing the mobilization of the transplanted MSCs to the bone injury sites, supplying growth factors and providing the ability for

differentiation into the osteoblast cells, and second, by inhibiting the functional activity of the osteoclasts and slowing the bone resorption process.

Figure 16 shows histological slices of the ulna inside the fracture zones 24 weeks after fracturing.. Many bone defects crossing the shear axes of the bones were also found in the control group images The defects were packed with irregularly spaced immature cartilage islets (arrows) containing a significant number of the flattened chondroblasts, primarily in the central region.. There were mild to extreme dystrophic variations in the bone tissue in the second group (polymer in PBS): single gaps with bone marrow components were evident (upper, arrow), and irregular growths of the immature cartilage tissue with no clear borders in the form of the tiny islets (lower, arrow).

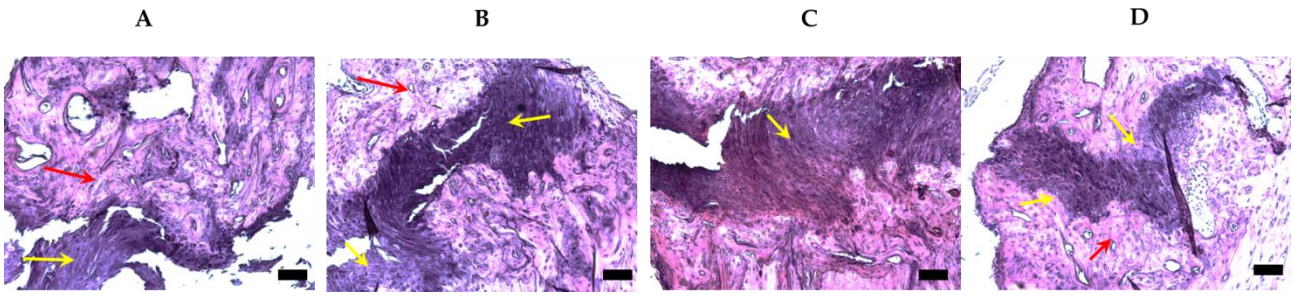

Figure 16. Histological analysis of longitudinal ulna sections in 24 weeks after the fracture. Staining with Hematoxylin and Eosin, 10X. A - PBS group, immature cartilaginous tissues in the fracture region (yellow arrow); B - Polymer group, irregular growth of immature cartilaginous tissue (yellow arrows), bone tissue with serious dystrophic modifications (red arrow); C - MSC group, significant number of the polymorphic chondroblasts are randomly distributed and cartilaginous tissue is overgrown (yellow arrow); D - Polymer modified MSCs, distinct bone formation and ossification (yellow arrows), no signs of dystrophy or degeneration of the bone tissue (red arrow). Calibration=100μm.

The group treated with plain MSCs had bone tissue fragments with mild dystrophic modifications on the histological slices: the trabeculae were small; they revealed the outgrowth of immature cartilaginous tissue without a visible transition region; cartilaginous fibers were thick and unequally colored. Finally, within the central zone and periphery, a significant number of polymorph shaped chondroblasts were chaotically scattered (arrows, Figure 16). Concurrently, no major dystrophic or degenerative alterations were found in the fourth group bone slices. The

trabeculae had uniform shape. Islets of immature hyaline and elastic cartilaginous tissue with a clear transition zone (arrows) were present; the fibers were thick and uniformly stained. The central areas contained a considerable number of the polymorph chondroblasts, while the periphery contained round-shaped chondrocytes.

The histological evaluation of ulnar fracture zones revealed a substantial variation in reactive and reparative processes between the experimental groups, with the most notable regenerative outcomes in the group with transplantation of the polymer modified MSCs, which was consistent with the micro-CT results.

4.4 SUMMARY

During the *in vivo* studies it was shown that the treatment with MSCs modified with osteophilic polymer has more pronounced effect in regeneration of the bone tissue in osteoporotic fracture condition according to the measurements of the bone mineral density. The fate of cells was monitored and observed for 1 week after transplantation to ensure the homing towards the bone tissue. Histological assessment was performed at 4 and 24 weeks to confirm the BMD findings and monitor the fracture healing. Thus, surface modification of MSCs allowed not only to increase the bone density in short-term perspective after the fracture by 27.4% (4 weeks) but also to maintain the regeneration effect after 6 months since the treatment at 21.5% compared to the control.

CONCLUSION

The development of novel approaches in osteoporotic fractures treatment remains of a great research interest and in particular mesenchymal stem cell therapy attracts a lot of attention in orthopedic field. Transplantation of MSCs in osteoporosis has been shown some results in animal model, but the use of the cell therapy in human in osteoporotic condition is still insufficient. Lack of the targeting potential and loss of homing receptors in expanded MSCs culture remains the limiting factor.

In this dissertation the idea of using the polymer to navigate the cells and target the particular tissue of interest was employed. The polymer was synthesized using ATRP technique in collaboration with Dr Alan Russell lab at Carnegie Mellon University and characterized for the presence of the functional groups. The NHS functional group interacts only with the surface membrane of the mesenchymal stem cells and does not affect the genetic apparatus or possess a cytotoxic effect *in vitro*. The bone-targeting potential was also confirmed *in vitro* using the control polymer without alendronate group and *in vivo* through the transfection of MSCs with luciferase lentiviral vector and bioluminescent imaging with IVIS Spectrum (Caliper, USA). The overall therapeutic utility of the approach was tested in osteoporotic fracture condition. Four transplantation (1 per week) of polymer coated MSCs ($1x10^6$ cells) resulted in a significant increase in BMD in short-term (4 weeks) and long-term (24 weeks) perspective by 27.4% and 21.5% correspondingly. The BMD results were found to be consistent with the post mortem histological analysis. The preclinical results provide this approach with a strong base for further potential use in clinical practice.

Considering the limitations imposed by the rat animal model, the transplantation of allogeneic MSCs instead of autologous and minimal number of the sample size further investigation

is recommended to be performed in larger animal that resembles the human bone metabolism in stronger manner and increased sample size. The *in vivo* tracking of the transplanted MSCs might be also improved by using cells isolated from the GFP transgenic animals and use of MRI facility instead of microCT imaging system.

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