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Investigation of Cell Derived Nanoparticles for Drug Delivery and Osteogenic Differentiation of Human Stem/Stromal Cells

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Abstract

The successful repair of bone defects and injuries is enhanced by the delivery of osteoinductive factors, such as drugs, growth factors, and genetic material that can promote the osteogenic differentiation of stem/stromal cells into osteoblasts. Nanoparticle delivery systems are being studied to enable the sustained release of these factors but suffer from limitations such as cytotoxicity issues, poor loading capacity, and poor cellular uptake. In this project, we developed cell-derived nanoparticles (CDNs), a biomimetic nanoparticle delivery system with high drug loading efficiency, to deliver a glucocorticoid drug, dexamethasone (Dex), to promote the osteogenic differentiation of stem/stromal cells. The synthesized Dex-loaded CDNs had a consistent size range of 30-920 nm, spherical shape, high drug loading efficiency, good cytocompatibility, and were internalized by human adipose-derived stem/stromal cells (hADSCs). Drug-loaded CDNs were able to induce the osteogenic differentiation of hADSCs *in vitro*, indicating their potential as an efficient drug delivery vehicle for bone regeneration and other applications.

Keywords

Cell-derived nanoparticles, osteogenic differentiation, stem/stromal cells, bone repair, drug delivery

Summary for Lay Audience

The use of bone tissue or grafts obtained from the same or different individual is the most widely used method for the repair of bone defects and injuries in clinics today. Successful bone repair requires three components: a scaffold that supports the growth of new bone, molecules that promote the differentiation of stem/stromal cells in the area surrounding the injury into bone or osteoblast cells, and the cells which can give rise to new bone. The molecules that promote osteoblast formation are called osteoinductive molecules and they can be growth factors, drugs, or genetic material. The focus of current research is to efficiently deliver these molecules to the site of injury for the enhanced repair and regeneration of bone tissue. Nanoparticle delivery systems are being studied to enable the sustained and prolonged release of these factors, which is essential for successful repair of the injury. However, they suffer from limitations such as toxicity to cells in high doses, inability to encapsulate large quantities of osteoinductive molecules, and inability to be taken up by cells which are essential to effectively deliver osteoinductive molecules to the target cells. In this project, we developed cell-derived nanoparticles (CDNs), which are synthesized from cell membranes of cells. During the preparation process, the cell membranes are broken into small fragments. These fragments can self-assemble to form nanoparticles. These nanoparticles mimic the properties of cell surfaces due to the presence of proteins, phospholipids, and carbohydrates carried over from the source cells. CDNs can encapsulate osteoinductive molecules within them during the self-assembly process. Here, CDNs were used to deliver dexamethasone (Dex), a well known osteoinductive molecule, to promote the osteogenic differentiation of stem/stromal cells. They were able to induce the differentiation of these stem/stromal cells to bone cells, indicating they could be used as efficient drug delivery vehicles.

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List of Abbreviations

AFM	Atomic force microscopy
ALP	Alkaline phosphatase
ANOVA	Analysis of variance
ARS	Alizarin red S
BMP	Bone morphogenic protein
CDN	Cell derived nanoparticle
DAPI	4',6-diamidino-2-phenylindole
DBM	Demineralized bone matrix
Dex	Dexamethasone
DiI	1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindocarbocyanine Perchlorate
DNA	Deoxyribonucleic acid
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
hADSC	Human adipose-derived stem/stromal cells
HEK293	Human embryonic kidney 293 cells
LPS	Lipopolysaccharide
MKP-1	Mitogen activated protein kinase phosphatase
MSC	Mesenchymal stem cell
MTS	(3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
NIR	Near infrared
PBS	Phosphate buffered saline
PDGF	Platelet derived growth factor

PGA	Poly-glycolic acid
PLA	Poly-lactic acid
PLGA	Poly-(lactide-co-glycolide)
PMA	Phorbol 12-myristate 13-acetate
RBC	Red blood cells
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
RUNX2	Runt-related transcription factor 2
SDF-1	Stromal derived factor-1
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SOX9	SRY-box transcription factor 9
TEM	Transmission electron microscopy
TGF-β	Transforming growth factor β
THP1	Human acute monocyte leukaemia cell line
TNF-α	Tumour necrosis factor alpha
VEGF	Vascular endothelial growth factor
WNT	Wingless and int-1

Chapter 1

1 Introduction

1.1 Overview

The incidence of bone defects, injuries, and diseases has increased worldwide in the past years [1–4]. Although bone has regenerative capacity, the repair of many bone injuries and defects in a timely and appropriate manner requires medical intervention [5–7]. The most commonly employed method in clinics for bone repair is the use of autografts and allografts, which are bone tissues from the same or different individuals respectively [3,8]. Despite their widespread use, these grafts have limitations such as issues with availability, increased pain (autografts from same patent), morbidity, disease transmission and infection, and adverse immune responses [4,9]. Synthetic grafts and biomaterial scaffolds are being developed and studied as alternatives to natural graft materials. The synthetic scaffolds typically have three main components: a) cells with the ability to form bone cells, b) drugs (proteins and small molecules) that can recruit or activate stem cells and other osteoinductive factors in the microenvironment, c) biomaterials that act as supports for the delivery of the other two components and provide an environment that withstands the forces exerted on the bone and supports the dynamic nature of the biological agents that play a role in bone regeneration [1,3,10–12]. In this project, we have focussed on the efficient delivery of small drug molecules to accelerate osteogenic differentiation and bone repair. Drugs and bioactive molecules can play a role in regulating cellular function and have an effect on intercellular communication through the regulation of signalling pathways involved in bone remodelling. These regulatory effects of such molecules can greatly increase the chances of effective bone repair and regeneration and are critical in the success or failure of the scaffolds [13,14]. Several types of therapeutic molecules such as growth factors, peptides, small molecule drugs, and genetic material have been tested and studied to this end [15–17]. In order for these drug molecules to be effective, they need to be released in a controlled manner from the support material. Nanoparticle-based drug delivery systems have the ability to do this and have been widely used to deliver a variety of therapeutic molecules for numerous applications [18–22].

1.2 Project motivation and hypothesis

The small size, flexibility in design, ease of synthesis, encapsulation of therapeutic molecules, stability, and the ability to be incorporated into biomaterials and scaffolds by physical and chemical interactions have led to the use of nanoparticle delivery systems in bone repair applications [23]. However, some of the common challenges of nanoparticle delivery systems include cytotoxicity issues, poor loading capacity, and poor cellular uptake [16,23]. Despite extensive research with natural and synthetic nanoparticles, reproducible strategies to treat bone injuries using nanoparticles still remain elusive under clinical settings. Cell-derived nanoparticles (CDNs) are a new class of nanoparticles produced from the cell membranes of erythrocytes, platelets, immune cells, cancer cells, or stem/stromal cells depending on their applications [24]. They can be used to coat or camouflage other nanoparticles in order to reduce their cytotoxicity. The surface receptors on the CDNs carried over from the source cells give them an advantage over other nanoparticles by allowing them to evade immune clearance. They offer several unique advantages: they are highly non-cytotoxic, exhibit increased cellular uptake compared to free drugs, are multicomponent particles (made of lipids, proteins, and carbohydrates) that mimic cells, and have inherent targeting abilities due to the surface proteins carried over from parent cells [25]. These properties, when applied to bone regeneration strategies, could allow for a sustained release of the osteoinductive molecules entrapped within the CDNs. Most importantly, the surface proteins on the CDNs could potentially encourage the interaction of the delivery system with the cells and biomolecules in the surrounding microenvironment, making the scaffold biologically active [26]. This could further promote its osteoconductivity and osteogenesis. The preparation method for these particles is such that a number of different types of drug/bioactive molecules could be encapsulated within the CDNs.

The primary goal of this project was to provide a proof of concept to show the ability of CDNs to independently deliver drug molecules. Our hypothesis was that CDNs could efficiently encapsulate dexamethasone (an FDA approved glucocorticoid drug shown to promote osteogenic differentiation) and deliver them effectively to human adipose-derived stem/stromal cells (hADSCs) to induce osteogenic differentiation (**Figure 1-1**).

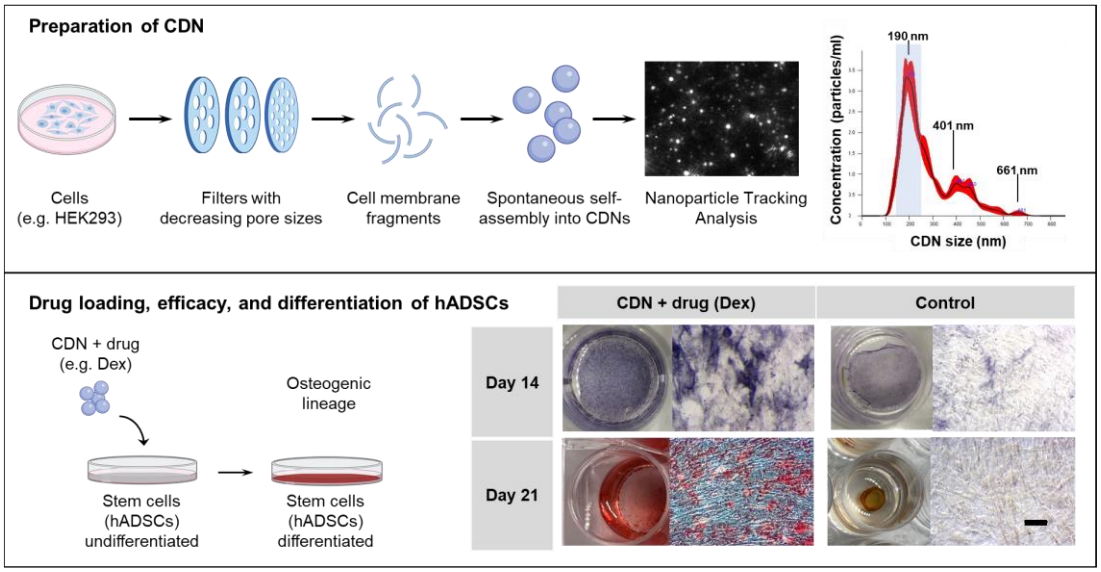


Figure 1-1. Preparation of CDNs by cell membrane fragmentation and shearing, followed by differentiation of stem/stromal cells (hADSCs) using CDNs loaded with drug (Dex).

1.3 Thesis objectives.

To test the ability of CDNs to entrap and deliver dexamethasone (Dex) to promote the osteogenic differentiation of hADSCs, the following specific objectives were developed (Figure 1-2):

Objective 1: Preparation, optimization, and characterization of CDNs loaded with drug molecules

Objective 2: Investigation of the cytocompatibility and biofunctionality of drug loaded CDNs for the osteogenic differentiation of hADSCs using in vitro analyses

As a follow up to these Objectives, in the future, CDNs-loaded Dex will be incorporated within a biomaterial or hydrogel scaffold or coated onto an implant material such as titanium to investigate its ability to release Dex in a controlled and sustained manner. By performing *in vitro* and *in vivo* analysis with these CDN incorporated/coated materials, we would be able to further prove their efficiency as drug delivery vehicles for bone repair and regeneration

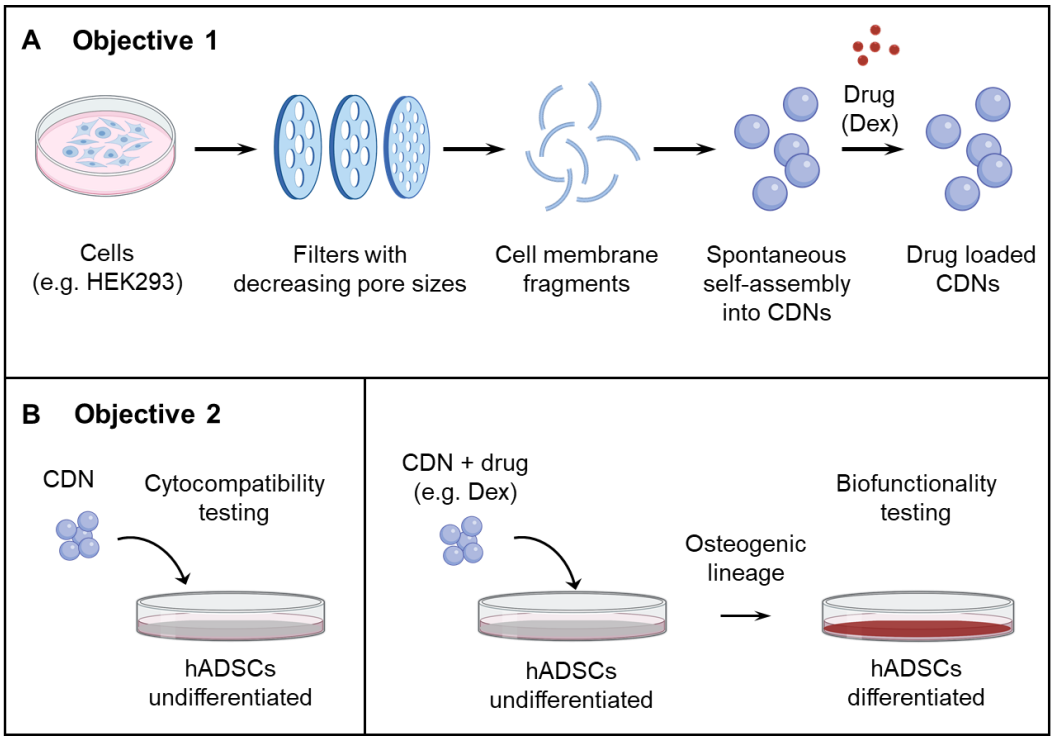


Figure 1-2. Schematic of Thesis Objectives.

Chapter 2

2 Literature Review

2.1 The skeletal system and bone formation

2.1.1 Functions, development, and maintenance of the skeletal system

The skeletal system provides the structural foundation to our body and plays an important role in both mechanical and non-mechanical function [27]. It is the point of attachment for tendons, ligaments, and muscles which together bring about the movement or locomotion of the body. The skeletal system can be broadly classified into the axial skeleton and the appendicular skeleton [27]. The appendicular skeleton includes the bones of the upper and lower limbs and their joints. The axial skeleton includes the bones of the skull, ribcage, and vertebral column. The fragile organs (i.e., brain, spinal cord, heart, lungs, etc.) present in the cavities of the axial skeleton and the pelvis, are offered crucial protection from external injury. The skeletal system also forms a reservoir for several minerals that are essential for the normal functioning of the body. Depending on the body's cues, these minerals are released into the blood stream or deposited in the bones for future use [28,29]. Thus, the skeletal system maintains mineral homeostasis. The inner cavities of bones are reservoirs of hematopoietic cells that differentiate and proliferate to form the blood cells. These blood cells are involved in the transport of gases, immune protection and responses, and the formation of clots [30]. Finally, bones harbour cells that are storage sites for energy, growth factors, and cytokines [31,32].

Bones are composed primarily of 3 types of cells: osteoblasts, osteocytes, and osteoclasts. Osteoblasts and osteocytes are derived from mesenchymal stem cells (MSCs), and osteoclasts are derived from hematopoietic stem cells as shown in **Figure 2-1**. MSCs differentiate into a series of precursors of osteoblast cells, and this process is directed by the expression of transcription factors and by signalling pathways [29]. During the bone development, MSCs first differentiate into osteoprogenitor cells due to the expression of the SRY-Box transcription factor 9 (SOX9). Osteoprogenitor cells can be directed to form both osteoblasts and chondrocytes. Chondrocytes are the cells that make up cartilage, a flexible connective tissue that cushions the joints of bones and protects them from erosion and high stress. The presence of Runt-related transcription

factor 2 (RUNX2) in osteoprogenitor cells indicates their differentiation to preosteoblast cells. Preosteoblasts further differentiate into osteoblast cells. Osteoblasts are responsible for the secretion of several extracellular matrix proteins and enzymes which form the components of bone tissue [27,33]. The presence of these proteins and enzymes *in vitro* are indicators of the fate of stem cells towards an osteoblast lineage.

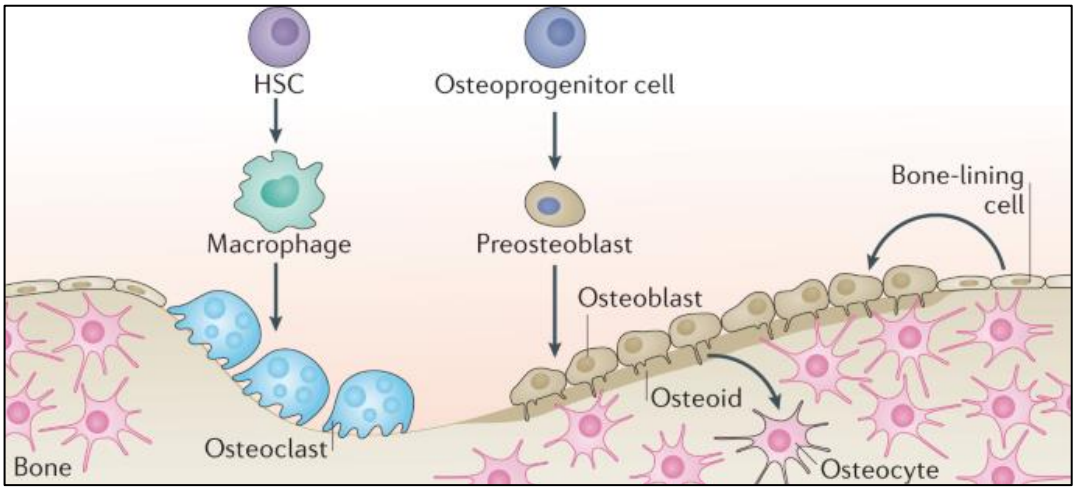


Figure 2-1. Cells involved in the development and remodelling of bone. Osteoblasts and osteocytes are formed by the differentiation of osteoprogenitor cells that in turn are differentiated from mesenchymal stem cells. Osteoblasts and osteocytes are responsible for matrix mineralization and the formation of new bone tissue during remodelling. On the other hand, osteoclasts arise from hematopoietic stem cells, and are responsible for bone resorption. Bone lining cells are quiescent osteoblasts that get activated after injury when bone repair is necessary. Figure reproduced with permission from ref [27].

Alkaline phosphatase is an enzyme that is secreted by bone cells during the early stages of their differentiation and plays a role in regulating bone mineralization [34]. Osteocalcin and osteopontin are found in the matrix towards the later stages of differentiation and they play a role in the regulation of several processes such including metabolism, proliferation, and homeostasis [35,36]. Mature osteoblasts go on to form osteocytes which are present in the mineralised matrix of the bone, or bone lining cells (quiescent cells) that surround the extracellular matrix [32,37]. The osteocytes are the most abundant cells in the bone and are responsible for the communication between the osteoblasts and osteoclasts during the remodelling and repair of bone [27,38].

After the development of the skeletal system in an infant, the bones of the body continuously undergo modelling throughout our lifetime. The majority of the modelling process takes place before and during the adolescent years and starts to slow down when

the individual is between 20 – 25 years of age. Hormones play an important role in the extent and efficiency of bone growth during this stage. After reaching adulthood, bone modelling stops in a healthy adult and bone remodelling begins. Bone remodelling occurs when there is a balance between bone formation and resorption (**Figure 2-2)**. It ensures that new bone tissue replaces older bone tissue for the proper functioning of the skeletal system. Osteoclast cells are responsible for the resorption of bone and are the first cell type to be activated during bone remodelling. Bone resorption and bone formation need to occur in a balanced, consecutive, and controlled manner for efficient remodelling to take place. The activation of osteoclasts is followed by the resorption of bone. The next stage is the reversal of the resorption phase, where the activity of the osteoclasts is reduced. Lastly, the differentiation of stem cells to osteoblast cells takes place during which the bone architecture is restored, and new bone is formed in its place [33,39].

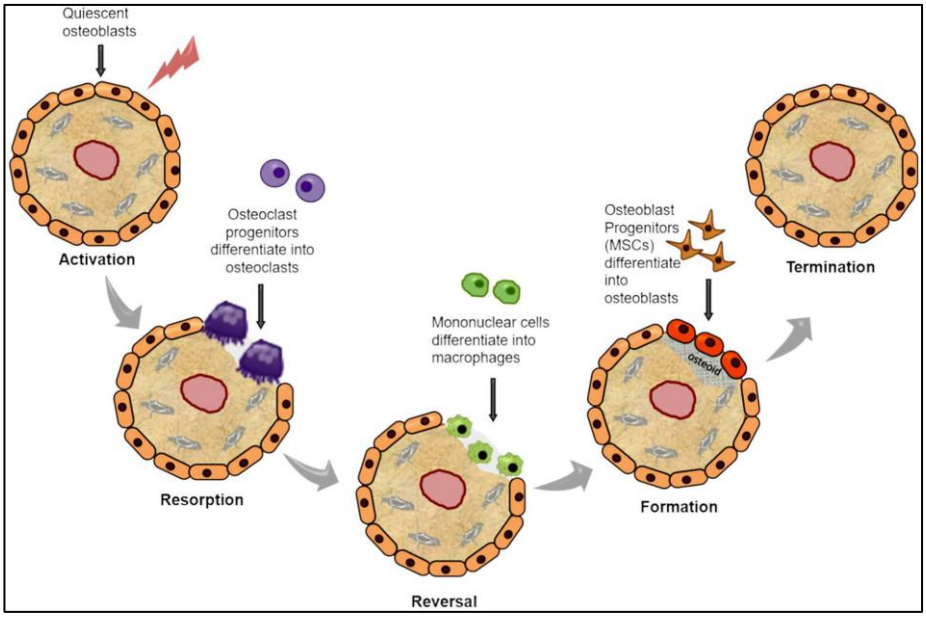


Figure 2-2. Process of bone remodelling and repair after injury. When there is an injury to the bone, the quiescent osteoblast cells that line the existing bone tissue get activated. This leads to the activation of osteoclast cells which are involved with the resorption of injured bone. Once the resorption process is complete, the activity of the osteoclasts is reduced during the reversal phase. This is followed by the differentiation of MSCs into osteoblasts cells which undergo proliferation and maturation to give rise to new bone tissue. Figure reproduced with permission from ref [39].

2.1.2 Signalling pathways involved in the development of bones

Several signalling pathways are involved in the differentiation of stem cells to the different types of bone cells. One of the first signalling pathways to play a role in limb development is the Hedgehog signalling pathway, which is responsible for the formation of the anterior-posterior body axis. Recent studies have shown that Sonic Hedgehog and Indian Hedgehog, two types of Hedgehog ligands, play a role in the activation of transcription factors, such as RUNX2, which drive the fate of MSCs towards an osteoblast lineage [40]. Promotion of osteogenic differentiation by several drugs and osteoinductive molecules have been shown to have occurred due to the upregulation of the signalling molecules of the Hedgehog signalling pathway [41–43].

The Notch signalling pathway is involved with both the promotion and inhibition of osteoblast differentiation depending on the stage of differentiation. The Notch receptor binds to the Jagged or Delta-like proteins. This binding initiates a series of downstream pathways that can inhibit or promote the differentiation of preosteoblast cells into mature osteoblasts [27,42]. Due to the varying effect of the Notch signalling pathway on the differentiation process, this pathway is not often targeted for bone repair applications.

The Wingless and Int-1 (WNT) pathway plays a central role in the differentiation of mesenchymal stem cells into osteoblast cells. WNT signalling pathways can be divided into the canonical WNT pathway, which is dependent on β-catenin function, and the non-canonical WNT pathways, which are independent of β-catenin function. While all these pathways affect bone formation and maintenance, the WNT/β-catenin pathway is the most widely investigated target for improving bone repair [44,45]. Studies have shown that, in addition to regulating osteoblast formation, the WNT/β-catenin pathway also plays a role in the formation of osteocytes from mature osteoblasts, which has a positive and essential role in bone remodelling. The two most prominent non-canonical pathways are the WNT-planar cell polarity pathway and the WNT-calcium pathway. These pathways play a role in the differentiation of osteoclast cells [46]. All the WNT signalling pathways intersect with the other signalling pathways through their downstream components to regulate the process of bone development and remodelling [33].

The transforming growth factor β (TGF β) and the bone morphogenic protein (BMP) mediated signalling pathways are involved in all the stages of bone differentiation [42]. They influence chondrogenesis, proliferation of osteoprogenitors, and differentiation of preosteoblasts into osteoblast cells. BMP2 has been shown to target RUNX2 to promote osteogenic differentiation and has critical functions during the bone repair and remodelling process [47].

2.2 Bone defects, diseases, and injuries

The incidence of bone diseases and defects are increasing worldwide, and bone tissue is becoming one of the most frequently transplanted tissues worldwide [1–4,48]. A better understanding of the different signalling pathways involved in bone development and remodelling has also led to improved awareness about the consequences of the dysregulation of these pathways [49]. Our body is capable of repairing minor injuries through its own regeneration mechanisms; however, this is not the case with severe injuries and defects [50]. These defects are called critical-sized bone defects and are defined as 'those that will not heal spontaneously within a patient's lifetime' [51]. There are several situations where our body's bone repair and bone regeneration rates fall short and require medical and surgical intervention: fractures and defects in the long bones, hip, spine, jaw, skull, wrist, or ankle due to trauma [52], bone defects due to removal of benign or malignant tumours [49], diseases and disorders of the bone resulting in fractures or defects such as osteoporosis, Paget's disease, and osteonecrosis [53,54], prosthetic revision surgeries [52], genetic and developmental factors [55], and dental implants/surgeries [49]. Many factors such as the location of the defect or fracture, the extent of damage, presence of infection, age of the patient, presence of comorbidities/metabolic disorders, alcohol/smoking habits of the patient, and inflammatory/immune disorders play a role in the rate and success of bone healing and remodelling [50,56,57]. Ongoing research is focussed on developing efficient methods of treating these defects while keeping in mind the complex nature of bone repair and regeneration.

2.3 Current treatment methods of bone repair and regeneration

The most commonly used method for bone repair is the use of grafts. Bone grafting is the method by which bone tissue from one region of the body is transplanted into the site of injury to promote the growth of new bone. The successful repair of bone defects

and the efficiency of grafts is characterized by three important processes. Firstly, the treatment method must be able to promote osteogenesis at the site of injury or defect. Osteogenesis is the process of formation of new bone, and this occurs from the presence of donor cells that can differentiate into bone cells. Secondly, it must have osteoinductive properties, which is the ability to induce the host stem cells to differentiate into bone cells. This further increases the efficiency of bone repair by recruiting host machinery to speed up bone remodelling in the area. Thirdly, grafts and bone substitutes must possess osteoconductive properties. This allows for the integration of the material with the surroundings by allowing the entry of the host cells, vasculature, and tissue [58–60]. In addition to these properties, the grafts must be non-toxic, and not cause the transfer of diseases and infections. Several types of materials, grafts, pharmaceuticals, and drugs have been explored for bone repair and regeneration (**Figure 2-3**). The following subsections discuss the current treatment methods being used in clinics worldwide.

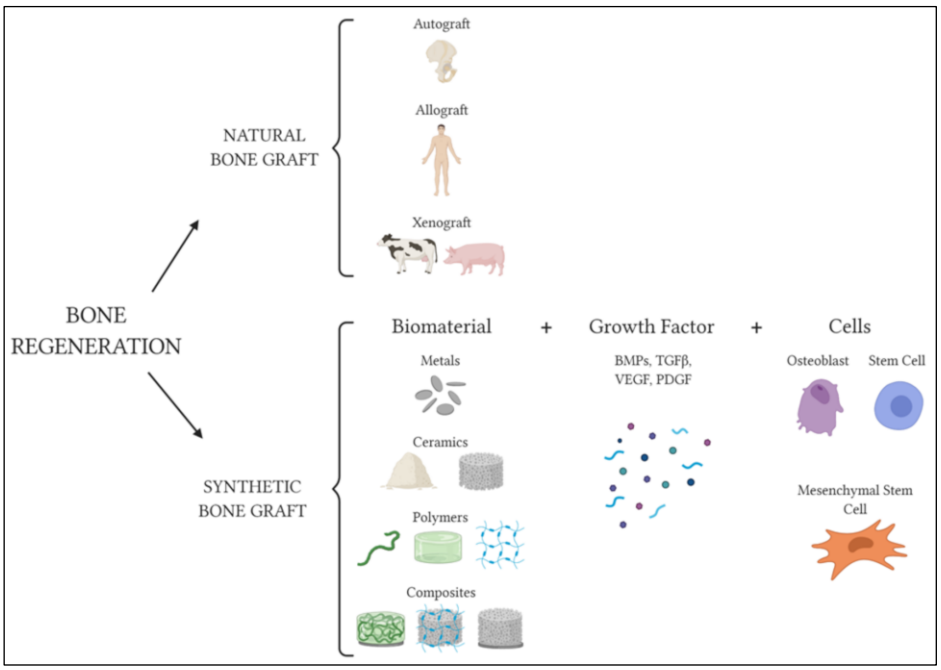


Figure 2-3. Bone regeneration strategies. The use of natural graft materials such as autografts and allografts are the most widely used method of treatment for bone injuries and defects in clinics. However, the use of synthetic bone substitutes and grafts are a good alternative to natural grafts. These substitutes can be metals, ceramics, natural polymers, synthetic polymers, or bioactive glass. They are usually combined with growth factors, drugs, genetic material, and/or cells to improve their osteogenic, osteoinductive, and osteoconductive properties. Figure reproduced with permission from ref [60].

2.3.1 Autografts and allografts

Autografts are the most commonly used method in clinics [49]. Bone from the patient's own body is harvested and used. They contain cells and growth factors from the patient and are osteoconductive, osteoinductive, and osteogenic [49]. As the graft is derived from the host, there is no risk of rejection or disease transmission of non-infectious origin. Cancellous bone, which is found inner to the cortical bone, is rich in bone forming cells. Their high osteogenic potential makes them the most used autologous graft material. Other bone tissues used as autografts include cortical bone, vascularized bone graft, and bone marrow aspirate [59,61]. Although this is the ideal method of treatment, the availability of autografts is limited. The operative time is increased as healing must occur at the donor site, and there is an increased risk of donor site pain [62]. Studies have shown that the rate of reabsorption of autografts can be higher than the rate of formation of new bone, decreasing its efficiency as a treatment method [49,63].

An alternative to autografts is allografts. Allografts are bone tissues that are obtained from human cadavers or a different individual. To make them safe for transplantation, all the cellular content is removed, and the tissue is sterilized. As a result, allografts do not have osteogenic potential due to the absence of viable bone cells. The present matrix proteins and growth factors confer the property of osteoinduction; however, studies have shown that the process of sterilization/disinfection using gamma irradiation or the use of reagents such as ethanol can have a negative effect on their osteoinductive properties [53,59,64]. The most important advantage of using allografts is their osteoconductivity. Despite the absence of viable cells and growth factors, several components of the matrix are retained which provides the mechanical support and acts as a scaffold on which new bone can be formed in the host [65]. Allografts are the second most commonly used treatment for bone repair. Cancellous bone, frequently used as autografts, are not ideal allograft materials. The loss of cells compromises their osteogenic property and their poor mechanical properties do not offer support at the site of injury. Cortical bones, on the other hand, have suitable mechanical properties and act as a weight-bearing support and a source of stabilization to the injured bones [66]. Demineralized bone matrix (DBM), which are bone tissues that have been treated with acid to remove the mineral content of the bone, are another type of allograft material used in clinics. The use of acids can lead to the continued presence of several growth

factors which give this graft the property of osteoinduction [64]. Nevertheless, there are several challenges associated with these grafts. The loss of osteogenic potential decreases their efficiency, and there is a greater risk of disease transmission [49,61,67].

2.3.2 Bone substitutes and biomaterials

Bone substitutes are synthetic or natural materials that are used in place of autografts and allografts. They mimic natural bone and can be designed to have desirable properties specific to the bone being repaired. The biggest advantage of bone substitutes is their abundant availability since they can be manufactured or synthesised based on the requirement [67]. The following are the different types of bone substitutes being explored and used for bone repair.

Natural polymers: Natural polymers can mimic the structure of bone extracellular matrix and connective tissues [67]. They are abundant, can be manipulated and modified to suit the specific purposes, and have excellent osteoconductive properties. Furthermore, they are non-cytotoxic, biodegradable, and can interact with the microenvironment [68]. Several osteoinductive factors can easily be incorporated into their network, and their extraction is relatively simple [69]. Examples of natural polymers used for bone repair are chitosan, collagen, alginates, cellulose, agarose, and starch. The main disadvantage of natural polymers is their low mechanical strength and the variability between the different batches of polymers. Moreover, their rate of degradation is often faster than the rate of bone healing [67].

Synthetic polymers: synthetic polymers such as poly-lactic acid (PLA), poly-glycolic acid (PGA), and poly-(lactide-co-glycolide) (PLGA) are versatile polymers and can be synthesised consistently. Like natural polymers, they are non-cytotoxic and biodegradable. In addition, their properties can be controlled, and they can be designed in several shapes and sizes. However, synthetic polymers need to have surface modifications in order to interact with the surrounding cells and molecules. Their degradation can lead to the release of compounds that have adverse effects on the bone healing process [48,68].

Metals: metals have mechanical properties suitable for use in repairing bones that have high load-bearing capacities. Their stiffness is similar to trabecular bone and their porosity and structure provide good osteoconductive properties that can support the

ingrowth of bone during the repair stages [67]. Titanium and its alloys have been used extensively for bone repair due to their strength and resistance to corrosion which is an important factor to consider during the selection of metals as bone implants [70]. Some of the limitations of metals is the necessity for a second surgery to remove the material after bone repair, risk of toxicity from the metal ions, and the requirement of surface treatments to improve the adherence of tissues and their interaction with the surrounding microenvironment [69]. Recently, other metals such as magnesium, zinc, iron, and their alloys are being studied to develop biodegradable implants with enhanced properties.

Ceramics: ceramics are made of clay, minerals, and metallic or non-metallic materials that are combined to form crystalline structures [50]. Their non-cytotoxic nature, favourable chemical composition, biological activity, corrosion resistance, and degradability have resulted in their extensive use for treating bone defects and fractures [71]. Hydroxyapatite is a natural ceramic made primarily of calcium phosphate and is commonly used in clinics due to its similarity to bone mineral composition [69]. Synthetic ceramics such as calcium phosphate, tricalcium phosphate, and biphasic calcium phosphate can be modified to incorporate osteoinductive properties. This, combined with their excellent osteoconductivity and resorbable nature, is used to develop tuneable and effective bone graft materials with a good balance between mechanical stability and biodegradability [49,53,59].

Bioactive glass: bioactive glass can be silica-based, borate-based, or phosphate-based. The commonly used silica-based bioglass material contains silica, calcium oxide, sodium oxide, and phosphorous pentoxide [53]. Like the other bone substitutes, bioglass is non-cytotoxic, and has good bioactivity due to the formation of a hydroxyapatite layer on its surface. A porous bioglass implant material can support the proliferation of biological molecules and can be used to deliver a number of therapeutic factors that can accelerate bone healing [67]. The main advantage of bioglass is that the degradation rate can be varied by modifying its composition to match the rate of bone formation [50,64]. This would ensure that the material can support bone repair during the remodelling phase while the degradation process would ensure that the new bone is fully formed in the area and the removal of the implant material is not necessary [49].

2.3.3 Delivery of growth factors, genes, therapeutic drugs, and cells

The use of grafts and bone substitutes as scaffold materials is the most common method to treat bone injuries and defects and has shown a degree of success in clinics worldwide [72]. These methods rely on the mechanical and osteoconductive properties of these materials to improve bone repair. However, knowledge about the different signalling pathways, bone development and remodelling processes, growth factors and transcription factors involved in bone development, and the different types of cells involved have opened new possibilities to improve bone repair and regeneration. The application of tissue engineering and genetic engineering principles to develop bioactive scaffolds that have osteoinductive and/or osteogenic properties is the focus of research efforts today. To this end, several biological molecules are being considered to improve regeneration by integration with the existing or new scaffold materials. Growth factors have been shown to have direct effects on the osteogenic differentiation process and are at the forefront of studies to incorporate osteoinductive molecules in scaffolds. Genes encoding for growth factors involved with bone formation, peptides, small molecules, hormones, antibiotics, and drugs are also being investigated [52].

The primary focus during the selection of bioactive molecules for bone repair is their ability to induce the differentiation and/or maturation of osteoblast cells which are key players in bone remodelling and repair [73]. Such molecules work majorly by targeting different components of the signalling pathways involved in osteoblast differentiation. Alternate strategies focus on other areas of bone remodelling such as the influx of progenitor/stem cells, proliferation of progenitor and preosteoblast cells, promotion of angiogenesis and vascularization at the repair site, promotion of chondrogenesis and other processes required for the proper formation of bone tissue, influx and proliferation of osteoclasts during the initial stages of bone remodelling, and osteocyte maturation. The bioactive molecules that have been studied to achieve these goals can be grouped into the following 3 broad categories and will be discussed in this section.

Growth factors: Growth factors are perhaps the most widely explored and characterized bioactive molecules for bone repair and regeneration [72,74]. Among the growth factors studied, BMPs have shown excellent osteoinductive properties. As a result of their extensive study, two recombinant BMPs, BMP-2 and BMP-7, were approved for clinical use [72]. BMPs work with the other members of the TGF-β signalling pathway

and the BMP signalling pathway to induce the formation of cartilage and bone. They have been incorporated into different scaffold materials to improve their delivery and availability at the site of repair. Numerous studies have shown that BMPs promote osteoinduction in several bone defects and have been used in multiple surgeries including long bone defects, spinal fusion, mandibular defects, and maxillofacial surgeries [72,75–79].

While the proliferation and maturation of osteoblasts is the primary step in bone remodelling, there are other crucial steps that need to occur for complete healing as we have seen in previous sections. Growth factors that target cells and processes in these later stages of bone repair have also been investigated. Platelet derived growth factor (PDGF), stromal derived factor-1 (SDF-1), and fibroblast growth factor (FGF) all play a role in the migration and influx of MSCs to the site of fracture. The presence of MSCs enables their differentiation into bone and cartilage cells ensuring the completion of the repair process [72,73]. These growth factors also work by interacting with the members and receptors of the signalling pathways involved with the migration of cells to the injury site and their proliferation, which further promotes bone regeneration.

Bone tissue is highly vascularized, with blood vessels supplying nutrients, oxygen, growth factors, and hormones to existing and developing bone cells. For bone remodelling to occur, the newly formed tissue must develop a vascular network of blood vessels along with bone tissue [80]. Angiogenesis, the formation of new blood vessels from pre-existing networks, is initially characterized by the influx of endothelial cells. Simultaneous osteogenesis and angiogenesis have been shown to result in the best bone repair outcomes [81]. Vascular endothelial growth factor (VEGF) is one of the key promotors of angiogenesis as shown in many studies and has also been implicated to have an indirect role in bone formation [52,73,74]. Dual delivery strategies where VEGF and BMP have been incorporated into engineered scaffolds, have shown higher efficacy when compared to the use of a single growth factor [82–86]. Thus, growth factors play an important role in bone repair by influencing the signalling pathways involved in bone remodelling towards bone regeneration and are the potent osteoinductive molecules.

Genetic materials: While the direct delivery of growth factors is desirable, there can be issues with preserving the function of these molecules *in vivo*, limiting the dosage and

localization so as to avoid side effects, and ensuring the sustained availability of the molecules over the period of bone repair. An alternate method to ensure the presence of osteoinductive growth factors is to use gene therapy [72]. Genes encoding BMPs, or other growth factors are being incorporated into the scaffold materials. This method has several advantages including localized and sustained secretion of the growth factor, cell specific delivery, and the use of lower doses [87]. Both viral and non-viral methods have been explored, with the genetic material delivered in the form of plasmid DNA, virally packaged DNA, or modified mRNA [88]. The types of viral vectors investigated are adenovirus, adeno-associated virus, and lentivirus. The main advantages of viral vectors are ease of production and scale up, high packaging capacity, lentiviral integration of the gene of interest into the genome enabling prolonged gene expression, and high efficacy while the prominent disadvantages are the probability of mutagenesis in the host cells, immunogenicity, and adverse off-target effects [88]. Non-viral vectors include polymer-based scaffolds and matrices, lipid-based vectors, and nanoparticles [87–89]. These are safer than viral vectors but have lower efficiency and require the use of excellent delivery principles to achieve the desired effect [89]. Similar to growth factor delivery, a combinatorial approach to involving the delivery of more than one therapeutic agent has favourable outcomes and is the focus of current research [90,91].

The strategies discussed so far make use of molecules to promote certain processes or pathways. However, osteogenic differentiation can also be increased by inhibiting molecules that interfere with this process. MicroRNA (miRNA) and small-interfering RNA (siRNA) have been used to this end, where they mediate the silencing of target genes that act as inhibitors in pathways like WNT, hedgehog, notch, and TGF-β [91,92]. Several miRNA and siRNA have shown promising results *in vivo* in pre-clinical studies in combination with biomaterial scaffolds and non-viral vectors [93–96].

Therapeutic drugs and antibiotics: small molecule drugs are commercially available to treat numerous diseases and disorders. They are chemically synthesized compounds that have low molecular weights and affect the production or function of biological molecules in our body [97]. The main advantages of using small molecule drugs are good structural stability, defined manufacturing processes, affordability, low immunogenicity, and availability of high throughput screening methods to test for the most effective drugs [98]. The following are small molecules that have shown success and effectiveness in bone repair: phenamil, a derivative of a diuretic, capable of

promoting osteogenesis by promoting BMP signalling [95,99], simvastatin, a cholesterol-lowering drug, that affects BMP expression and angiogenesis leading to increased bone formation [100,101], dexamethasone, a glucocorticoid drug, shown to increase osteoblast differentiation and maturation [102,103], resveratrol, a natural polyphenyl compound, which stimulates the WNT and BMP signalling to increase matrix mineralization [56], and a number of other small molecules that promote or inhibit the expression of the members of the WNT, BMP, TGF-β, and notch signalling pathways and key transcription factors involved in osteogenesis [97,104–106].

Lastly, bone injuries are characterized by open wounds that can get infected, and this can lead to problems in bone healing. This is a major challenge faced in clinics and is a parallel area of focus for bone regeneration [107,108]. The incorporation of antibacterials within transplanted scaffolds, along with osteoinductive factors, can ensure the prevention of serious infection and subsequent complications during the bone healing process. To this end, several antibiotics and antimicrobial agents such as vancomycin, tetracyclines, tobramycin, silver, and antimicrobial peptides are being studied [109–112].

Dexamethasone (Dex): Dex is a glucocorticoid anti-inflammatory drug that has been used for many decades to promote the differentiation of stem/stromal cells towards an osteoblast lineage [113]. Due to its widespread and well documented use over the decades and its ability to successfully promote osteogenic differentiation, we choose to use Dex as the therapeutic drug driving stem cell differentiation for this project. Dex modulates this process by the overexpression of RUNX2 which then influences the canonical WNT/β-catenin pathway and the BMP pathway. The addition of Dex leads to the upregulation of 3 molecules: a) FHL2, a LIM domain protein which shuttles between the nucleus and cytoplasm, that binds to β-catenin and transports it to the nucleus leading to the expression of RUNX2 [114], b) TAZ, a β-catenin-like protein, that activates RUNX2 mediated transcription [115], and c) MKP-1, a mitogen activated protein kinase phosphatase, that de-phosphorylates RUNX2 leading to its enhanced activation [116,117]. This upregulation of RUNX2 then leads to increased osteoblast formation [118,119]. Studies have shown that Dex administration through the systemic route has adverse side effects, such as Cushing's syndrome, due to off target delivery and increased doses [120]. The delivery methods that have been investigated for Dex delivery involve the use of polymeric scaffolds/hydrogels made from several types of

materials [102,121], microparticle or nanoparticle delivery vehicles [120,122–124], or extracellular vesicles [103]. Dex is usually delivered alongside β-glycerophosphate, a source of phosphates, and ascorbic acid, which promotes the secretion of collagen [125]. The presence of osteogenic differentiation *in vitro* is typically determined by the secretion of alkaline phosphatase, presence of calcium deposits indicating matrix mineralization, and the expression of osteocalcin, osteopontin, and RUNX2 [34,126–130].

Cells: A parallel strategy is the use of stem/stromal cells or differentiated osteoblast cells to provide osteogenic properties to the scaffolds [131]. This strategy focusses on improving the osteogenic potential of the scaffolds. Instead of completely relying on the body to make use of its undifferentiated cells, they would be directly introduced to the site of injury. One method of achieving this is to incorporate stem/stromal cells into the scaffold, along with factors that would induce their differentiation. These cells would then promote the differentiation of cells in the surrounding area. The second method is to differentiate stem/stromal cells *ex vivo* using one of the many available growth factors/drugs and incorporate these cells into the scaffold. Thus, this would result in the presence of osteoblast lineage cells at the repair site, promoting further osteogenic differentiation. While these strategies would provide the best results, achieving this in a large scale in clinics has proven to be difficult [132]. Current limitations of using cells are large-scale manufacturing, storage, survival of cells *in vivo*, and the choice of cell types to provide the best results. Different types of stem/stromal cells isolated from various tissues in the body are currently being studied, and efforts are being made to overcome these limitations to develop highly effective bone regeneration scaffolds [56].

There is abundant availability of bioactive molecules and approaches that show excellent potential to be used in osteogenesis and bone repair, as seen in the above section. However, real-time clinical use of these molecules requires the combined application of the principles of engineering, biology, biochemistry, and mechanics to understand the effects of their use on the highly complex process of bone regeneration and remodelling.

2.4 Current challenges and emergence of nanoparticle delivery systems to improve the efficiency of bone repair drugs

When we investigate the delivery methods of bioactive molecules and therapeutic drugs, simply incorporating them into the scaffold material has not shown the desired level of efficacy. Growth factors and genetic material, used as osteoinductive molecules, are susceptible to degradation by proteases, DNases, RNases, or other enzymes that are naturally present in our bodies [133]. This implies that their stability is poor *in vivo*. Their premature degradation and short-lived nature reduce their bioactivity at the site of injury and prevents them from working to their full potential. To overcome this, some research groups immobilized these molecules on implant surfaces or in the scaffold network by covalent bonding or affinity binding [134]. While this showed a measure of success in terms of increasing the stability of the molecules, it decreased their ability to diffuse into the microenvironment, thereby limiting its access to cells in the surrounding area. When immobilization strategies were not used, the bioactive molecules or therapeutic drugs were incorporated into porous scaffolds. This was done with the intention to ensure a controlled release of the osteoinductive factors to the injury/defect site by remaining in the scaffold until the scaffold material degrades or the diffusion of these osteoinductive molecules from the scaffold in a sustained manner. However, some limitations of this method were the fast release of the molecules from the scaffold leading to the requirement of higher doses, which could cause adverse side effects or inhibition of osteogenesis [135]. The scaffold material, in this case, not only has to have mechanical properties to match the bone tissue, but also have a degradation rate conducive to the sustained release of the osteoinductive molecule. In the case of small molecules and therapeutic drugs, they must be non-cytotoxic, have good cell permeability, and high bioavailability. Incorporation into scaffolds, while enabling controlled release, cannot change the characteristics of these molecules [136]. Most importantly, all these osteoinductive substances work by targeting different components of signalling pathways involved in bone formation and remodelling. However, many of these components and pathways are also involved in other functions and demonstrate crosstalk with other pathways. Off target effects and/or undesired upregulation or downregulation can have serious adverse effects which lead to the increase in unwanted inflammatory reactions or hampered bone repair. Therefore,

it is important to deliver these molecules using a strategy that makes the most of their osteoinductive potential [135–139].

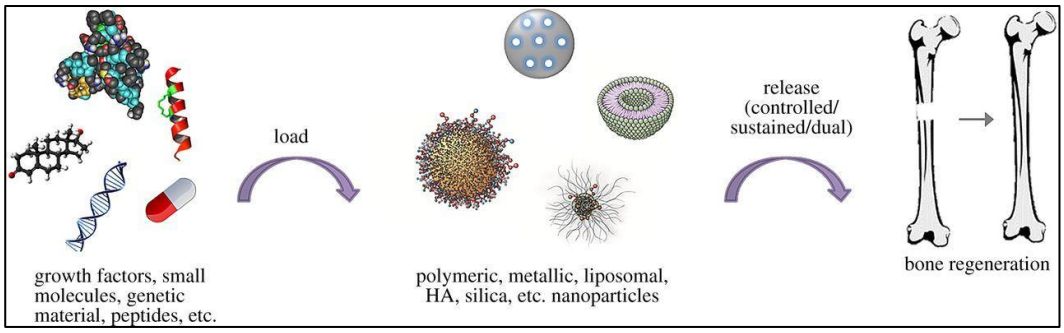


Figure 2-4. Nanoparticle delivery systems to promote bone regeneration and repair. Several growth factors, small molecules, genetic material, peptides, and hormones have excellent osteoinductive properties. However, their stability *in vivo*, or bioavailability might be lacking. The use of nanoparticle delivery systems has been shown to improve the efficiency of delivering these osteoinductive molecules by enabling the sustained release of these molecules. Silica nanoparticles, lipid-based nanoparticles, polymeric nanoparticles, calcium phosphate nanoparticles, and exosomes have been investigated as delivery vehicles for bone regeneration. Figure reproduced with permission from ref [139].

Nanoparticles have been used to encapsulate osteoinductive molecules and are in turn incorporated into biomaterial scaffolds and grafts to improve the efficiency of bone regeneration and repair (**Figure 2-4**). The small size of nanoparticles, high surface-to-volume ratio, and the ability to be internalized by cells have prompted their use, in pre-clinical and clinical scenarios, for the treatment of numerous diseases [139,140]. Specifically in bone repair and regeneration, these properties play an important role in ensuring the efficient employment of osteoinductive molecules. The following are the prominent nanoparticles explored in this field.

Mesoporous silica nanoparticles: Mesoporous silica nanoparticles have a stable, porous, and honeycomb-like structure with a large surface area [141]. Their low toxicity, high stability, and high drug loading capacity make them good drug delivery systems. Their tuneable nature allows for the encapsulation of various types of drug and bioactive molecules. Furthermore, their surface can be functionalized to bind to targeting molecules for cell specific delivery. Several studies have used them in combination with other materials to deliver Dex and growth factors like BMP [120,142–144]. The major limitations of these particles are the presence of high density

of silanol groups on the surface leading to haemolysis and adverse metabolic changes leading to issues with toxicity is certain cases [135,141].

Lipid-based nanoparticles: Lipid-based nanoparticles are widely employed in drug delivery and vaccine applications with several commercial formulations available. Liposomes are perhaps the most studied lipid-based nanoparticles and are composed of a bilayer of phospholipids. Their similarity to the plasma membrane structure is their biggest advantage making them non-cytotoxic and biodegradable. Because of their tuneable nature, they have been investigated for the delivery of several osteoinductive molecules such as Dex, siRNA, and growth factors [124,145,146]. Some determinants to consider are the control of dosage (sometimes show toxicity), size characterization to ensure that they lie in the favourable size range, and surface modifications required for targeting or improved bioavailability (could increase complexity of manufacturing and quality control procedures).

Polymeric nanoparticles: A number of polymers can be synthesised into nanoparticles that encapsulate and deliver therapeutics. They have versatile architecture, are non-cytotoxic, biodegradable, and can be functionalized through the polymer network. Both synthetic, including PLGA, polyethylene glycol, poly-(ε-caprolactone) and natural polymers, including chitosan, gelatine, hyaluronic acid, alginate, and collagen, have been used as drug delivery vehicles for bone repair and have been utilized for the encapsulation of growth factors and small molecule drugs [147–152]. The challenges of using these materials comes from lack of reproducibility when using natural polymers, burst release, premature degradation, requirement of additional surface modifications, and the lack of interaction with cells in the microenvironment [147,153].

Calcium phosphate and hydroxyapatite nanoparticles: These nanoparticles are primarily explored for bone repair due to the presence of calcium and hydroxyapatite in natural bone tissue. This makes them non-cytotoxic and increases the osteoconductivity of the scaffold. Hence, these particles have a dual function; the delivery of osteoinductive factors and the increase in osteoconductivity of the biomaterial. These particles, individually or in combination with other materials, have been used to encapsulate and deliver small molecules like Dex and growth factors [123,154]. Some of the limitations of these particles are the non-uniformity and size,

non-homogenous distribution, difficulty in uniform production, and the absence of extensive biocompatibility analysis [155].

Exosomes and self-assembling peptides: Biomimetic nanoparticles have emerged as efficient candidates for drug delivery with excellent potential for many applications. Exosomes are a class of biomimetic nanoparticles that are released by certain types of cells. They typically have a size range of 50 – 120 nm and are carriers of biological cargo such as proteins and genetic material. They mediate cell-to-cell communication naturally and are specific to the cell types that produce them [57]. Studies on exosomes have shown that they are directly involved with the osteogenic differentiation of stem/stromal cells, proliferation and maturation of osteoblasts and osteoclasts, and have angiogenic properties. They are being investigated for the delivery of growth factors for bone regeneration due to their inherent osteogenic properties and targeting capabilities [57,156–158]. The source cell plays and important role when using exosomes as the origin of their characteristics. However, due to lack of sufficient data and the difficulty in the production of exosomes, they have not yet progressed beyond the pre-clinical stage [57]. Another class of biomimetic nanoparticles are self-assembling peptides which have ordered structures held together by non-covalent interactions. Self-assembling peptides like ferritin and peptide amphiphile molecules have been explored to deliver growth factors for bone applications. Like exosomes, they are still in the nascent stages of development and study but are promising candidates for future drug delivery applications for bone regeneration [159,160].

From the above discussion and examples, we have seen that nanoparticle delivery vehicles have an important role to play in improving bone regeneration and osteogenesis. Keeping in mind the advantages and disadvantages of the existing delivery systems, we have focussed on the development of a class of biomimetic nanoparticle delivery vehicle called cell-derived nanoparticles (CDNs) for my project. The emergence, use, synthesis, and examples of CDNs will be discussed in the following section.

2.5 Introduction of Cell Derived Nanoparticles for enhanced drug delivery

Cell-derived nanoparticles (CDNs) are prepared from the cell membranes of cells. Cell membranes inherently have surface proteins, lipids, and other molecules on its surface that are involved with numerous biological functions [161]. Amidst the endeavour to develop the ideal drug delivery vehicle, CDNs emerged as promising candidates due to the absence of cytotoxicity. Their surface properties, carried over from the source cells, have been shown to increase their circulation time in the body, when compared to the free drug or nanoparticles prepared from other materials, leading to enhanced retention and delayed clearance [162,163]. Attempts are being made to utilize the surface properties for targeted delivery of therapeutics for various diseases. The cell membranes can be isolated from any cell, and the preparation methods used so far can be applied indiscriminately to all cell types [164,165]. The following are the cell sources that have been investigated so far.

Red blood cells (RBCs): RBCs are characterized by their long circulation times in the body, where they perform the function of gaseous transport. Their ability to avoid immune clearance, lack of nucleus, and abundant and ready availability made them the source cells for the first CDNs synthesised. They have been utilized for the delivery of small molecule drugs, and their response to stimuli such as pH and near infrared (NIR) light has been studied and exploited. Their efficiency has been tested and proven *in vivo* for cancer, imaging, and immune modulatory applications [162,166–168].

Cancer cells: Cancer cells are perhaps the most widely explored cell source for CDN synthesis. Cancer cells have surface markers that enable targeting with other cancer cells and this property has been taken advantage of to deliver drug molecules to tumour sites. Stimuli responsive CDNs and hybrid CDNs prepared from the membranes of two different cell types have both been shown to improve drug delivery by prolonged circulation and increased targeting to the desired site [163,164,169,170].

Platelets: Platelets play an important role in haemostasis and are one of the first responders in case of injury. Their homing to the site of injury, inherent immunomodulatory property, and ability to bind to pathogens were sought after when developing CDNs. They have been investigated for promoting angiogenesis, cancer

therapy, and target specific antibacterial delivery. Successful drug delivery promoted by platelet derived CDNs have been shown *in vivo* in mouse models [166,171].

Stem/stromal cells: Stem/stromal cells have a number of diverse functions and are specific to different cell types and molecules. The unique surface properties of stem/stromal cells have been utilized by using them as the source cells for CDN synthesis. Bone marrow mesenchymal stem/stromal cells, cardiac stem/stromal cells, and umbilical cord mesenchymal stem/stromal cells have been investigated so far [172–177].

Immune cells: Immune cells have unique surface properties specific to the type of immune cells being considered, since each type of immune cell has different functions. They have the ability to identify non-self-cells and molecules and are responsible for mediating the protection of the body by fighting infections and disorders. They have been used to prepare CDNs and encapsulate drugs to treat cancer and inflammation [25,166,178].

Other cells: Other cells used as source materials for CDN preparation include nerve cells, lung cells, endothelial cells, and bacterial cells [177,179,180].

The majority of the research so far has used cell membranes as a nanoparticle cloaking mechanism. When materials such as polymers and metals are used for drug delivery, cytotoxicity can become an issue in increased doses. By cloaking or camouflaging these particles with cell membranes, their biocompatibility is greatly increased. The presence of the surface markers would prolong circulation time and promote the internalization of the nanoparticles [25,181,182]. Since the cytosolic content is removed during the synthesis, they are no longer responsive, are not affected by signalling, and do not show any metabolic activity. Thus, cell membranes are extraordinary tools with specific and unique properties that have the potential to upgrade drug delivery [161,172].

The cell membranes have been isolated using several different methods. The principle, however, is the same for all the strategies used. The first step involves the disruption or breaking open of the cell to remove the cytosolic content. This is a crucial step since the presence of proteins or genetic material from certain cells, like cancer cells, is not desirable and has the potential to show adverse effects. The disruption of the cell is followed by the fragmentation of the cell membranes into smaller fragments. This is done so that smaller vesicles can be obtained. This is followed by the isolation of the

cell membranes and cloaking of nanoparticles/entrapment of drugs. The cloaking can also be done by multiple methods: extrusion sonication, electroporation, and physical interaction [163,165,183].

When CDNs are used for cloaking other nanoparticles, the size of the final product is influenced by the nanoparticle core. When cell membranes are used to make vesicles that entrap drugs without the presence of a core nanoparticle carriers, careful consideration should be given to the preparation process so as to obtain particles of desired size. The characterization of CDNs is done by imaging, size distribution analysis, surface characterization, and drug loading quantification. CDNs, like other nanoparticles having a similar size range, are internalized through the clathrin-mediated or the caveolae-mediated pathways. Their size and shape play a role in this internalization process [184–188].

Chapter 3

3 Materials and Methods

3.1 Cell culture

Human embryonic kidney 293 (HEK293) cells were purchased from Sigma Aldrich (Cat. No. 85120602) and cultured in Minimum Essential Medium Eagle (Sigma Aldrich, Cat. No. M5650) supplemented with 10% v/v fetal bovine serum (FBS), 1% v/v penicillin-streptomycin, and 2 mM L-Glutamine. Human adipose-derived stem/stromal cells (hADSC) were purchased from Lonza (Cat. No. PT-5006) and cultured in ADSC basal medium (Lonza, Cat. No. PT-3273) supplemented with the ADSC-GM Singlequots^{TM} Supplement Kit (Lonza, Cat. No. PT-4503). Cells were incubated at 37 °C at 5% CO_2. THP1 monocytes were a gift by Dr. Lauren Flynn (Western University, Canada). They were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% v/v fetal bovine serum (FBS), 1% v/v penicillin-streptomycin, and 2 mM L-Glutamine.

3.2 CDN preparation by cell fragmentation and shearing

HEK293 cells were cultured in petri dishes (VWR, Cat. No. CABD53003). When cells reached 80% confluency, they were trypsinized and pelleted by centrifugation at 200 *g* for 5 minutes, counted, and washed twice with ice-cold Phosphate Buffered Saline (PBS). A cell suspension of 30 x 10^6 cells in 450 µL of ice-cold PBS was placed in a spin cup containing two 10 µm filters and centrifuged at 4 °C at 14,000 *g* for 10 minutes. The supernatant was discarded, the pellet was resuspended in 450 µL of ice-cold PBS, reintroduced into the same spin cup, and centrifuged for the second time at 14,000 *g* for 10 minutes. The supernatant was discarded, and this pellet was then resuspended and introduced into a new spin cup containing two 8 µm filters. Centrifugation was repeated twice, as done in the previous step [189,190].

The pellet obtained from the above step was again resuspended in ice-cold PBS. To obtain a drug loaded sample, the pellet was instead resuspended in a 2 mg/mL Dex (Sigma Aldrich, Cat. No. D2915) solution. Here, the Dex used was a water-soluble form od Dex. These solutions were then introduced into a spin cup containing three 0.2 µm filters and centrifuged at 14,000 *g* for 30 seconds. The pellet obtained was

resuspended in the same PBS solution and the process of centrifugation and resuspension was repeated 9 more times.

3.3 Quantification of proteins, DNA, and drug loading

The protein and DNA concentrations of the samples before and after the CDN preparation process were quantified using NanoDrop Lite (Thermo Scientific, Canada). The drug loaded CDNs were passed through a protein concentrator (Thermo Fisher Scientific, Cat. No. 88527) with a 10 kDa molecular weight cut off to separate the unloaded Dex from the Dex-loaded CDNs. The unloaded Dex was quantified using a Geneses 10S UV-Vis Spectrophotometer (Thermo Scientific) by measuring absorbance at 242 nm, and this was used to calculate the amount of Dex loaded.

3.4 Size characterization of CDNs

NanoSight NS300 (Malvern Pananalytical, Canada) was used to measure the size of the CDNs. 150 µL of the CDN samples were made up to 1 mL using PBS. The best concentration for measurement was determined by trial and error. The sample was then diluted 10 times and passed through the sample chamber using a 1 mL syringe and sample pump. A green laser (532 nm) was used for sample detection and the detection threshold was set to 40. Measurements were carried out at 24.4 – 24.5 °C.

3.5 Atomic Force Microscopy and Transmission Electron Microscopy

The CDNs were fixed using 4% paraformaldehyde at a 1:1 ratio of the fixative and CDNs for Atomic Force Microscopy (AFM). A drop of this mixture was deposited onto a freshly cleaved muscovite mica sheet (VWR International, Cat. No. 103302-472) for 10 minutes, dried, and imaged using an MFP-3D Origin^{TM} Atomic Force Microscope (Asylum Research Oxford Instruments, USA). Image visualization and analysis was performed using the Gwyddion software [191].

A fixative made up of a mixture of 2% paraformaldehyde and 1% glutaraldehyde in 0.1 M Cacodylate was used to fix the CDNs for Transmission Electron Microscopy (TEM). A 1:1 ratio of the fixative and CDN sample were mixed. A drop of the fixed sample was placed onto a 200-mesh copper grid precoated with carbon and allowed to deposit for 1-2 minutes before the solution was removed using a filter paper. The sample was stained using 0.5% uranyl acetate and washed with water before being dried for 30 minutes. Imaging was done using a Philips 420 Transmission Microscope.

3.6 Western blotting

CDN Dex and CDN samples were denatured at 95 °C with 4x Laemmli SDS sample buffer (Bio Rad, Cat. No. 1610747) and run on a 12% SDS-PAGE gel (BioRad, Canada) at 120 V. The gel was blotted onto a nitrocellulose membrane using Turbo-Blot Turbo transfer system (Bio-Rad, Canada). The membrane was immunoblotted with Rabbit Caveolin 1 Polyclonal Antibody (Thermo Fisher Scientific, Cat. No. PA1064), followed by Goat anti-Rabbit IgG (H+L) Secondary Antibody conjugated to HRP (Thermo Fisher Scientific, Cat. No. 656120). Bands were visualized by chemiluminescence using Clarity™ Western Blotting Substrate (Bio-Rad, Cat. No, 170-5061) detected with a ChemiDoc™ XRS+ imaging system (Bio-Rad, Cat. No. 1708265).

3.7 Cytocompatibility studies

hADSCs were seeded onto 96-well plates with a seeding density of 3000 cells per well and cultured overnight. Fresh media was added, and the cells were treated with different doses of CDNs suspended in PBS (42 ng, 420 ng, and 4200 ng) for 24 h, 48 h, and 72 h. Here, the amounts of CDNs for each of the doses was added to 200 µl of the media in each 96 well. This was followed by an MTS assay (Promega, Cat. No. G3580) to check for cell metabolic activity. Furthermore, the cellular viability of the above groups was also tested qualitatively using Calcein AM staining (Thermo Fisher Scientific, Cat. No. C1430).

The cytocompatibility was further tested by quantifying TNF-α secretion by TH1 differentiated macrophages when treated with different doses of CDNs. THP1 monocytes were seeded into 24-well plates with a seeding density of 2 x 10^6 cells per well. They were treated with 200 ng/mL of Phorbol 12-myristate 13-acetate (PMA) (Sigma Aldrich, Cat. No. P1585-1MG) for 2 days to differentiate them into macrophages. This was followed by the removal of PMA and a rest period of 1 day in fresh media. The macrophages were treated with 42 ng, 420 ng, and 4200 ng of CDNs for 24 hours. The positive control group was treated with 1 µg/mL of lipopolysaccharides (LPS) from *E.coli* (Sigma Aldrich, Cat. No. L6529-1MG) [192]. TNF-α secretion by the macrophages was quantified using a Human TNF-alpha Quantikine ELISA Kit (R&D Biosystems, Cat. No. DTA00D) according to the manufacturer's protocol.

3.8 CDN internalization

A fluorescent lipophilic membrane dye, DiI (Sigma Aldrich, Cat. No. 42364), was used to stain the CDNs. 100 µL of CDN solution was added to 100 µL of dye solution (1:200 dilution of 1 mg/mL dye solution) and incubated for 1 hour at 37 °C. The sample was then centrifuged twice at 14,000 *g* for 10 minutes to remove free dye molecules. The labelled CDNs were resuspended in PBS. hADSCs were cultured in 24-well plates and treated with the stained CDNs for 6 hours at 37 °C. Cells were then washed twice with PBS and stained using Calcein AM stain (Fisher Scientific, Cat. No. C1430) for 20 minutes. After the removal of Calcein AM, the cells were washed, fixed for 5 minutes using 4% paraformaldehyde, stained using DAPI (Fisher Scientific, Cat. No. 5087410001) and mounted onto a coverslip. The cells were imaged using a Nikon Eclipse Ti2-E microscope (Nikon Instruments Inc., Canada), and image analysis was done using the Imaris Microscopy Image Analysis software (Oxford Instruments, USA).

3.9 Human adipose-derived stem cell differentiation using drug loaded CDNs

hADSCs were seeded onto 24-well plates with a seeding density of 0.1 x 10^6 cells per well, with 3 technical replicates. The cells were cultured until 100% confluency. The media was replaced with hADSC growth medium supplemented with 10 mM β-glycerophosphate (Sigma Aldrich, Cat. No. G9422-10G) and 50 µM L-ascorbic acid (Sigma Aldrich, Cat. No. A4544-100G), and this was used as the control group for all the differentiation experiments. Experimental groups were treated with CDN Dex (10 µM Dex), Dex (10 µM), or CDN (concentration corresponding to the amount of CDNs added to the CDN Dex groups) samples [193]. The media was replaced every 3-4 days.

3.10 Alkaline phosphatase staining

Alkaline phosphatase (ALP) activity was visualized using a BCIP®/NBT Liquid Substrate System (Sigma Aldrich, Cat. No. B1911-100ML) on Day 7 and Day 14. The cells were washed twice with PBS and fixed with 4% paraformaldehyde for 15 minutes. This was followed by 3 washes with Tris-buffered saline (Bio Rad, Cat. No. 1706435) with 0.1% Tween® 20 (Sigma Aldrich, Cat. No. P9416-100ML) detergent (TBST) to remove the excess fixative. The wells were then covered with the BCIP®/NBT Liquid

Substrate solution (Sigma Aldrich, Cat. No. B1911-100ML) and incubated for 30 minutes at room temperature. After colour development, the cells were washed 5 times with TBST and imaged using an Olympus IX2-ILL100 (Olympus Life Science Solutions, Canada) microscope.

3.11 Alizarin red S staining

Calcium deposits were visualized by staining with Alizarin red S (ScienCell, Cat. No. 8678) on Day 14 and Day 21. The cells were washed twice with PBS and fixed with 4% paraformaldehyde for 15 minutes. This was followed by 3 washes with distilled water. The wells were covered with the Alizarin red S and incubated for 45 minutes at room temperature. The stain was removed, the cells were washed 5 times with distilled water to remove the excess stain and imaged using an Olympus IX2-ILL100 (Olympus Life Science Solutions, Canada) microscope.

3.12 Reverse transcription quantitative polymerase chain reaction (RT-qPCR) analysis

qPCR analysis was done for hADSCs differentiated for 21 days. The cultured cells were washed twice with PBS before RNA isolation. RNA isolation was performed using the RNeasy mini kit (Qiagen, Cat. No. 74104) according to the manufacturer's protocol. Briefly, the cells were lysed using 350 µL of the lysis buffer, transferred to a 1.5 mL tube, and homogenized by vortexing for 30 seconds. 350 µL of 70% ethanol was added to the lysate and the solution was transferred to a RNeasy spin column placed in a 2 mL collection tube and centrifuged at 8000 *g* for 15 seconds leading to the binding of the RNA to the silica membrane in the spin column. This was followed by 3 centrifugation steps at 8000 *g* with wash buffers to wash away the contaminants. The RNA was finally eluted from the column in 50 µL RNase free water by centrifugation at 8000 g for 1 minute. The extracted RNA was quantified using NanoDrop (Thermo Scientific, Canada). One hundred and fifty nanograms of total RNA was used to synthesise cDNA using a High Capacity cDNA Reverse Transcription Kit (Thermo Fisher, Cat. No. 4368814). The reaction mixture consisted of 10 µL of RNA, 2 µL of RT Buffer, 0.8 µL of dNTP mix, 2 µL of RT Random Primers, 4.2 µL of nuclease-free water, and 1 µL of MultiScribe^{TM} Reverse Transcriptase. Reverse transcription was done in a Chromo4TM Real Time PCR Thermocycler (BioRad, Canada).

Amplification of the target late-stage markers of osteogenic differentiation was done using TB Green Advantage qPCR premix (Takara Bio, Cat. No. 639676). The RT-qPCR reaction mix was prepared using 2 µL of cDNA, 10 µL of TB Green, 0.4 µL of forward primers (See Table 3-1 for primer sequences), 0.4 µL of reverse primers, and 7.2 µL of RNase free water. The target genes were *RUNX2* [194], osteocalcin (*OCN*), and osteopontin (*OPN*), and the reference gene was *GAPDH* (housekeeping gene). The RT-qPCR reactions were carried out in a LightCycler® 96 System (Roche, Canada). Amplification conditions were as follows: 2 min of preincubation at 95 °C, and 45 cycles at 95 °C denaturation for 10 s, 55 °C annealing for 10 s, and 72 °C extension for 15 s. The quantification was carried out using the Pfaffl method [195] where *GAPDH* was the reference gene and the control group (basal media supplemented with β-glycerophosphate and L-ascorbic acid) was the calibrator.

Table 3-1: RT-qPCR forward and reverse primer sequences

Genes	Forward primer (5' → 3')	Reverse primer (5' → 3')	Tm (°C)
GAPDH	AAC AGC GAC ACC CAC TCC TC	CAT ACC AGG AAA TGA GCT TGA CAA	84
RUNX2	AGA TGA TGA CAC TGC CAC CTC TG	GGG ATG AAA TGC TTG GGA ACT	85
OCN	CAA AGG TGC AGC CTT TGT GTC	TCA CAG TCC GGA TTG AGC TCA	86
OPN	GTG CAG AGG AAA CCG AAG AG	TGT TTG CAG TGG TGG TTC TG	82

3.13 Statistical analysis

All the experimental data was calculated as mean ± standard deviation. The variations among the experimental groups were quantified using a student's t-test and one way ANOVA with Tukey post hoc comparisons. Values of $p<0.05$ were considered to be statistically significant (* = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$).

Chapter 4

4 Results and Discussion

4.1 Preparation of CDNs by cell membrane fragmentation and shearing

Firstly, CDNs were prepared by passing HEK293 cells through a series of filter membranes with smaller pore sizes used in each subsequent step (**Figure 4-1 A**). HEK293 cells show rapid growth, low batch to batch variation, and are emerging as an promosing cell line for a number of biomanufacturing processes [196,197]. While we use HEK293 cells for CDN preparation because of these advantages and ease of use, our preparation method can be applied to other cell lines or primary cells to obtain CDNs with similar physical characteristics [198,199]. After each centrifugation step using the 10 µm and 8 µm pore sized filters, the supernatant was discarded to remove the cytosolic content, including DNA, and the pellet was resuspended in fresh, ice-cold PBS. The pellet at the end of the 8 µm filter step was resuspended in PBS to prepare the unloaded CDN sample, or PBS with Dex to prepare the drug loaded CDN sample. At this point, the solution contained large fragments of cell membranes.

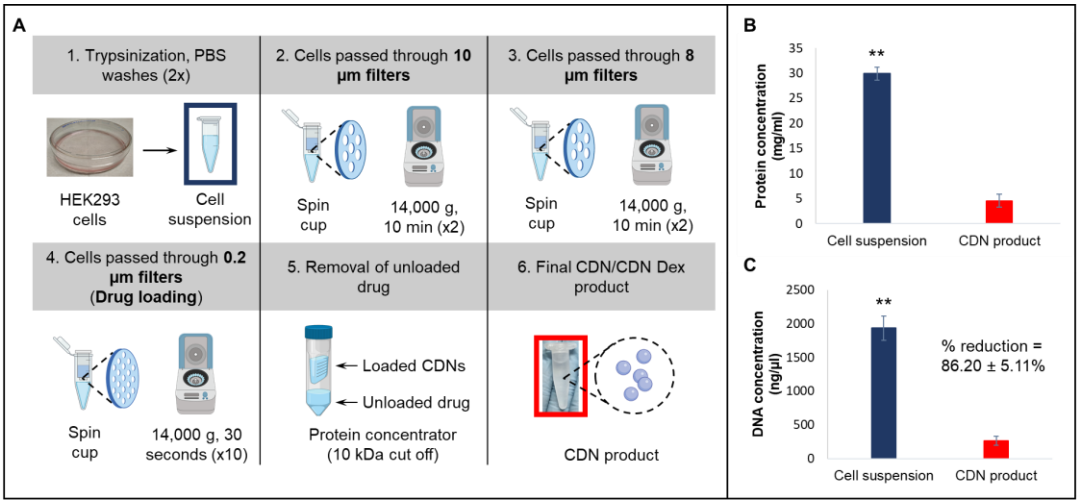


Figure 4-1. Preparation and characterization of CDNs. A) Illustration shows the steps involved with the preparation and drug loading of CDNs. B) Protein concentrations of the samples at the first and last stages of the preparation process were measured. The final protein concentration was found to be 4.59 ± 1.3 mg/mL. Results are shown as mean ± S.D. (n=3). C) Similarly, DNA concentrations were also measured. The final DNA concentration was found to be 263.07 ± 66.29 ng/µL, showing a reduction of 86.2 ± 5.11% compared to the first step. Results are shown as mean ± S.D. (n=3). (**=$p<0.01$)

The next step of passing the membranes through 0.2 µm filters was done to entrap the drug molecules within the CDNs and to further break the membrane fragments so that smaller vesicles could be formed. Cell membranes have the ability to self assemble spontaneously due to the presence of hydrophobic interactions [200] between the phospholipid groups, thus formed vesicles/particles which entrapped the drug molecules. These vesicles were then passed through a protein concentrator (10 kDa molecular weight cut off) to remove the unloaded Dex. The final protein concentration of the CDNs was found to be 4.59 ± 1.3 mg/mL (**Figure 4-1 B**). The final DNA concentration was found to be 263.07 ± 266.29 ng/µL and showed a reduction of 86.2 ± 5.11% compared to the lysate indicating the removal of a significant quantity of DNA using this method (**Figure 4-1 C**). Overall, this preparation method makes use of commonly available lab equipment and resources, making it easy to employ in an everyday lab setting [201].

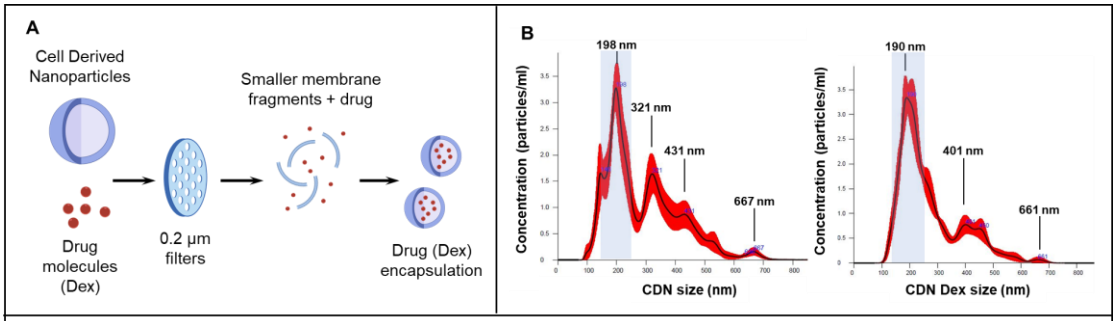
4.2 Characterization of CDNs and quantification of drug loading

The drug loading of the CDNs occurred when they were passed through the 0.2 µm filter membranes in the last step of the preparation process. The cell membrane fragments, and the subsequent particles formed from the previous steps were presumably larger in size due to passing through filters with pore sizes in the micron range. Upon passing through the 0.2 µm filter, the membranes were fragmented further, and the size of the particles was reduced (**Figure 4-2 A**). The small molecules of Dex were encapsulated within the particles by physical entrapment.

4.2.1 Determining the CDN size distribution

The drug loaded CDN Dex and the unloaded CDNs were characterized in terms of size using NanoSight NS300 based on nanoparticle tracking analysis. Individual samples (3 CDN Dex samples and 3 CDN samples) were prepared on different days and their sizes were analysed on Days 1, 4, and 7. The graphs generated with particle size vs particle concentration showed that there was a distribution of differently sized particles in each of the 6 samples tested, with graphs of the Day 1 samples shown in **Figure 4-2 B**. This confirmed that the CDN preparation process results in the formation of particles in the nanometer range. The maximum number of particles were between the size range of 180-230 nm, shown by the highest peaks in the graphs (**Figure 4-2 C**). The particle concentration ranged from 0.99×10^{10} to 5.95×10^{10} particles per 150 µL of the sample

(**Figure 4-2 C**) indicating that a high concentration of differently sized CDNs can be obtained. Furthermore, the size range of the CDNs from different days consistently fell between 20 - 730 nm (**Figure 4-2 D**). Studies have shown that nanoparticles with sizes between 20 – 200 nm are internalized effectively by cells using different endocytosis pathways [140,202].



C

	Day 1		Day 4		Day 7	
	Peak	Conc. (x 10^{10})	Peak	Conc. (x 10^{10})	Peak	Conc. (x 10^{10})
CDN	180	0.99 ± 0.09	200	1.05 ± 0.13	179	5.95 ± 0.09
CDN Dex	190	3.34 ± 0.07	233	1.00 ± 0.14	198	3.54 ± 0.29

Peak – highest peak (nm) **Conc.** – concentration of particles per 150 µL of sample

D

	Day 1	Day 4	Day 7
CDN (nm)	50 - 700	40 - 720	20 - 600
CDN Dex (nm)	80 - 700	40 - 720	50 - 730

Figure 4-2. Size characterization of CDNs using Nanoparticle Tracking Analysis. A) Schematic shows the process of drug (Dex) loading of CDNs using 0.2 µm filter membranes. B) Size vs concentration graph obtained using nanoparticle tracking analysis shows the distribution of nanoparticle sizes in Day 1 CDN and CDN Dex samples. A similar size distribution was seen for both samples. C) Table showing the size of the highest peaks and the concentration of particles per 150 µL of sample. CDN and CDN Dex samples were tested on Days 1, 4, and 7 (n=1). The size of the particles with the highest peaks were around 200 nm and a high concentration of particles was obtained for each sample. D) Table showing the size range of particles in in CDN and CDN Dex samples, which were all consistently between 20 – 730 nm.

While all of the particles obtained using this preparation method do not fall in this range, the majority of the particles (~30-40%) do, as seen by the highest intensity peaks, which would promote their internalization. In order to obtain a more homogeneously sized CDN sample, the particles can be purified using a Sephadex G50 size exclusion column which has been previously used to obtain particles of 200 nm size [198,201,203]. Furthermore, previous work has shown that increasing or decreasing the initial number of cells used for CDN preparation can have increase or decrease the size of the particles due to the change in the shear forces acting on them

when they are forced to pass through the pores on the filter membranes [198]. This, in combination with an appropriate size exclusion column, can be used as a method to vary the final size of the CDNs for different applications. Analysis of the sizes of 6 different samples prepared on different days, stored at 4 °C, showed that the size range of the CDNs remained constant over time (**Figure 4-2 D**). This agrees with previous work which showed that CDNs were stable and maintained their sizes for up to 3 weeks [201]. Hence, CDNs are stable in PBS indicating the possibility of manageable storage conditions in the event of large-scale production.

4.2.2 Characterization of CDN shape

The shape of the CDNs was confirmed by TEM and AFM. TEM of CDN and CDN Dex samples showed that the particles were uniformly spherical in shape and well dispersed (**Figure 4-3 A**). Similar results were seen in the AFM images (**Figure 4-3 B**).

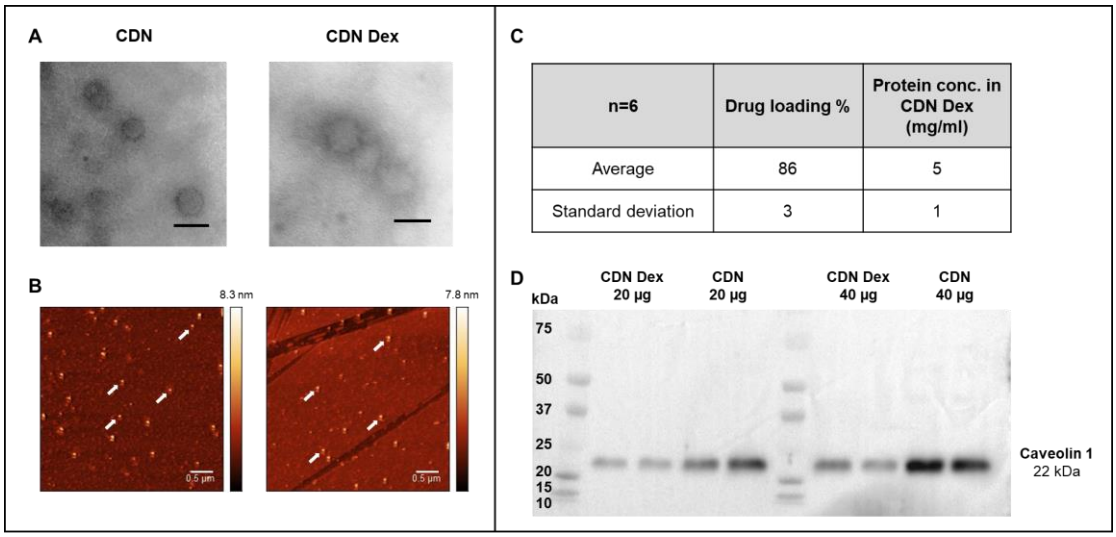


Figure 4-3. Imaging, quantification of drug loading, and membrane characterization of CDNs. A) TEM images showing the size and shape of CDN and CDN Dex samples. Particles were circular and appear to be dispersed. (Scale bar = 100 nm). B) AFM images showing the size and shape of CDN and CDN Dex samples. (Scale bar = 500 nm). C) Table showing the percentage of drug loading and the protein concentration in CDN Dex samples. Drug loading of 85.49 ± 2.95 % and protein concentration of 5.01 ± 0.92 mg/ml was obtained. Results are shown as mean ± S.D. (n=6). D) Western blot showing the presence of Caveolin-1, a membrane protein, bands at 22 kDa for different amounts of CDN and CDN Dex samples.

4.2.3 Quantification of drug loading of CDN Dex samples

The separation of the CDN Dex from the unloaded Dex in the solution was performed using a protein concentrator with a 10 kDa molecular weight cut off. The samples were loaded into the protein concentrator and centrifuged at 3000 rpm for 10 minutes. Dex molecules are small, with a size less than 1000 Da, and hence readily passed through the filter. Drug loaded CDNs, being larger, remained in the top portion of the protein concentrator. The solution that passed through was collected and diluted to measure the amount of Dex that remined unloaded. This was done using a UV-Vis Spectrophotometer by measuring absorbance at 242 nm (absorbance of Dex). This was then used to measure the amount of Dex that was loaded into the CDNs. It was found that an average of 85.49 ± 2.95% of the total Dex (2 mg) added to the solution was loaded into the CDNs as shown in **Figure 4-3 C**. Therefore, a high drug loading percentage was obtained indicating that CDNs can encapsulate the small molecule drug, Dex, with high loading efficiency.

4.2.4 Confirmation of the presence of membrane protein using western blotting

Cells express a number of proteins and receptors on their surface and on the cell membranes which are responsible for mediating the interaction of the cells with other cells and molecules in the microenvironment surrounding them and within them. When CDNs are prepared using the membrane fragmentation and cell shearing approach, these surface proteins and membrane proteins are carried over to the nanoparticles formed [198,201]. The presence of one such membrane protein, Caveolin-1, on the CDNs was determined using SDS-PAGE and western blotting. Caveolin-1 has a molecular weight of 22 kDa, and is the primary protein present on caveolae, which are invaginations present on the plasma membrane [204,205]. For the western blot, 20 µg and 40 µg of the CDN Dex and the CDN samples were used. From the blot obtained (**Figure 4-3 D**), clear bands can be seen at a molecular weight of 22 kDa indicating the presence of Caveolin-1 in both CDN Dex and CDN samples. The band intensity increased twofold when the concentration of the samples was doubled. However, the band intensity of the CDN Dex samples was found to be less than the band intensity of the CDN sample. This could be due to the high quantities of Dex present in the drug

loaded sample causing interference during the measurement of protein concentration, leading to reduction in the band intensity compared to the CDN group. This could be resolved by using a more direct method of quantification to measure the amount of Dex and protein in the sample. The presence of bands in all the wells this indicated that the preparation process and the presence of Dex did not have an effect the presence of Caveolin-1 on the cell membrane.

4.3 Studies to test the cytocompatibility and internalization of CDNs

4.3.1 Cell viability and cell proliferation studies with CDNs

The cytocompatibility of the CDNs *in vitro* was tested by treating hADSCs with different amounts of CDNs. The doses of CDNs used were 42 ng, 420 ng, and 4200 ng added to 200 µl of media in each 96 well. The amounts used correspond to the average amounts of CDNs used for the osteogenic differentiation of stem/stromal cells in the

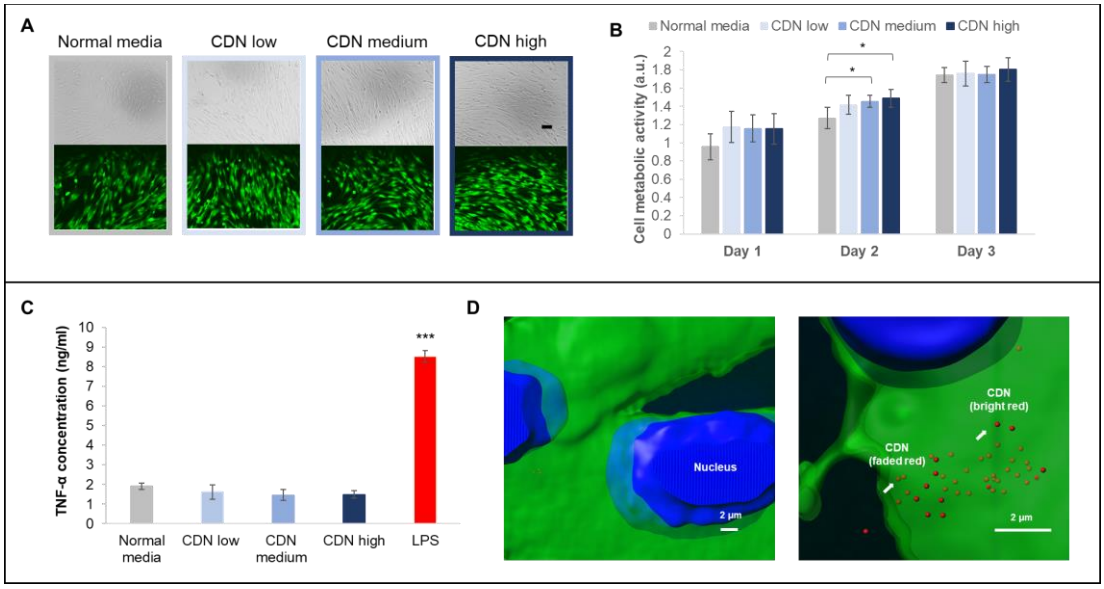


Figure 4-4. Cytocompatibility analysis and internalization of CDNs. A) Brightfield and fluorescence images of hADSCs treated with different concentrations of CDNs for 24 hours and stained with Calcein AM. Viable cells were observed in all the treated groups. (Scale bar = 100 µm). B) MTS assay showing the cell numbers of hADSCs treated with different doses of CDNs for 24, 48, and 72 hours. One way ANOVA analysis: No significant difference in cell metabolic activity between the groups was observed after 72 hours. Results are shown as mean ± S.D. (n=5). C) Cytokine assay quantifying the secretion of TNF-α by macrophages after treatment with different doses of CDNs for 24 hours. No significant difference in TNF-α secretion was observed in the experimental groups when compared to the negative control. Results are reported as mean ± S.D. (n=3). D) Internalization of CDNs by hADSCs. The image on the left does not have any CDNs and the image on the right has CDNs that are internalized (faded red) and CDNs that are not internalized (bright red). (Scale bars = 2 µm and 1 µm). (*=$p<0.05$, ***=$p<0.001$).

subsequent experiments. Cell viability of hADSCs treated with the 3 doses of CDNs for 24 hours were tested qualitatively using a live/dead stain to show the absence of cytotoxicity. The cells were stained using Calcein AM, which is a marker of cellular viability. Fluorescence and brightfield images taken after 24 hours show the presence of viable cells in the control and experimental groups (**Figure 4-4 A**), indicting the absence of intrinsic cytotoxicity of the CDNs.

The cytocompatibility was further confirmed by quantifying the cell metabolic activity observed in hADSCs after treatment with different doses of CDNs using an MTS assay. Three treatment periods were tested: 24 hours, 48 hours, and 72 hours. From the graph in **Figure 4-4 B**, it was seen that the control and all the experimental groups showed no significant difference in metabolic activity after 24 hours. A significant difference in absorbance was observed between the CDN medium-normal media and the CDN high-normal media groups after 48 hours, however, the cell proliferation normalized after 72 hours. The absence of significant difference in cell proliferation after 72 hours indicates that CDNs had no adverse or cytotoxic effects on the proliferation of hADSCs.

4.3.2 Analysis of inflammatory response in macrophages on treatment with CDNs

The presence of adverse inflammatory responses in THP-1-derived macrophages due to treatment with different doses of CDNs for 24 hours were tested using a cytokine ELISA assay. The doses of CDNs tested were 42 ng, 420 ng, and 4200 ng, as used in the cell viability and proliferation assays. The negative control used for this experiment was macrophages in basal media (no treatment) and the positive control was macrophages treated with LPS, which is known to induce the production of pro-inflammatory cytokines in macrophages. The presence of TNF-α, a pro-inflammatory cytokine produced in response to infection, in the supernatant after 24 hours was tested using a TNF-alpha Quantikine ELISA Kit [206]. It was found that the experimental groups treated with different doses of CDNs showed no significant difference in TNF-α production compared to the negative control (**Figure 4-4 C**), while the LPS group showed significantly high TNF-α concentration in the supernatant. This further proves that the CDNs are cytocompatible and do not induce the production of pro-inflammatory cytokines.

4.3.3 CDN staining and internalization

The internalization of the CDNs by hADSCs was tested by staining the CDNs with a lipophilic fluorescent membrane dye, DiI. hADSCs were cultured overnight and treated with stained CDNs for 6 hours. The cells were then stained using Calcein AM (green fluorescence). They were then fixed and stained with DAPI, washed, and mounted onto a coverslip. Fluorescent images were analysed using the Imaris software, which resolves the diffracted light from individual points, to visualize the presence of particles inside and outside the cells. The analysed image on the right in **Figure 4-4 D** shows the presence of bright and faded red spots, where the bright spots indicate particles outside the cell and the faded red spots indicate particles inside the cells. The image on the left does not have any red spots indicating the absence of CDNs. These images indicate that the CDNs were internalized by hADSCs. This is similar to results seen in literature that tested the uptake of CDNs synthesized from HEK293 cells using a similar method and found that they were capable of being internalized [199]. The ability to be internalized by cells is an important property of nanoparticles to enable efficient delivery of its payload. It can allow for the nanoparticle to deliver membrane impermeable drugs, hydrophobic drugs, and drugs with low bioavailability [198].

4.4 Alkaline phosphatase activity in hADSCs

After confirming the cytocompatibility of CDNs, we tested the biofunctionality of CDNs loaded with Dex in promoting the osteogenic differentiation of hADSCs. The first step of this process was to check for the presence of alkaline phosphatase (ALP) activity, which is an indicator that the fate of the hADSCs is progressing towards the osteoblast lineage (**Figure 4-5 A**).

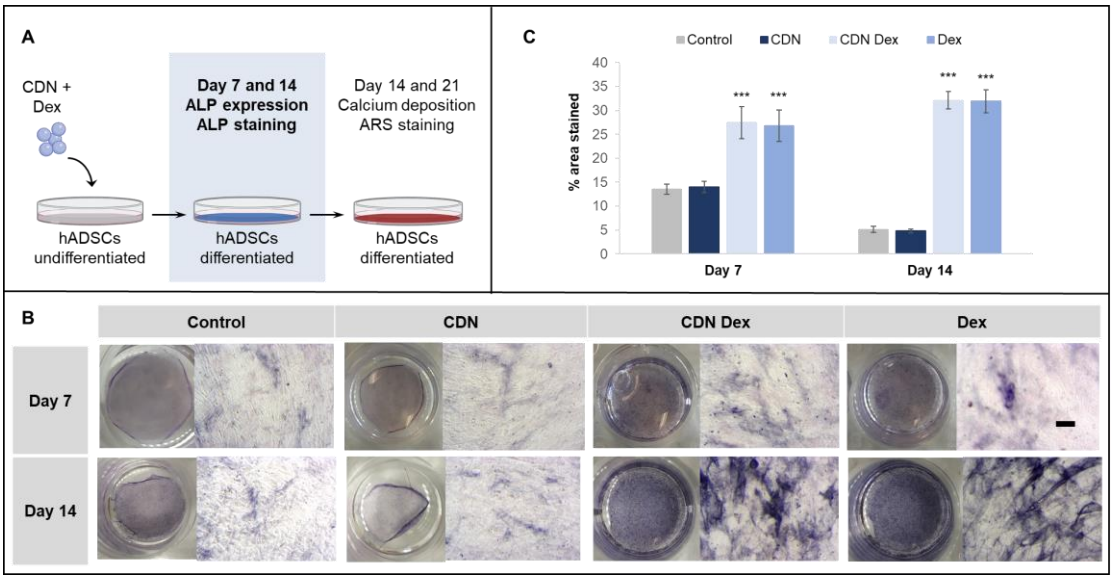


Figure 4-5. Determination of alkaline phosphatase activity in hADSCs treated with CDNs. A) Schematic showing the timeline for the differentiation of hADSCs and determination of ALP activity. B) ALP activity in hADSCs treated with CDN Dex (10 µM), Dex (10 µM), CDN, and control on Days 7 and 14. ALP activity was observed in CDN Dex and Dex groups on both days. (Scale bar = 100 µm). C) Quantification of ALP staining from the images using ImageJ. One way ANOVA analysis: CDN Dex and Dex groups showed significant increase in ALP activity compared to the CDN and control groups. The significant differences between the CDN Dex group and the control group are shown as ***=$p<0.001$ Results are shown as mean ± S.D. (n=3, technical replicates). (***=$p<0.001$).

ALP activity was checked on Day 7 and Day 14 using a chromogenic solution that forms a purple substrate in the presence of ALP as shown in **Figure 4-5 B**. It was seen that ALP expression was significantly higher in the CDN Dex and the Dex groups on both days when compared to the control and CDN groups. Moreover, there was no significant difference between the ALP activity in the CDN Dex and Dex groups indicating that the CDN Dex shows comparable activity to the positive control group (**Figure 4-5 C**).

4.5 Alizarin red staining to assess matrix mineralization

The presence of osteogenic differentiation was further confirmed by detecting the presence of calcium deposits, an indicator of matrix mineralization by osteoblast cells is occurring (**Figure 4-6 A**). This was done using alizarin red S (ARS) staining on Day 14 and Day 21. It was seen that both CDN Dex and Dex showed significantly higher mineral deposition compared to the control and CDN groups as shown in **Figure 4-6 B**. This trend was seen on both Day 7 and Day 14 indicating that the CDN Dex group consistently shows higher osteoinductive potential. Furthermore, between the CDN Dex and Dex groups, the CDN Dex group shows significantly higher mineralization (**Figure 4-6 C**). This is an indicator that CDN Dex could be performing better than the free Dex, showing that encapsulation within the CDNs can increase the efficiency of the drug molecule. This could be a result of the internalization of the particle, leading to the direct delivery of the drugs to the cells. Further analysis of the internalization pathways and markers is necessary to provide more evidence and insight to this observation.

4.6 qPCR analysis of osteogenic differentiation markers

The results of the ARS staining were further confirmed by running qPCR analysis to test the expression of 3 osteogenic genes: *RUNX2*, *OCN*, and *OPN* on Day 21. *RUNX2* was expressed in all the experimental groups on Day 21, with the CDN Dex group showing a significant increase in expression compared to the control group (**Figure 4-6 D**). Furthermore, there was no significant difference in expression levels between CDN Dex and Dex, and the CDN and control. This indicates that the CDN Dex and Dex groups are showing comparable osteoinductive activities. A similar trend was seen in the expression of *OPN* and *OCN* on Day 21, where there was a significant increase in the expression of these markers in the CDN Dex group when compared to the control

group. This further supports the previous results, confirming that CDN Dex is able to effectively deliver Dex and promote the osteogenic differentiation of hADSCs.

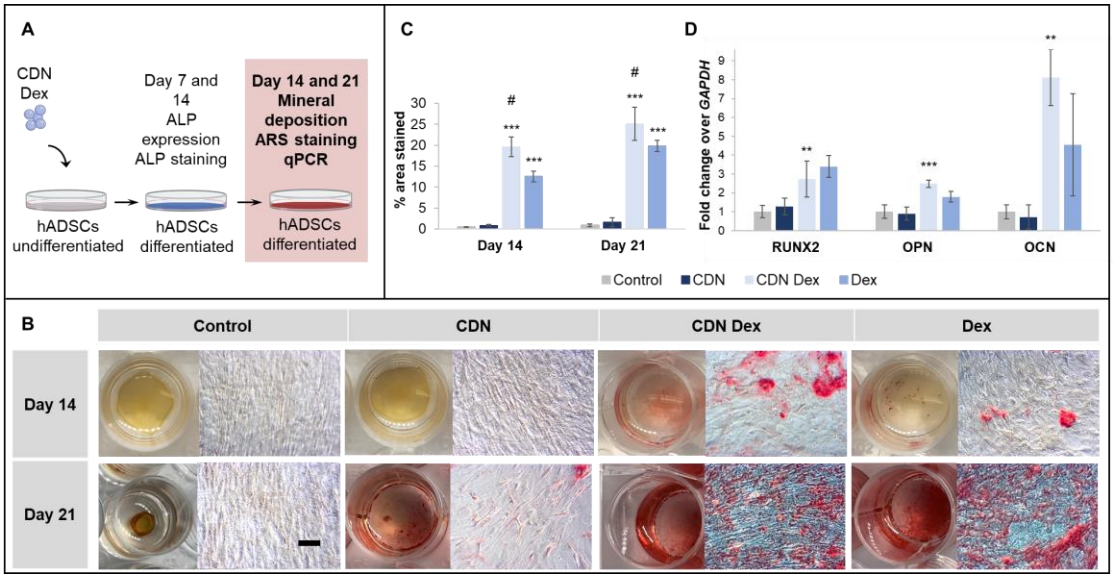


Figure 4-6. Determination of calcium deposition and expression of osteogenic genes in hADSCs treated with CDNs. A) Schematic showing the timeline for the differentiation of hADSCs, determination of mineral deposition, and osteogenic gene expression. B) ARS staining to detect calcium deposition in hADSCs treated with CDN Dex (10 µM), Dex (10 µM), CDN, and control on Days 14 and 21. Mineral (calcium) deposits were found in both CDN Dex and Dex groups on Days 14 and 21. (Scale bar = 100 µm). C) Quantification of ARS staining from the images using ImageJ. One way ANOVA analysis: CDN Dex and Dex groups showed significant increase in mineralization compared to the CDN and control groups. The significant differences between the CDN Dex group and the control group are shown as ***=$p<0.001$ and the significant differences between the CDN Dex and the Dex group are shown as #=$p<0.001$. Results are shown as mean ± S.D. (n=3). D) qPCR analysis to determine the expression of osteogenic markers *RUNX2*, *OPN*, and *OCN* on Day 21. CDN Dex showed significant increase in shown as mean ± S.D. (n=3). D) qPCR analysis to determine the expression of osteogenic markers. One way ANOVA analysis: CDN Dex showed significant increase in *RUNX2*, *OPN*, and *OCN* expression compared to the control group, whereas there was no significant difference in the expression of these markers between the CDN Dex and Dex groups. The significant differences between the CDN Dex group and the control group are shown as **=$p<0.01$ and ***=$p<0.001$. Results are reported as mean ± S.D. (n=3, technical replicates) (**=$p<0.01$, ***=$p<0.001$).

Chapter 5

5 Conclusions and Future Directions

5.1 Conclusions

In this project, we have developed a cytocompatible and biofunctional drug delivery system using cell-derived nanoparticles that could successfully deliver dexamethasone to promote the osteogenic differentiation of hADSCs. The prepared CDNs consistently had a distribution of sizes between 20 – 730 nm. The concentration of the particles in the samples were found to be high, in the order of 10^{10} particles per 150 µL of the CDN sample. A high percentage (~ 86%) of the DNA content of the cells were removed during the preparation process indicating the efficiency of the process in removing the cytosolic content. Both the CDN and CDN Dex particles had a spherical shape, as shown by AFM and TEM. The drug loading process, which involved passing the CDNs repeatedly through 0.2 µm filters, resulted in the formation of CDNs with high drug loading efficiency (~ 86%). This property is desirable in nanoparticle drug delivery vehicles as it allows the encapsulation of high dosage of drug molecules within a small number of particles, ensuring the least possible cytotoxic effects at the delivery site [207]. Additionally, the presence of a membrane marker, Caveolin-1, on the CDNs was tested using western blotting. The presence of clear bands of Caveolin-1 were obtained indicating that the preparation method preserved the proteins on the membrane.

Following the characterization of CDNs, *in vitro* analyses were conducted to test the cytocompatibility and biofunctionality of CDNs. Cell viability and cell metabolic activity analyses showed the absence of cytotoxicity in hADSCs due to CDNs. Furthermore, a cytokine assay testing the presence of TNF-α secretion (pro-inflammatory cytokine) showed the absence of TNF-α in the CDN experimental groups. This confirmed that CDNs had no cytotoxic effects. Biofunctionality studies involved the analysis of differentiation in hADSCs treated with CDN Dex. Results from ALP staining and ARS staining, on Days 7 and 14 and Days 14 and 21 respectively, showed that CDN Dex was able to induce comparable, if not better, osteogenic differentiation in hADSCs compared to free Dex. RT-qPCR analyses on Day 21 quantifying the expression of osteogenic markers showed similar results, confirming that CDN Dex was able to promote osteogenic differentiation in hADSCs.

It is important to note that the CDN preparation method of cell fragmentation and shearing employed in this project made use of common laboratory instruments and resources. This ensures that this method is accessible to the wider scientific community and can be reproduced easily by many research groups. Furthermore, unlike a majority of the previous work which use CDNs as a coating material for synthetic nanoparticles, this method involves the utilization of the vesicles alone to deliver drugs. This simplifies the synthesis process while still exploiting the full potential of cell membranes. The “nano” size, high loading efficiency, good stability, non-chemical preparation method, and highly non-cytotoxic nature of the CDNs offer unique advantages over synthetic nanoparticles. Taken together, these findings indicate that CDNs have excellent potential as efficient drug delivery vehicles.

While this project proves that CDNs can be used to successfully deliver a small molecule drug, further studies and optimization is required to overcome the current limitations of CDNs. While our studies show that there is a reduction in the DNA content by ~87%, the safe use of CDNs for a wide range on applications requires the complete removal of genetic material or proof of the absence of adverse reactions [208,209]. This will prevent the presence of undesired immunogenicity due to the CDNs. Additionally, we have confirmed the presence of one membrane protein on the CDNs. Further characterization of the surface and membrane proteins is necessary to understand the effects of the preparation method on the surface properties of CDNs. Likewise, understanding the internalization pathways of CDNs by hADSCs can give a better understanding of the effect of size on their internalization. Lastly, our project and other studies make use of CDNs to encapsulate drugs or other nanoparticles respectively by physical entrapment. A better understanding of the interactions of CDNs with different types of molecules with enable its use for the delivery of numerous therapeutics and drugs.

5.2 Outlook and Future directions

For future work, keeping in mind the limitations of CDNs from the previous section, DNases and RNases will be used during the preparation process to completely remove the genetic material. A more through characterization of pro-inflammatory cytokines and the activation of innate immunity will de performed to prove the absence of immunogenicity due to CDNs. The purification of CDNs based on size using a Sephadex column, a size exclusion chromatography method, would allow us to obtain CDNs of a specific size. This could be valuable when targeting internalization pathways of nanoparticles, or for applications that require particles of 20 – 200 nm. A more detailed characterization and understanding of the surface proteins on the CDNs can be obtained by flow cytometry or mass spectrometry, and this information could be utilized when targeting CDNs towards specific cells or molecules is necessary. Additionally, studies have shown that trypsin treatment during the subculturing step could lead to the cleavage of surface proteins [210,211]. To avoid this, a milder treatment to detach cells, the use of cell scraping to prevent the proteolytic cleavage of surface proteins, or the use of cells grown in suspension could be explored. In terms of drug loading, the quantity of drug loaded will be confirmed directly using more sensitive methods such as chromatography. Lastly, *in vivo* studies with CDNs prepared

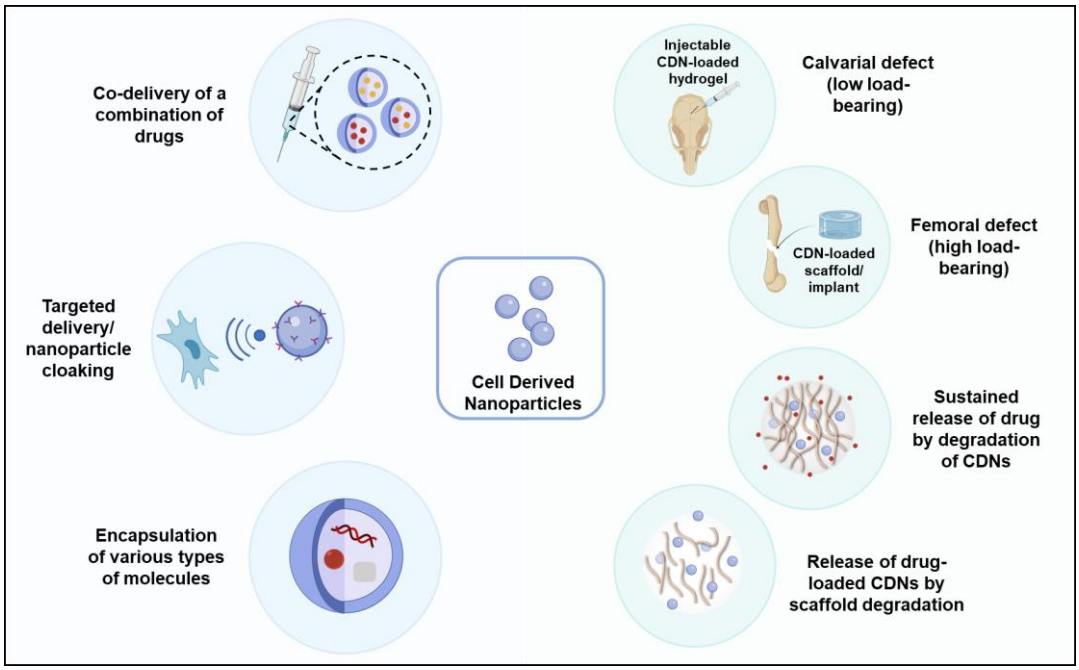


Figure 5-1. Future directions for the use of CDNs in drug delivery applications.

from HEK293 cells will be done to confirm the cytocompatibility and bioavailability of these CDNs.

In order to further investigate the use of CDNs for bone repair, the CDNs can be incorporated into a polymer network to form a scaffold material or coated onto implants (**Figure 5-1**). The mechanical properties of these scaffolds could be adjusted to suit the required application. For example, a scaffold with low stiffness can be used in low load bearing conditions such as the calvarial defect [212–214], whereas a scaffold with high stiffness and mechanical strength could be used in high load bearing conditions such as a femoral defect [215–218]. When CDNs are a part of a scaffold material, their release can occur through two ways: the scaffold material can degrade leading to the release of CDNs from the network leading to their internalization by cells, or the CDNs could break open within the intact scaffold leading to the sustained release of the encapsulated drugs. Both strategies have potential to improve the efficiency of drug delivery and could be investigated for bone repair applications. A recent trend in the combined use of biomaterials and nanoparticle delivery systems is to engineer a material that can sequentially deliver two osteoinductive factors [212,213]. CDNs could play a role in effectuating this process due to their potential to encapsulate a variety of molecules, and the simple drug loading process that could allow for the encapsulation of more than one drug/growth factor into the particles.

Different cell sources, such as stromal cells, can be explored for the preparation of CDNs for different applications. The differential expression of surface proteins on these cells could be used to target the prepared CDNs to specific cell types or biomolecules. The development of targeted nanoparticles is being widely explored to ensure that a majority of the therapeutic molecules reach the intended site, so as to avoid adverse off-target effects and to increase the efficiency of the therapeutic molecule [26,218–221]. Lastly, CDNs could be used to co-deliver more than one molecule, such as BMP2 and VEGF. This could be beneficial in a number of applications, including bone regeneration which often requires both osteogenesis and angiogenesis for complete repair of bone [214].

Collectively, these studies would enable the improvement and optimization of CDNs in encapsulating and delivering therapeutic molecules for the treatment of different diseases and disorders.

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