THE POTENTIAL OF PLASMA-GENERATED CULTURE SURFACES FOR STEM CELL-MEDIATED TISSUE REPAIR

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ABSTRACT

A major drawback of cartilage tissue engineering is that human mesenchymal stem cells (hMSCs) from osteoarthritic (OA) patients express high levels of type X collagen. Type X collagen is a marker of late stage chondrocyte hypertrophy, linked with endochondral ossification. It has been shown that a novel plasma-polymer, called nitrogen-rich plasma-polymerized ethylene (PPE:N), is able to inhibit type X collagen expression in committed MSCs. The aim of this study was to determine if the decreased expression of type X collagen, induced by PPE:N, is maintained when MSCs are transferred to pellet cultures, an arrangement of cells which mimics embryonic condensation of mesenchymal stem cells, which results in prehypertrophic chondrocytes. hMSCs were obtained from the bone marrow of donors undergoing total hip replacement for OA. hMSCs were cultured on polystyrene dishes and on two different PPE:N surfaces: high (H) and low (L) pressure deposition for 7 days. Cells were transferred for 7 additional days in serum free media in pellet culture. RNA was extracted using a standard TRIzol protocol. RNA was subjected to RT-PCR using primers specific for type I and X collagen. As observed in previous studies, type X collagen mRNA level was suppressed when cultured on both H- and L-PPE:N. HPPE:N was more effective in decreasing type X collagen expression than LPPE:N. Results also showed that the decreased type X collagen expression was maintained when cells were removed from the PPE:N surfaces and transferred to pellet cultures. Culturing hMSCs from OA patients on PPE:N surfaces and in pellet culture had however no effect on the level of type I collagen mRNA. The present study confirmed the potential of PPE:N surfaces in suppressing type X collagen expression in hMSCs from OA patients. More importantly, when these cells are transferred to pellet cultures, type X collagen suppression is maintained. These results show a promising future for tissue engineering using autologous hMSCs.

RÉSUMÉ

Un inconvénient majeur de l'ingénierie tissulaire du cartilage est dû aux niveaux de collagène de type X élevés exprimés dans les cellules souches mésenchymateuses (CSM) des patients arthritiques. Le collagène de type X est un marqueur de l'hypertrophie avancée des chondrocytes, qui est lié à l'ossification endochondrale. Il a été démontré que l'expression du collagène de type X par les CSM différenciées peut être inhibée en présence des polymères plasma enrichies d'azote (PPE:N, nitrogen-rich plasma-polymerized ethylene). Le but de cette étude était de déterminer si la diminution de l'expression du collagène de type X, induite par PPE:N serait maintenue lorsque les CSM sont transférée dans des cultures culots. Les dernières sont un arrangement de cellules mimant la condensation embryonique des CSM résultant ainsi en des chrondrocytes prehypertrophiques. Les CSM ont été obtenus à partir de la moelle osseuse des donneurs subissant une arthroplastie totale de la hanche. Les CSM ont été cultivées sur des boîtes de polystyrène, ainsi que sur deux surfaces de PPE:N différents; à haute (H) et à faible (L) pression de dépôt pendant 7 jours. Les cellules ont été transférées pour 7 jours supplémentaires dans des milieux sans sérum dans la culture culot. L'ARN a été extrait selon un protocole standard TRIzol. L'ARN a été soumis à la RT-PCR avec des amorces spécifiques pour les collagènes de type I et X. Tel qu'observé dans des études antérieures, la quantité de ARNm de collagène X été supprimée lorsque les CSM était cultivées sur les surfaces HPPE:N et LPPE:N. Le HPPE:N était plus efficace pour diminuer l'expression de collagène de type X que le LPPE:N. De plus, la diminution d'expression du collagène de type X a été maintenue bien que les cellules ont été retirées de la surface PPE:N et transférées à des cultures culots. Cependant, le niveau de ARNm du collagène de type I récupéré dans les patients atteints d'arthrose n'a pas été influencé par les surface PPE:N, ni par la culture culot. Cette étude a établie le potentiel des surfaces PPE:N en supprimant l'expression de collagène de type X par les CSM chez les personnes souffrant d'arthrose. Plus important encore, lorsque ces cellules sont transférées à des cultures culots, la suppression du collagène de type X est maintenue. Ces résultats montrent un

avenir prometteur pour l'ingénierie tissulaire en combinaison avec les CSM autologues.

PREFACE AND ACKNOWLEDGEMENTS

The successful completion of these experiments and analysis of data would not be possible without the intellectual contribution and guidance of Alain Petit, and Laura Epure. The contribution of time and effort in producing the various surfaces in which the experiments were carried out on as well as the characterization of the surfaces was done by Juan Carlos Ruiz Buico. Most techniques used in the experiments that were included in both manuscripts were demonstrated by Lara Ajemian. The completion of this thesis, as well as the courses and lab work required for the degree of Master of Science would not have been possible without the assistance, guidance and encouragement of Michael Grant.

The guidance and supervision for this research project was made possible by Drs Fackson Mwale, John Antoniou and Michel Wertheimer, who took the time to correct both manuscripts, and also aided in the discussion and interpretation of the results. Financial support for the candidate was provided by Dr John Antoniou. Other authors contributed their suggestions for the results and the discussion of both manuscripts.

CONTRIBUTION OF AUTHORS

This thesis has been composed of two manuscripts outlining research that has been done towards the Masters of Science degree. The first manuscript, written by the candidate is based on data obtained and analyzed by the first author (the candidate). The candidate was responsible for carrying out each experiment, and looking at type X collagen expression on various nitrogen rich surfaces. The second manuscript, written by the author and the candidate (as co author) is based on data that was collected and analyzed by the candidate.

ABBREVIATIONS

A = acetylene

AGG = aggrecan

Al = Aluminum

ALP = alkaline phosphatase

BMP = bone morphogenic protein

BOPP = Biaxially-Oriented Poly(Propylene)

BSP = bone sialoprotein

C = Carbon

C_2H_4 = Ethylene

COL I = type I collagen

COL X = Type X Collagen

DBD = Dielectric Barrier Discharge

DEPC = diethylpyrocarbonate

DMEM = Dulbecco's modified eagle medium

DNA = Deoxyribose Nucleic Acid

F = Fluorine

FE-SEM = Field-emission scanning electron- microscopy

FGF = fibroblast growth factor

H = High Pressure

H = Hydrogen

HV = High Voltage

ICAM-1 = intercellular adhesion molecule-1

IGF = insulin growth factor

IL-1β = interleukin-1-β

IVD = Intervertebral Disc

L = Low Pressure

M = methane

MSC =Mesenchymal Stem Cell

N = Nitrogen

N_2 = Nitrogen

NH_2 = Amine

NH_3 = Ammonia

O = Oxygen

OA = Osteoarthritis

OC = osteocalcin

PBS = phosphate-buffered saline

PCR = Polymerase Chain Reaction

PE:N = N-Coating "Polyethylene"

PET = Polyethylene Terephthalate

PP = Plasma Polymerization

PPARγ = peroxisome proliferators-activated receptor-γ

PPE:N = N-Rich Plasma-Polymerized Ethylene

PS = polystyrene

PTH = parathyroid hormone

RNA = Ribonicleic Acid

RT = Reverse Transcriptase

RT-PCR = Chain Reaction PCR

SCCM = Standard Cubic cm per Minute

slm = standard litres per minute

TFBA = (Trifluoromethyl) Benzaldehyde

TGF-β = transforming growth factor β

TiO_2 = titania

TNF-α = tumour necrosis factor-α

UV-PE:N = Vacuum-Ultraviolet Photo-Polymerization

VUV = Vacuum Ultraviolet

XPS = X-Ray Photoelectron Spectroscopy

SECTION 1: INTRODUCTION

A) Rational and Objectives

Mesenchymal stem cells (MSCs) are pluripotent progenitor cells with the ability to generate cartilage, bone, muscle, tendon, ligament and fat. However, recent evidence indicates that a major drawback of current cartilage- and intervertebral disc-tissue engineering is that human MSCs isolated from arthritic patients (a clinically relevant source of stem cells) express type X collagen (a marker of chondrocyte hypertrophy associated with endochondral ossification) and osteogenic markers. We recently addressed the possible effect of the chemical composition of the substratum on chondrocyte hypertrophy and osteogenesis. We examined the growth and differentiation potential of human MSCs cultured on extremely N-rich plasma polymer layers, which we call "PPE:N" (N-doped plasma-polymerized ethylene, containing up to 36% [N]). We showed that PPE:N almost completely suppressed the expression not only of type X collagen, but also of osteogenic marker genes such as alkaline phosphatase (ALP), bone sialoprotein (BSP) and osteocalcin (OC). In contrast, neither aggrecan nor type I collagen expression were significantly affected. In the present research project, we wanted to determine if MSCs pre-cultured on PPE:N coatings then transferred to pellet culture can maintain their suppression of type X collagen.

B) Review of the Literature

Articular Cartilage

Articular cartilage is a durable tissue structurally organized in a manner that allows it to function fully within the synovial joint, withstanding high levels of mechanical stress (Figure 1). Being a very metabolically active tissue, articular cartilage has the capacity to maintain its structural integrity, physical and mechanical competence as well as its response to load transmission and absorption [1,2].

Its large extracellular matrix is composed mainly of collagen fibers arranged in a dense network, as well as proteoglycans found in high concentrations [3]. Other matrix proteins

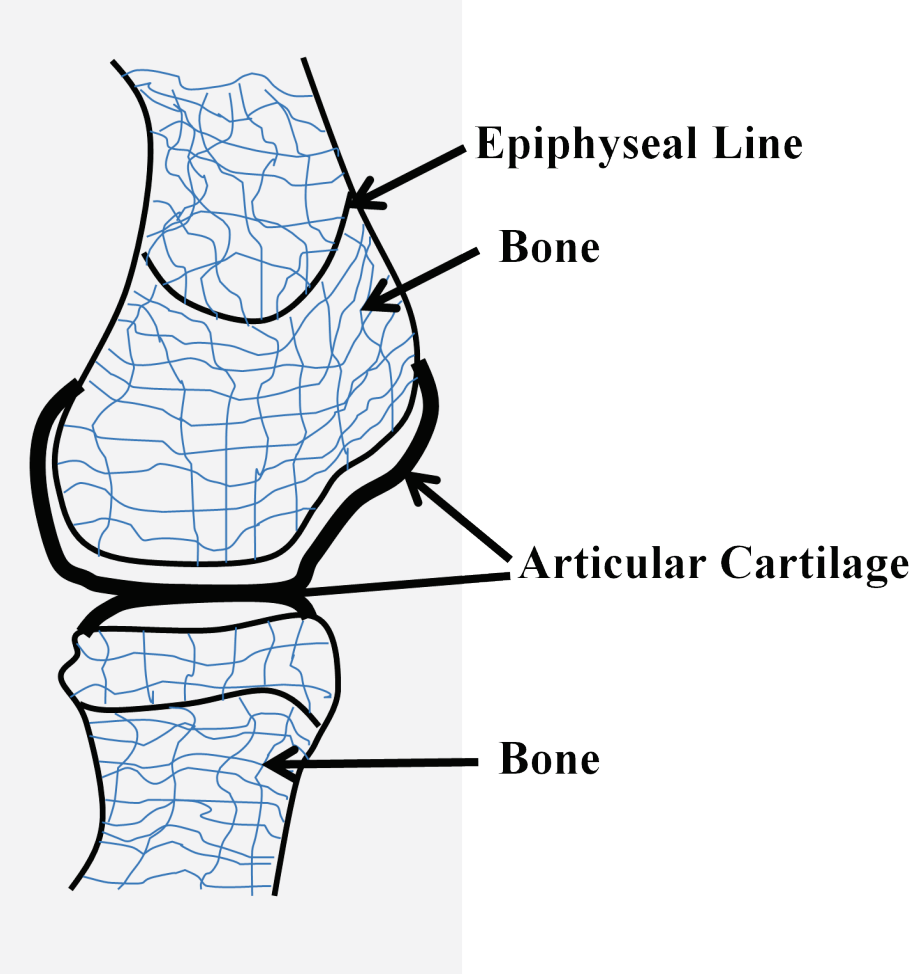

Figure 1: Articular Cartilage seen covering the ends of two long bones at the articulation.

are also found in articular cartilage such as fibromodulin and fibronectin [4]. Chondrocytes are found dispersed in the extracellular matrix. These chondrocytes synthesize the components and maintain the integrity of the matrix [5]. Within the matrix, collagen fibers help maintain the structure of the tissue, while

proteoglycans provide the tissue with its compressive properties, necessary for load bearing [6]. Attached to a protein core, these high molecular weight proteoglycans contain close to 100 chondrotin sulphate chains, and about 50 keratin sulphate chains [7]. Formed from glycosaminoglycan chains, containing many sulphate and carboxylate groups, these proteins found immobilized in the matrix in high concentrations draws water into the matrix, creating a large osmotic swelling pressure [8]. The quality of the collagen network, as well as the high concentration of proteoglycans within the matrix play an important role in the physical and mechanical properties of articular cartilage [9]. Articular cartilage is capable of modifying its structure to meet the functional demands of its environment, where *in vivo* moderate loading in a cyclic manor has been seen to stimulate proteoglycan synthesis [10].

On the other hand, immobilization, or reduced joint loading has been shown to lead to an increased loss and decreased synthesis of extracellular matrix macromolecules [11]. It is this halt in cartilage matrix protein turnover that may lead to the thinning and loss of articular cartilage, which ultimately leads to joint pain, decreased mobility, as well as morbidity [12]. Both reduced loading as well as over loading of joints can lead to the degradation of cartilage. Damage and ultimately degradation of cartilage can develop from chronic pathological incorrect joint loading patterns, such as joint instability or misalignment, as well as acute chronic high intensity loading, seen often by athletes and individuals often participating in high impact sports [13]. On the other hand, however, joints that have been immobilized for prolonged periods *in vivo* are also seen to undergo cartilage thinning, tissue softening, and reduced proteoglycan content [14]. Cartilage damage due to overuse or disuse is irreversible, due to the avascular nature of the tissue, which prevents the inflammatory response by damage tissue followed by its repair to occur in an untimely fashion [15].

Many techniques have been established in hopes of repairing damaged articular cartilage. Some methods of repair include changing the load on articular cartilage, by mechanically altering the joint, penetrating the subchondral plate, which

introduces traditional elements needed for the healing process, such as fibrin clot, blood and marrow cells, growth factors and cytokines. Vascular invasion also occurs. These are accomplished by various techniques such as; multiple drill holes, microfractures, spongialization and abrasion of a small section of the subchondral cortex [16]. Tissue transplantation of cartilage tissue and isolated chondrocytes is another therapeutic option for the repair of articular cartilage. Autologous tissue used for grafting is favorable, because this tissue will not confer an immunologic response. The risk of transmitting an infection is also quite low, and the long term viability of the tissue also seems to be better with an autograph than an allograft. One disadvantage of using autologous tissue is its limited supply [17]. When considering allografts, two main advantages can be put forth. First, tissue can be used from younger donors, and second, a graft taken from the exact location on the donor as the recipients' deficiency is a possibility, therefore a graft that is physiologically relevant, with the same thickness, contour and compliance can be used [18]. Not only are these methods used to treat cartilaginous lesions, due to wear and tear and trauma, but they are also implemented in other genetic and metabolic conditions that affect the joint, such as Paget's disease, acromegaly and osteoarthritis [19].

Osteoarthritis

Osteoarthritis is the most common form of arthritis, which affects millions of people in the United States alone [20]. It most commonly affects the hip and knee joints [21]. Osteoarthritis is a disease whose prevalence is age related [22]. Osteoarthritis is the most common cause of total knee and total hip replacement [23]. The differences in the disease state can be seen between men and women. For example, before the age of 50, osteoarthritis in joints is more prevalent in men, than women. After 50 years of age, women are more affected than men, with the disease found mostly in the hand, foot and knee [24,25].

Pathology and symptoms are used to define osteoarthritis. In pathological terms, the whole joint is affected, where hyaline articular cartilage is thinned [26], catalyzing changes in the bone underneath the cartilage which include the development of marginal outgrowths, osteophytes as well as an increased thickness of the bone envelope [27]. Other soft tissues, such as the synovium, ligaments and adjacent muscles are also affected, becoming either inflamed or weak [24,25].

Not only is whole tissue affected by the onset of osteoarthritis, but the change in the activity of the underlying cells, in particular, chondrocytes and MSCs are also affected by the disease process. The proliferative abilities, as well as osteogenic potential of MSCs are significantly reduced with the onset of osteoarthritis in older adults [28]. In addition, the chondrogenic, and adipogenic activity of MSCs from OA patients is significantly reduced, compared to that of normal individuals [28]. In addition, MSCs from patients with osteoarthritis express high levels of aggrecan [29], as well as type X collagen [30]. This phenomenon is unusual, because MSCs in the bone marrow do not have requirements for aggrecan, because in the bone marrow space, they do not need to resist compressive loading. Similarly, type X collagen is only expressed in chondrocytes prior to hypertrophy [29].

Type X collagen expression is found in the matrix of hypertrophic chondrocytes, which suggests that it may be important in endochondral bone development and growth [31]. Type X collagen has been associated with endochondral ossification, being expressed by terminally differentiating chondrocytes, namely, hypertrophic chondrocytes [32]. This short chain collagen is arranged as a homotrimer with each unit consisting of three α-chains separated by a 38 residue linker, followed by a 463 residue triple helix, and a highly conserved 161 residue non-collagenous domain at its carboxyl terminus [33]. This gene, as well as protein has been highly conserved throughout evolution [34]. The endogenous function of type X collagen is believed to aid in calcification, perhaps by inducing matrix reorganization. Type X collagen has been used as a marker of terminally

differentiated, hypertrophic chondrocytes. The presence of the type X collagen message is used to study chondrocyte growth and differentiation [32].

Mesenchymal Stem Cells

Until recently, it was believed that the only pluripotent cells capable of differentiating into ectodermal, endodermal and mesodermal tissue were embryonic stem cells [35]. It was known that adult stem cells were found in various tissues throughout the body, and the belief was that these stem cells served the purpose of replenishing and repairing its neighboring tissue [36]. MSCs, an adult stem cell found in the bone marrow, has been shown to be able to differentiate into many different mesodermal tissues, with their differentiation potential including and not limited to osteogenic, adipogenic, chondrogenic and neural differentiation [37]. In addition, other sources of stem cells, such as cord blood, and peripherial blood stem cell collections are not able to differentiate into MSCs in culture [38]. Therefore, for the purposes of tissue repair and tissue engineering, bone marrow derived MSCs are the best source of MSCs.

The differentiation potential of bone marrow derived MSCs contribute to the regeneration of mesenchymal tissue, such as bone, cartilage, muscle, ligament, tendon, adipose and stroma [39] (Figure 2). Under controlled *in vitro* conditions that favor adipogenic, chondrogenic and osteogenic conditions, MSCs are able to differentiate into the desired tissue, which consistently express respective marker proteins, and clearly showed distinguishable phenotypes among the three lineages [40].

MSCs appear to be the most attractive candidate for the purposes of regenerating injured tissue, not only because of their vast differentiation potential, but also because they can be easily isolated from the bone marrow, and can be expanded ex vivo, while still maintaining their multipotent characteristic [41]. When encased in tissue specific scaffolds, and implanted into respective tissue sites, MSCs are capable of differentiating into different phenotypes of the mesenchymal

lineage [42]. Because of this property of MSCs, the reformation of tissue has been vastly explored and employed. To date, there are at least three different modes of delivering MSCs embedded in scaffolds. The first method involves seeding the scaffold with MSCs *in vitro*. After a short incubation period, to endure cells have been embedded in the matrix, the cell-scaffold composite is implanted *in vivo*. A second method involves incubating the cell-scaffold composite in a differentiation medium, stimulating mesenchymal stem cell differentiation, prior to implantation. The third method involves implanting a naked scaffold, into the desired *in vivo* site, where cells are able to attach to docking sites on the scaffold [43].

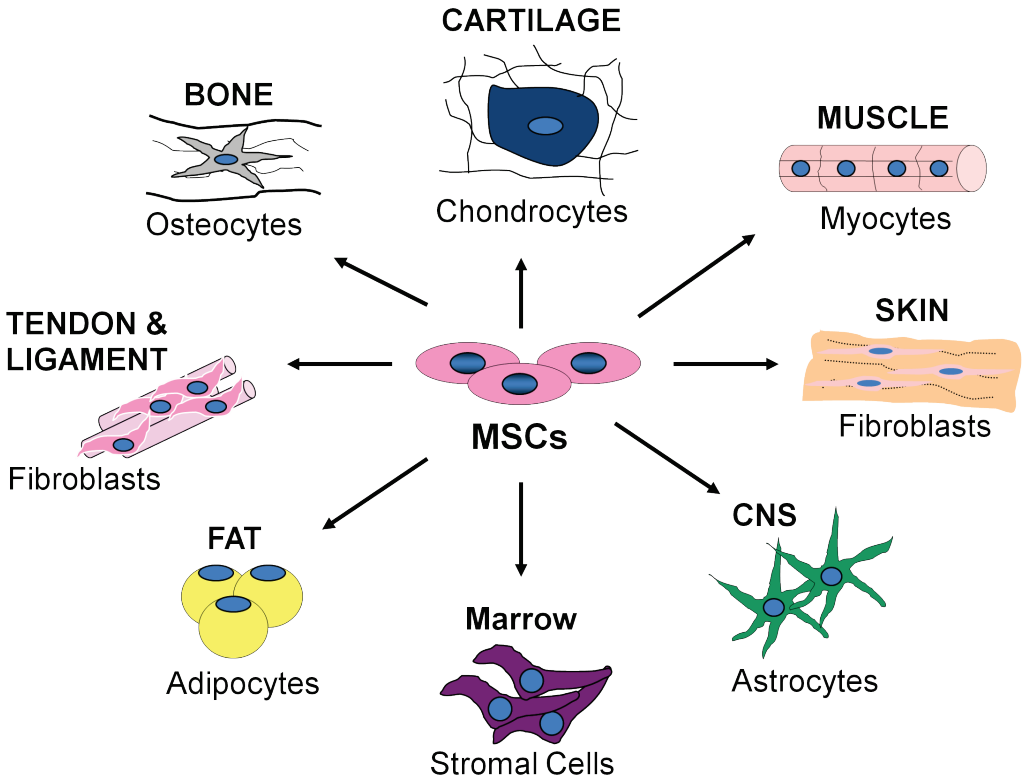

Figure 2: Differentiation potential of bone marrow derived MSCs.

In vitro Chondrogenesis

For undifferentiated stem cells to differentiate into a specific cell type, requires the cooperative effort of both the genomic potential of the cell, the intrinsic factor,

and information given from the local microenvironment, the extrinsic factor. These extrinsic factors include signaling molecules such as cytokines, hormones and growth factors, which regulate cellular metabolism and development in both an autocrine and paracrine fashion [44]. The most important growth factors required for *in vitro* chondrogenesis of MSCs are transforming growth factor β (TGF-β), insulin growth factor-1 (IGF-1) and bone morphogenic protein (BMP) [45]. When added to a basal media, some growth factors, such as TGF-β are able to activate sox9, the transcription factor involved in type II collagen and aggrecan expression, markers for chondrogenesis [46]. In addition, in collaboration with sox9, BMP is involved in the maintainance of chondrogenic differenciation [47]. IGF-1 has been suspected of being able to enhance the effect of TGF-β on chondrogenic differentiation, in addition to regulating cartilage differentiation [6]. Chondrogenesis induction also can be realized using a combination of growth factors, such as TGF-β3+ BMP6 or TGF-β3+ IGF-1, where in combination these growth factors promote a significant expression of sox 9 as well as the genes involved in the production of cartilage extracellular matrix [48]. It is speculated that BMP-6 and IGF-1 may synergize to increase the effects of TGF-β on the induction of MSCs to chondrogenesis [48]. Another key element required for chondrogenesis is the glucocorticoid, dexamethasone, which at different concentrations has been reported to influence both chondrogenesis and osteogenesis [49]. Another important requirement for *in vitro* chondrogenic induction is the microenvironment in which this process takes place. Chondrogenic differentiation is induced by culturing MSCs in a high density three dimensional system, which enhances cell to cell interactions, essential for chondrogenesis, which in fact mimic the processes involved in embryonic mesenchymal stem cell chondrogenesis [50]. It has also been reported that MSCs begin displaying chondrogenic properties when placed in a pellet culture [51].

Pellet culture systems were originally developed to prevent phenotypic regulation of chondrocytes. This method of cell culture involves the formation of an aggregate of cells, using a centrifugation method involving one step [52]. It has been demonstrated that when MSCs are cultured in a high glucose, serum free,

growth factor rich media, chondrocyte like extra cellular matrix proteins such as type II collagen and proteoglycan production is maximized [53]. The pellet culture system is merely an arrangement of cells that allows for the maximization of cell to cell interactions, which are similar to the processes undertaken during embryonic development, involving precartilage condensation of MSCs, ultimately leading to prehypertrophic chondrocytes [54].

Type X Collagen

Type X collagen is a short chain collagen composed of three identical subunits, which assemble into a hexagonal meshwork [55]. The transient expression of type X collagen is tightly controlled both spatially and in a developmental fashion. This expression is localized to the hypertrophic zone of the epiphyseal growth plate in long bones (Figure 3), ribs, and the vertebrates during endochondral ossification [56]. The precise function of type X collagen still remains a mystery, however, because of its integral association with areas of primary cartilage calcification as well as metaphyseal bone marrow invasion, it has been speculated that type X collagen may play an important role in endochondral ossification [57]. The gene for type X collagen is regulated at the transcriptional level [58]. Type X collagen has also been found in the extreme upper layer of the surface of articular cartilage in adult human, growing pig and fetal rat. It has also been found to be present in the pericellular region of chondrocytes making up the ‘tidemarks’ of adult human cartilage [59].

Type X collagen is also a marker for the pathology of osteoarthritis [56]. Whereas normal chondrocytes secrete large amounts of type II collagen, chondrocytes isolated from articular cartilage of patients with osteoarthritis showed increased synthesis of type X collagen, which may suggest that the metabolism of this collagen is severely altered by the disease process [60]. The altered expression of type X collagen also seems to be related to the severity of the disease [61]. Other

studies have shown that type X collagen is irregularly distributed around chondrocyte clusters in fibrillated OA cartilage, suggesting that premature chondrocytes may differentiate into hypertrophic chondrocytes in osteoarthritic cartilage [62].

Chondrocytes from osteoarthritic patients are not the only cells that express type X collagen, as a result of the disease pathology. It was found that MSCs from osteoarthritic patients, also express higher than usual levels of type X collagen [29]. More troublesome, however is that the expression of type X collagen also seems to be upregulated when MSCs are placed in pellet culture in the presence of standard chondrogenic media [29]. These findings pose many drawbacks in terms of tissue engineering of cartilage using autologous MSCs from osteoarthritic patients, whom would require a source of autologous stem cells for the biologic repair of articular cartilage. For example, the initial expression of type X collagen in MSCs is unfavorable, as type X collagen is a marker for chondrocyte hypertrophy [63].

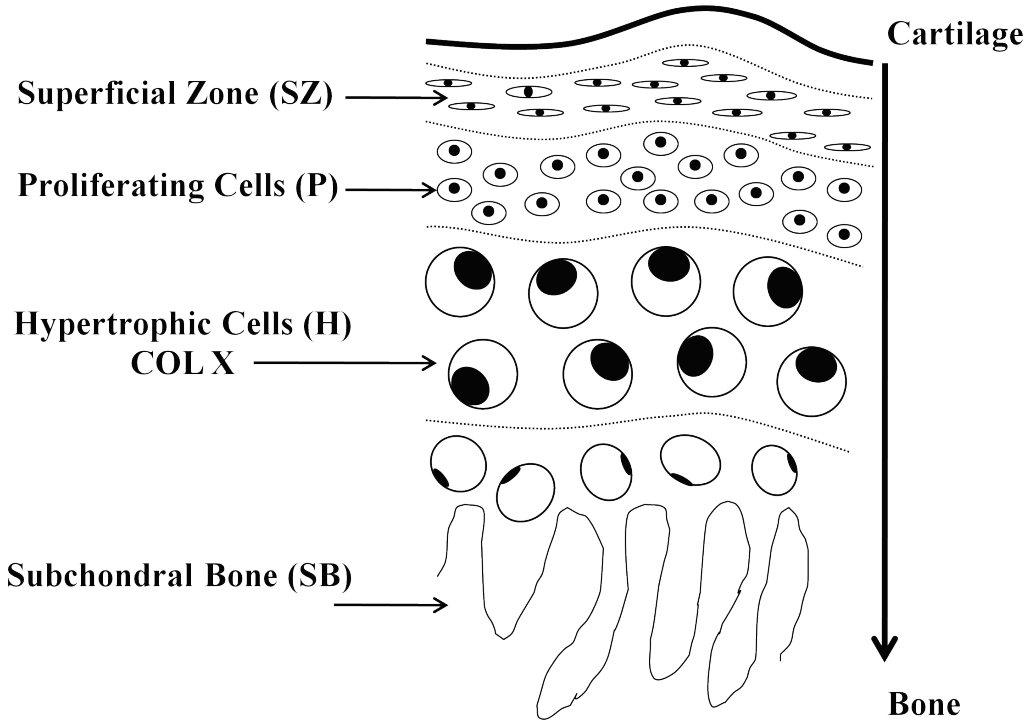

Figure 3: Diagram of the growth plate with its respective zones.

Nitrogen Rich Plasma Polymers

Cells are very sensitive to the surroundings which they reside in. Within the extracellular matrix lies the cell microenvironment, a structure which is highly defined and specialized which is critical for the correct development of tissue, as well as other processes a cell might undergo [64]. Many applications in cell biology, such as tissue engineering require the use of materials to help advance the formation of new tissues. This application can be performed *in vitro* and *in vivo* [65]. Tissue engineering relies heavily on the materials used to make up the scaffold or surface cells will reside on, and eventually differentiate [66,67]. It is these surface properties that must be tailored to fully maximize the favourable events that will take place at the cell-material interface. Cells are capable of detecting small differences in chemical properties of surfaces [68]. The chemical properties of the outermost functional groups of a surface as well as the charge of these functional groups can alter the orientation of anchoring proteins, which influence the binding of cells to a surface [66]. Research has shown that MSCs can be cultured on certain substrates and surface chemistries which enhance specific differentiation pathways, such as osteogenc and chondrogenic pathways. In addition, the density of cell adhesion is also critical in order for mesenchymal stem cell to differentiation into certain tissue types [69].

With the knowledge of surface chemistry and its potential to alter cellular processes, the manufacturing of synthetic polymers for such purposes has increased in recent years. Synthetic polymers with specific surface chemistries has been used in many fields of medicine and basic science, such as the production of cell culture plates [70], heart valves [71], hip joint prostheses [72], and drug delivery systems [73], only to name a very few. The modification of the surface chemistry is often done using electrical discharges, with two different methods. The first is grafting, where macromolecules in the near-surface region are modified with the addition of new functional groups. The second method involves the addition of an organic coating to an inert surface, also known as plasma polymerization. These newly created surfaces exhibit new physio-chemical

properties, such as chemical functionality, hydrophilicity, roughness and surface charge, which will determine its interactions within a given biological environment [74]. Surfaces with a functional group of interest are those that are rich in nitrogen. Nitrogen rich surfaces have been shown to increase cell adhesion [75], and to increase the differentiation of MSCs [76]. The positive charge of the primary amines is speculated to be the cause of these observed results. When in aqueous solution, under physiological pH, negatively charged biomolecules, such as proteins and DNA and living cells are attracted [74].

There are two methods available to produce a plasma polymerized surface. These plasma polymerized surfaces, coined PPE:N (N-rich plasma polymerized ethylene) can be produced at lower than atmospheric pressure, in a vacuum, and are called low-pressure polymerized ethylene (L-PPE:N), or can be produced at atmospheric pressure, and are called high-pressure polymerized ethylene (H-PPE:N). The surfaces produced by both methods not only vary in the method in which they are produced, but their chemical components are also somewhat different. When preparing L-PPE:N surfaces, the flow rate of ethylene C_2H_4 is kept constant at 20 standard cubic centimeters per minute (sccm), while the gas flow ratio (NH_3/C_2H_4) is varied from 0 to 3, by varying the flow rate of NH_3 between 0 and 60 sccm. In the preparation of H-PPE:N surfaces, the gas flow rate of N_2 is kept constant at 10 standard liters/minute (slm), while the ethleyene flow rate, C_2H_4 is varied from 5 to 60 sccm [77]. Despite the fact that there are many similarities between both H- and L-PPE:N surfaces, with respect to chemical composition, one advantage to preparing the H-PPE:N surface is that a larger surface can be used for plasma polymerized deposition [78].

These plasma polymerized surfaces are capable of inducing mesenchymal stem cell differentiation. In one study, the genes related to osteogenesis were affected when MSCs were cultured on certain plasma polymerized coated surfaces [76]. The adherence of MSCs was also affected positively by these surfaces. These results show that the differentiation of committed MSCs may be influenced by the physio-chemical properties of these surfaces, without using growth factors [76].

Therefore the prospect of using autologous MSCs for the purposes of tissue engineering may be possible, which will avoid the risk of immunocompatibilty issues.

For the purposes of engineering cartilage, for patients with osteoarthritis, autologous MSCs have been proposed as a starting material [79]. However, MSCs from these patients rapidly express type X collagen, a marker of chondrocyte hypertrophy, and endochondral ossification [80]. Decreasing the expression of type X collagen has been attempted using growth factors, as well as using plasma polymerized surfaces. In one study, when MSCs were cultured on three different H-PPE:N surfaces with different concentrations of bound nitrogen, were able to decrease the expression of type X collagen, as well as other osteogenic markers, such as alkaline phosphatise (ALP), bone sialoprotein (BSP), and osteocalcin (OC) in committed MSCs. These surfaces were selective, in that they did not alter the expression of other genes, such as aggrecan, or type I collagen, suggesting that these surfaces may be suitable for differentiating MSCs to a cartilage like phenotype [81].

SECTION 2: MANUSCRIPTS

A) Amine-Rich Cell-Culture Surfaces for Research in Orthopaedic Medicine

The physio-chemical properties of synthetic polymers that have been grafted, or plasma polymerized with an organic coating, are believed to influence favorable cell-surface interactions. Nitrogen rich coatings in particular were previously shown to regulate the phenotype of nucleus pulposis cells, as well as the phenotypic profile of notochordal cells. We were interested in investigating the potential of different plasma polymerized surfaces to increase cell adhesion of U937 monocytes, as well as mesenchymal stem cell differentiation. In terms of U937 monocyte adhesion to the surface, a critical value of [NH_2], deposited into a surface was determined to be necessary for cell adhesion. However, when considering mesenchymal stem cell gene expression on these surfaces, it was found that out of two nitrogen rich plasma-polymerized surfaces tested, the one with a lower concentration of nitrogen was more suitable for the suppression of type X collagen gene expression. It is speculated that this observed phenomenon could be due to the drastically differing surface textures of both surfaces examined.

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Amine-Rich Cell-Culture Surfaces for Research in Orthopaedic Medicine

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Abstract

We report results of ongoing research relating to cell-immobilisation and -culture on bio-active organic coatings deposited using three different techniques, two plasma-assisted, the third based on vacuum-ultraviolet photo-polymerisation. We start by briefly comparing those three methods and by describing some of the key characteristics of the resulting coatings; all are designed to be rich in primary amines, a functional group known to be highly bio-active. Next, we focus on two cell-types of importance to long-term objectives in our orthopaedic research laboratory: (i) the first, non-adherent human U937 monocytes, a cell line that has been widely used as a model of the mammalian cellular response to various inflammatory stimuli, and in understanding the clinical relevance of elevated cobalt and chromium levels in patients with metal-on-metal total hip arthroplasty and hip resurfacing arthroplasty. (ii) The second cell-type, adherent human mesenchymal stem cells (MSCs), derived from patients suffering from osteoarthritis (OA), are important in biological repair of cartilage and of the degenerate intervertebral disc (IVD) from the patients' autologous cells. Much progress has been achieved in both cases, as illustrated with results based to a considerable extent on real-time Reverse-Transcription (RT) Polymerase Chain Reaction (PCR), a key methodology used in this type of research.

1. Introduction

In recent years, plasma technologies have been extensively used to alter the surface properties of synthetic polymers, so as to elicit desired responses toward biological environments [1-3]. Those studies comprised either surface functionalisation (so-called grafting) [1-6], or plasma polymerisation (PP), which involves adding a thin organic coating on top of the original surface [1-3, 6-11]. Physico-chemical properties of both types of newly-created surfaces, namely chemical functionality, surface energy, roughness, surface charge, etc., are believed to govern their interactions with a given biological environment. Henceforth, we shall focus exclusively on thin film coatings. An important class of functional coatings are nitrogen (N)-rich ones [1,2,6-16], which have been found to promote cell adhesion [6,7,12-16], and even to influence differentiation of human mesenchymal stem cells (MSCs) [14,15], topics to be addressed in the present article. This is presumed to be due to the presence of primary amine groups, C-NH_2, and their associated positive charges that may, in aqueous solutions at physiological pH values, attract negatively-charged bio-molecules (proteins, DNA) and thus, directly or indirectly, living cells [1-3,6,12-16]. Moreover, such amino groups are chemically reactive, and they are used in biochemistry for covalent coupling of proteins in aqueous environments [1-3,6]. In the category of graft-modified polymer surfaces, commercial polystyrene culture dishes are generally pre-treated in atmospheric air plasma, and they therefore comprise oxygen (O)-based functionalities with typical concentrations, [O], in the vicinity of 18 atomic % (at. %).

If one elects to work with an aminated organic coating (e.g. a plasma polymer), the deposit must have a sufficiently high density of primary / secondary amines to become positively charged in aqueous media, and to enable imine and enamine coupling [11]. In addition, such a surface layer must be stable in the aqueous media routinely used for cell culture. These two requirements are frequently in conflict, and numerous earlier attempts to increase the density of amine groups, [-NH_2], by lowering average plasma power led to water-soluble films [1,6,17].

Well-known routes for obtaining coatings with high surface concentrations of nitrogen, [N], are plasma polymerisation of suitable N-containing precursors (e.g. allylamine, AA) [1,2,6,9-11,17-21], or the mixture of hydrocarbon and N-containing gases [7,8,22-27]. In a recently-published article [28], we focused on fabrication of "PE:N" (N-containing "polyethylene") films from binary feed-gas mixtures comprising ethylene (C_2H_4) and N_2 or ammonia (NH_3), leading to the most stable deposits. More precisely, we examined two kinds of "PPE:N" (N-rich plasma-polymerised ethylene), namely those obtained either by conventional low-pressure ("L") radio-frequency (r.f.) glow discharge, hereafter designated "L-PPE:N" [25,29], or in a dielectric barrier discharge (DBD) reactor operating at atmospheric ("H", for "high") pressure (hereafter "H-PPE:N") [7,8,29]. A third category of coatings, designated "UV-PE:N", results from vacuum-ultraviolet (VUV) photo-polymerisation reactions in the same gas mixtures as those used for producing the "L-PPE:N" films. UV-PE:N films are also prepared at low pressures, but in a reactor dedicated for VUV-photochemical research, mostly using a commercial Kr lamp emitting monochromatic radiation at 123.6 nm [26]. L-PPE:N and UV-PE:N films are *a priori* more costly to produce on account of the more expensive vacuum processing and -equipment and, in the photo-chemical case, expensive light source. However, in high value-added processes, for example those involving bio-materials and substrates for tissue engineering, production costs are not necessarily the dominating consideration, usually less so than performance characteristics of the types that are the focus of investigations described hereunder.

As already briefly mentioned above, we shall address two important applications of PE:N coatings in the remainder of this text, namely:

Adhesion of U937 monocytes [7,13];

Regeneration of cartilage via the use of mesenchymal stem cells (MSC) from osteoarthritic (OA) patients [14,15].

Both of these topics are of very great interest within our orthopaedics research program, but for quite different reasons and within different contexts: In the former case, the blood-borne human U937 cells, precursors for the formation of macrophages, were non-adhering to all then-known culture surfaces prior to investigations in these laboratories. This had been a distinct obstacle when pursuing research into the mechanisms of periprosthetic osteolysis, a loosening of artificial hip-implants due to apoptosis or necrosis of osteoblasts [30,31]. Thanks to their adherence to PE:N under certain conditions described later in this text, that obstacle has now been largely overcome.

Regarding the second topic listed above, several studies have been directed toward using MSCs from OA patients for the repair of cartilage and of the degenerate intervertebral disc (IVD), not only because these are the patients that will require a source of autologous stem cells if biological repair of cartilage or IVD lesions is to be a therapeutic option, but also to help further understanding of stem cell differentiation. However, recent evidence indicates that a major drawback of current cartilage or IVD tissue-engineering is that human MSCs from these patients express type X collagen (COL X), [14,15,32] a marker of chondrocyte hypertrophy associated with endochondral ossification. [33,34]

We have earlier published (or submitted) many of our important findings regarding both (i) and (ii) above; however, in the present article we present hitherto unreported views regarding the likely mechanisms underlying the adhesion and colonisation of these two cell-types of importance in orthopaedic medicine, when they are cultured on PE:N surfaces.

2. Experimental Methodology

2.1. Deposition of PE:N Films

PE:N films were deposited in the three reactor systems depicted in Figure 1, on two types of polymer substrates, namely biaxially-oriented poly(propylene) (BOPP, 50 μm thick, isotactic polymer film, graciously provided by 3M Company), and 50 μm thick PET film obtained from Goodfellow Corp. (Oakdale PA). Surface analyses (see below) were conducted to verify that the deposition rates and chemical compositions of PE:N deposits were independent of their host surfaces.

The L-PPE:N coatings were deposited in a cylindrical aluminium / steel vacuum chamber, approximately 20 cm in diameter and 20 cm in height, Figure 1a. [25,29] Flows of high-purity feed gases (ethylene 99.5% and ammonia 99.99%, Air Liquide Canada, Montreal, QC) were admitted into the chamber using electronic flow meter/controllers (Vacuum General Inc., San Diego, CA), and a "shower head" gas distributor (10 cm in diameter). While the flow rate of C_2H_4 "monomer", F_{C2H4}, was kept constant at 20 standard cubic centimeters per minute (sccm), its NH_3 counterpart, $F_{NH3,}$ could be varied between 0 and 60 sccm. [25] Based on our previous experience, we chose to deposit L-PPE:N coatings using rather mild plasma conditions (P = 10 W, resulting in a negative d.c. bias voltage, V_B =-40 V); under these conditions, polymer-like films with maximum nitrogen and amine concentrations were deposited. [25,29] Based on previous work relating to stability of the deposits in air and water, [28] we chose in the present studies to prepare particular coatings with F_{NH3} = 15 and 20 sccm, that is, with ratios R= F_{NH3}/ F_{C2H4} of 0.75 and 1, respectively (hereafter identified as L0.75 and L1). Indeed, unless otherwise specified, all of the data relating to experiments with MSCs were carried out with the L0.75 coating.

For the case of H-PPE:N, deposition was carried out in a DBD reactor, as also described elsewhere. [7,8,28,29] Briefly, this system (Figure 1b) comprised a cylindrical, dielectric-coated stainless steel high-voltage (HV) electrode, and a

horizontal, grounded planar aluminum (Al) electrode, covered by a 2 mm-thick glass plate that served as a second dielectric layer. The precursor gas mixture, $X = F_{N2}/F_{C2H4}$, composed of pure nitrogen (N_2) and ethylene (C_2H_4), was introduced into the discharge zone, an adjustable gap (usually 1 mm) between the HV electrode and the glass plate. The N_2 flow rate was fixed at 10 standard liters per minute (slm); as mentioned above, deposits were made using C_2H_4 flow rates of 20 or 50 sccm for reasons of their good to superior physicochemical stability (hereafter identified as H20 and H50). [28] Again, unless otherwise specified, MSC-related experiments were carried out with the H50 coating.

The third experimental set-up, used for VUV photochemical experiments (see Figure 1c) has also already been described in detail elsewhere [26]. Briefly, it consists of a stainless steel "cross" chamber, which was first evacuated to a base pressure of about 5×10^{-6} torr (~7×10^{-4} Pa) using a combination of turbo-molecular and rotary vacuum pumps. In the present VUV photo-polymerisation experiments with 123.6 nm monochromatic radiation, we irradiated flows of binary gas mixtures comprising C_2H_4 and NH_3 at low pressure, typically 100 mTorr (13.3 Pa), similar to those used for depositing L-PPE:N, described above. The flow rate of ethylene, F_{C2H4}, was kept constant at 50 sccm, while that of ammonia, F_{NH3}, could be varied between 0 and 75 sccm, yielding values of their ratio, R ($\equiv F_{NH3}/F_{C2H4}$), between 0 and 1.5. Also based on previous work relating to stability of the deposits in air and water, [28] we chose in the present study to prepare particular coatings with values of R ranging from $R = F_{NH3}/F_{C2H4}$ of 0.4 to 1, respectively.

For all coating processes, deposition durations were selected so as to create approximately 100 nm-thick films. Elemental compositions of PE:N samples and their primary amine concentrations were determined by X-ray photoelectron spectroscopy (XPS) in a VG ESCALAB 3MkII instrument, using non-monochromatic Mg Kα radiation, as described in detail elsewhere. [7,8,25,26,28,35] The surface-near concentrations of primary amines, [-NH_2], were determined after chemical derivatisation with 4-

(trifluoromethyl)benzaldehyde (TFBA, Alfa Aesar, Ward Hill, MA) vapour. With this method, [$-NH_2$] values can be deduced accurately from the measured fluorine concentrations, [F], also by XPS, [25,28,29]. As reported previously, values of [N] and [$-NH_2$] increase significantly with increasing NH_3/C_2H_4 or N_2/C_2H_4 ratios.

In certain experiments involving the U937 cells, micro-patterned arrays of PE:N islands were deposited on BOPP surfaces that were partially masked with 50 μm-thick Kapton™ polyimide films. These masks comprised square arrays (200 μm pitch) of small (ca. 50 μm diameter) holes that had been eroded through the Kapton with the help of a powerful KrF excimer laser [7].

2.2. Field-emission scanning electron microscopy (FE-SEM).

Selected coatings were examined by field-emission scanning electron-microscopy (FE-SEM) using a JEOL model "JSM-7600 TFE" instrument (JEOL Ltd., Tokyo, Japan). For this purpose, relatively thick (≥ 0.5 μm) H-PPE:N and L-PPE:N films were deposited on glass and on silicon wafers, respectively, in order to allow small (~ 1 cm^2) representative pieces to be fractured in ambient air, then introduced into the microscope's sample-holder. The purpose was to examine both top- and side-views of their micro-morphologies [28], but only top-views are presented and discussed here.

2.3. Cell culture and real-time RT-PCR

2.3.1. U937 Monocytes

Non-adherent human U937 monocytes (ATCC, Rockville, MD) were expanded in suspension in Dulbecco's modified eagle medium (DMEM) high glucose supplemented with 10% foetal bovine serum (FBS; HyClone, Logan, UT), 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were counted with a

hemacytometer; 100 µl volumes of cell suspension (5 x 10^5 cells) were carefully pipetted onto 1 cm^2 PE:N surfaces of varying [N] values, which had previously been placed face-up on the flat bottoms of wells in a 24-well cell-culture plate. Cells were then left in contact with the substrate surfaces in humidified air enriched with 5% CO_2 at 37°C for 1h before careful removal of the medium. We had previously demonstrated that the maximal adhesion of U937 cells to PE:N surfaces was reached within 1h. [7,13] Non-adherent cells were then removed rapidly to avoid their interaction with adherent ones that might modify cell-surface interactions and gene expression. Following this, fresh medium was pipetted into the wells (1 ml in each well) and again carefully removed to wash the cells. This washing procedure was repeated twice. All of the adhesion experiments were performed at the same time with the same cell suspension, to avoid variations due to the cells.

Real-time Reverse-Transcription (RT) Polymerase Chain Reaction PCR (RT-PCR) experiments were carried out for the case of adhering cells as follows: Cells were incubated for durations ranging from 3 to 24h on PE:N-coated BOPP, following which the total RNA was extracted with TRIzol reagent (Invitrogen, Burlington, ON). All of the RT-PCR experiments were performed at the same time with the same cell suspension, to avoid variations due to the cells. After centrifugation for 15 min at 12,000 x g at 4°C, the aqueous phase was precipitated in 1 volume of isopropanol, incubated for 45 min at room temperature, and centrifuged again for 15 min at 12,000 x g at 4°C. The resulting RNA pellet was washed with 75% ethanol, centrifuged, and air-dried. The pellets were suspended in 50 µl of diethylpyrocarbonate (DEPC)-treated water and assayed for RNA concentration and purity by measuring A_{260}/A_{280}. The reverse transcription (RT) reaction was performed using 1 µg total RNA after DNase I digestion according to the Invitrogen protocol. In a total volume of 20 µl, the reaction solution contained 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 50 µM each of dATP, dGTP, dCTP and dTTP, and 200 units of Superscript II - RNase H^- reverse transcriptase (Invitrogen). The expressions of tumour necrosis

factor-α (TNF-α), interleukin-1-β (IL-1β), peroxisome proliferators-activated receptor-γ (PPARγ), intercellular adhesion molecule-1 (ICAM-1), and transcription factors Erg-1 and PU.1 were measured using a Roche Diagnostics (Laval, QC) LightCycler™, as described elsewhere [13]. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as housekeeping gene. U937 cells in suspension served as a control. Salt-free primers for the target genes (TNF-α, IL-1β, PPARγ, ICAM-I, Egr-1, PU.1) and for the GAPDH housekeeping gene were generated by Invitrogen; their sequences are presented elsewhere, along with other procedural details [13]. In the present article, we shall report data pertaining only to TNF-α, IL-1β.

2.3.2. Human MSCs

Human MSCs were obtained from 10 to 25 ml bone marrow aspirates from the femoral intramedullary canal of donors undergoing total hip replacement using a protocol approved by the Research Ethics Committee of the Jewish General Hospital. [14,15,32] The marrow donors included both males and females ranging in age from 52 to 88 years (mean, 65 years). Isolation of MSCs was carried out using methods previously described. [14,15,36] Briefly, each aspirate was diluted 1:1 with Dulbecco's Modified Eagle Medium (DMEM; Wisent, St-Bruno, QC) and gently layered 1:1 over Ficoll (1.073 g/ml Ficoll-Plaque; GE Healthcare, Baie d'Urfé, QC) and centrifuged at 900 x g for 30 min. After centrifugation, the low-density MSC-enriched fraction was collected from the interface, supplemented to 40 ml with DMEM, and centrifuged at 600 x g for 10 minutes. After two washes, the cells were re-suspended in 20 ml of DMEM supplemented with 10% fetal bovine serum (FBS; Wisent), 100 U/ml penicillin, 100 µg/ml streptomycin and cultured under pre-confluency in 150-mm culture dishes at 37^{o}C with 5% humidified CO_2. After 72 h, the non-adherent cells were discarded when changing the medium.

One million of passage 3 or 4 MSCs were cultured for up to 7 days on either H-PPE:N or L-PPE:N coatings in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin. Commercial polystyrene (PS) tissue culture dishes (Sarstedt, Montreal, QC) were used as controls. The medium was changed every 2 days.

At the end of incubations, cells were washed with phosphate-buffered saline (PBS) and total RNA was isolated by a modification of the method of Chomczynski and Sacchi [37] using TRIzol reagent (Invitrogen, Burlington ON). After centrifugation for 15 min at 12,000 x g at 4°C, RNA in the aqueous phase was precipitated with isopropanol and recovered by centrifugation for 15 min at 4°C. The resulting RNA pellet was air dried, re-suspended in 40 µl diethypyrocarbonate-treated water, and the purity of the RNA was assessed by measuring the A_{260}/A_{280} ratio. The RT reaction was performed using 1 µg total RNA and 200 units Superscript II RNAse H^- reverse transcriptase (Invitrogen) as previously described. [14,15,32] PCR was performed in a total volume of 25 µl with 2.5U Taq polymerase (Invitrogen), also as previously described. [14,15,32] Primers used in the study and other procedural details are described elsewhere [14,15]. Amplified products were analyzed by electrophoresis on 1% agarose gels and visualized by ethidium bromide staining. Quantification was carried out using Quantity One software on a VersaDoc image analysis system (Bio-Rad Laboratories, Mississauga, ON) equipped with a cooled CCD 12 bit camera.

3. Results

3.1. Characteristics of PS control and of PPE:N surfaces

As already mentioned, the surfaces of commercial polystyrene (PS) tissue culture dishes are modified by plasma treatment at the manufacturer, and they typically comprise about 18 atomic % (at. %) of bound oxygen, so as to enhance wettability and cell-adhesion.

Chemical characterisations of the three families of PE:N films have been the subjects of previous articles, some dedicated to a specific family [7,8,25,26,29],

another to all three [28]. Figure 2 shows the compositions of two types of PE:N deposits used in this work, namely L-PPE:N and H-PPE:N. The total concentrations of nitrogen, [N] (in at. %) increased as a function of the gas mixture ratios, *X* and *R*, for both H- and L-PPE:N (Figure 2a). Primary amine [-NH_2] content also increased in function of the gas mixture ratio for L-PPE:N, but remained roughly constant for H-PPE:N (Figure 2b). The reader is reminded that for reasons of their superior chemical stability (low solubility in aqueous cell-culture media) [28], the following compositions were used here preferentially, unless otherwise specified:

L-PPE:N: $R = 0.75$ (L0.75);

H-PPE:N: $X = 200$ (H50).

Although [N] values ranged from about 16 to about 24 at % among the different materials illustrated in Figure 2, their primary amine concentrations, [NH_2], varied from 5.1 to 8.6 at. %, being near-constant for the case of H-PPE:N, but significantly higher for the two L-PPE:N coatings. Figure 3 shows that L- and H-PPE:N coatings differed not only in their compositions, but also in their surface-morphologies. Indeed, scanning electron microscopy images show that H-PPE:N coatings (Figure 3A and 3B) possess rough surface-morphologies, while their L-PPE:N counterparts, in sharp contrast, are extremely smooth (Figure 3C). The latter also applies to UV-PE:N coatings, not illustrated here [28].

3.2. U937 Monocytes

3.2.1. "Critical" surface conditions for cell adhesion

In earlier articles [7,13], we have already demonstrated and reported the existence of a "critical" nitrogen concentration, $[N]_{crit}$, a quite sharply-defined [N] value at PE:N film surfaces, below which U937 monocytes were found not to adhere at all. This is illustrated in Figure 4A for the case of H-PPE:N, where cells are seen to adhere only on the small PE:N islands deposited through openings in the Kapton mask, but not on the adjacent bare BOPP. Indeed, for the case of H-PPE:N, $[N]_{crit}$

was found to be 24% [13]: for all values of [N] > $[N]_{crit}$, U937 cells adhered to the coatings, but not when [N] values were lower.

Having stated this, let us now turn to Figure 4B: The first three of five different coatings families examined represent PE:N, while the fourth and fifth represent deposits based on the use of methane (M) and acetylene (A) "monomers", respectively [8,13]. All these were seen to also exhibit sharply-defined values of $[N]_{crit}$, just like H-PPE:N (with the exception of H-PPA:N, discussed below). However, the exact "critical" values varied among the different materials, ranging from 24% for H-PPE:N to 7.5% for VUV-PE:N. Thanks to the chemical derivatisation experiments described earlier and illustrated in Figure 2b, we could determine the corresponding values of "$[NH_2]_{crit}$", and these are also shown plotted in Figure 4B. Very strikingly, we note that these values of the "critical" primary amine concentrations are the *same* for all materials, $[NH_2]_{crit}$ = 4.2 ± 0.5%, within the limits of experimental precision. It might be added that we have verified our procedures for measuring $[NH_2]$ with the help of a commercially-available coating material that possesses a precisely-known primary amine concentration, and in which *all* of the bound nitrogen occurs in the form of this functionality, i.e. $[NH_2]/[N]$ = 100%. This material is Parylene diX AM (aminomethyl- [2-2]paracyclophane, Kisco Conformal Coating LLC)*, and we found the value $[NH_2]/[C]$, 6.5 ± 0.6 %, that almost exactly corresponds to the calculated one, 6.3 %, based on this material's chemical structure.

The key inference to emerge from Figure 4B is that primary amine groups play a dominant role in the mechanism of U937 adhesion to N-rich organic surfaces. This statement receives further convincing support from the observation, also illustrated on Figure 4B, that H-PPA:N (that can have [N] up to ~40%) has $[NH_2]$ values that never exceed 2% [8,13]; in other words, these are always below $[NH_2]_{crit}$. Therefore, this material is inherently incapable of inducing the adhesion of U937 monocytes. These results strongly support the view that $[NH_2]$, rather than simply [N], which is general and does not take into account the distribution of functional groups, should be considered in film design for such bio-

applications. Further circumstances that strongly support the above-stated inference are the facts that the films' other characteristics, for example, differing [O] and [H] values, but primarily their very different surface morphologies (mean surface roughness, see Figure 3) appeared to play no major roles. Possible

underlying cell adhesion mechanism(s) will be addressed below.

3.2.2 RT-PCR studies

Quantitative real-time RT-PCR experiments were conducted using U937 cells that had been made to adhere on PE:N materials for up to 24h, the purpose being to provide insight into the mechanism(s) of adhesion. We now examine the responses of genes that are known to play important roles in the cell biology of monocytes and macrophages. It is important to note that mRNA expression does not always correlate to protein expression. However, the expression of TNF-α and IL-1β, [38] as well as PPAR-γ, [39] ICAM-1, [40,41] and Egr-1 [42] is primarily regulated at the level of transcription, and their mRNA expression therefore represents a good indicator of their protein expression.

Since cytokines play a central role in the macrophage host defence, [43] we investigated the effect of U937 monocyte attachment to PE:N surfaces on the expression of tumour necrosis factor-alpha (TNF-α) and interleukin-1 beta (IL-1β), two cytokines secreted as part of immediate early response genes of these cells. The results of these RT-PCR experiments are presented in Figure 5, and they show a rapid (3h) but transient stimulatory effect of the amine-rich surfaces on the expression of these genes, attaining 10-15 times and 7-9 times the control values for TNF-α and IL-1β, respectively. However, the return to control values within 24h suggests that major inflammatory reactions were thereby not induced,

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**Footnote*: Parylene diX AM coatings were graciously provided by Drs. B. Elkin and C. Oehr, Fraunhofer Institute for Interfacial Engineering and Biotechnology, Stuttgart Germany (a joint publication is currently in preparation).

contrary to what was observed for corrosion products of orthopaedic implants that induced the expression of TNF-α in U937 cells for at least 24h under similar *in vitro* conditions. [30] However, further long-term adhesion studies are required to prove beyond doubt the low inflammatory reaction of these surfaces toward U937 cells.

3.3. Human MSCs

A current drawback of cartilage- and intervertebral disc (IVD)-tissue engineering is that human MSCs from OA patients express COL X, [14,15,32] a marker of chondrocyte hypertrophy associated with endochondral ossification. [33,34] Until very recently, no study had addressed the possible effect of the culture-substratum on the expression of genes related to hypertrophy. As will be shown presently, results of the study briefly sketched here, but reported in far more detail elsewhere [15], strongly suggest that MSCs obtained from these patients, a clinically relevant source of stem cells, can be used to engineer cartilage and IVD *in vitro* when pre-cultured on PE:N surfaces and transferred to pellet cultures.

The results of RT-PCR analyses of mRNA expression of type X collagen (COL X) on the different surfaces are shown in Figure 6. Expression of COL X, a definitive marker for the hypertrophic chondrocyte phenotype, [14,15,33] was consistently detectable in MSCs cultured on control (PS) culture dishes (Figure 6, Ctl). The expression of COL X did not change significantly throughout the 7-days culture period on PS control (results not shown). In contrast, its expression was decreased when cultured on L-PPE:N (61 ± 19% of control, $p = 0.02$) and on H-PPE:N (19 ± 27% of control, $p = 0.001$) coatings. However, L- and H-PPE:N coatings had no significant effect on the expression of type I collagen (COL I; $p = 0.57$ and 0.60 for L- and H-PPE:N, respectively) and aggrecan (AGG; $p = 0.86$ and 0.14 for L- and H-PPE:N, respectively). As previously reported, [32] type II collagen was not expressed in MSCs from OA patients cultured on the different surfaces.

In the more detailed version of this study [15], we showed for the first time that the inhibition of COL X can be maintained in pellet culture after "re-programming" MSCs on PE:N surfaces. Importantly, "re-programming" was found to have little or no effect on COL I, suggesting that these kinds of coatings show promise for tissue engineering of cartilage and disc tissues. However, the observed decrease of AGG expression remains to be addressed and ongoing investigation is presently looking at the addition of specific growth factors to maintain AGG expression without increasing COL X expression. Finally, the present results strongly suggest that not only the chemical composition but also the surface morphology of plasma-deposited coatings, more specifically of PE:N films (compare Figs. 3B and 3C), affect the behaviour of MSCs (unlike the case of U937, where these had no observable effects, as noted in section 3.2.1 above); they also suggest that these surfaces offer promising opportunities for tissue engineering of cartilage and disc.

4. General Discussion and Conclusions

In the present article we focused our attention upon the culture of two particular cell types of interest in our orthopaedics research program, U937 and MSCs, on different PE:N substrates. In numerous earlier articles from these laboratories that are cited in the list of references, we had already shown that a particular PE:N sub-group, namely H-PPE:N, was also capable of regulating the phenotype of IVD (nucleus pulposus) cells, [44] of maintaining the phenotypic profile of notochordal cells, [45] and of promoting healing around stent-grafts after endovascular aneurysm repair, [46] among other applications. In all cases, as in the literature, [2] those abilities of the coatings were largely attributed to the films' primary amine constituents. We therefore inferred that coatings from another PE:N sub-group, L-PPE:N, should in principle show even more promise than their "H" counterparts, on account of their significantly higher amine concentrations, $[NH_2]$, [26,28] clearly illustrated in Figure 2.

First, regarding culture of U937, Figure 4B clearly shows that the value of $[N]_{crit}$ varied widely among the different PE:N materials examined, along with similar coatings based on monomers other than ethylene, from a maximum of 24 at.-% to a minimum of 7.5 at.-%. The ability to distinguish among the numerous functionalities that contribute to [N], particularly the primary amine ($-NH_2$) groups via the use of chemical derivatisation, allowed us to identify the key common feature among those various coatings, namely the fact that in all cases $[N]_{crit}$ corresponded to a constant value of $[NH_2]_{crit}$, 4.2 ± 0.5 (per 100 atoms measured by XPS). Therefore, at least for the case of U937 monocytes, this confirmed the critical role of this ($-NH_2$) group in cell-surface interactions, something that had earlier been a topic of much uncertainty and debate in the literature. [1,2] Nevertheless, further studies will be necessary to investigate this critical role of ($-NH_2$) for the adhesion of other non-adherent cell types.

The data presented in Figure 5 indicate that the adhesion of U937 monocytes to PE:N surfaces with $[NH_2] \geq [NH_2]_{crit}$ induced a transient expression of TNF-α and IL-1β, two cytokines secreted as part of immediate early response genes of these

cells, suggesting that major inflammatory reactions were not induced. In contrast, PPARγ, implicated in the adhesion and retention of monocytes, had a more sustained expression (data shown elsewhere [13]). It was reported in the literature that PPARγ can be induced in various cell types by TNF-α and IL-1; [47,48] this would suggest that the transient expression of TNF-α and IL-1 may induce adhesion of monocytes via the activation of PPARγ, but that was not investigated directly.

Now turning to the second example, MSCs, referring to Figure 6 and to the preceding discussion regarding U937 cells, it was surprising to note that COL X suppression was *less* prominent in the case of the [NH_2]-richer (L0.75) L-PPE:N coating than on (H50) H-PPE:N (see Figure 2). In other words, our anticipated result that the extent of COL X suppression should be directly related to [NH_2] was not borne out by the observed data. However, as shown in Figure 3, the L- and H-PPE:N coatings differed not only in their compositions, but also in their surface-morphologies. It is well-known from the literature that the behaviour of cells adhering to solid surfaces can be strongly affected both by the surfaces' chemistries as well as their topological characteristics, particularly by micro-roughness. [49] Surface roughness-related effects are encountered not only in the case of plasma polymers, but they were, for example, also suggested as a key factor for inducing a favourable osteoblastic behaviour of MSCs cultured on different hydroxyapatite deposits on titania (TiO_2) powder. [50] Now, since the chemical compositions (in terms of [NH_2]) of L0.75 and H50 coatings are not *drastically* dissimilar, and since the latter manifested enhanced capability to reduce COL X expression (Figure 6), it is conceivable that the morphological differences observed (Figure 3) play a significant, possibly dominant, role in the control of COL X expression. The apparent synergy between surface composition and -morphology on MSC regulation remains to be elucidated and this is now the object of further in-depth investigation.

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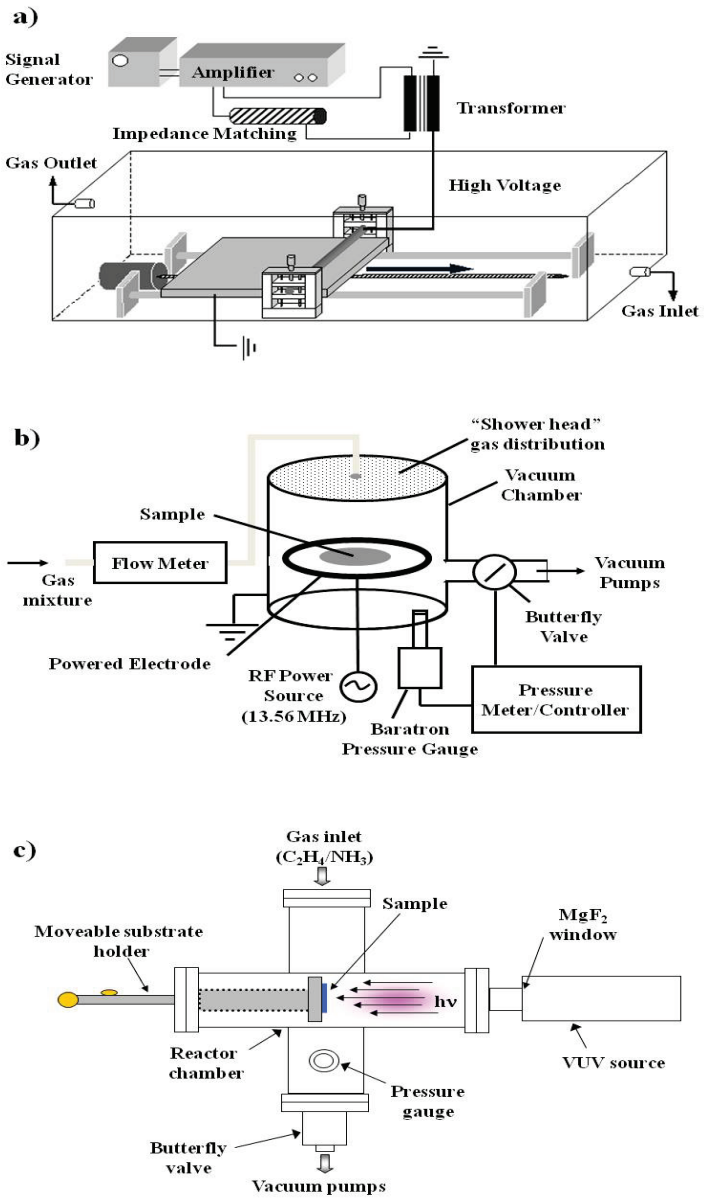


Figure 1: Three reactor systems used in the present research: (a) Low-pressure plasma (LP-PECVD), 13.56 MHz r.f. discharge; (b) Atmospheric-pressure dielectric barrier discharge (AP-DBD) plasma; (c) Vacuum ultraviolet photo-polymerisation (VUV-CVD), see text. (From ref. [28], with permission).

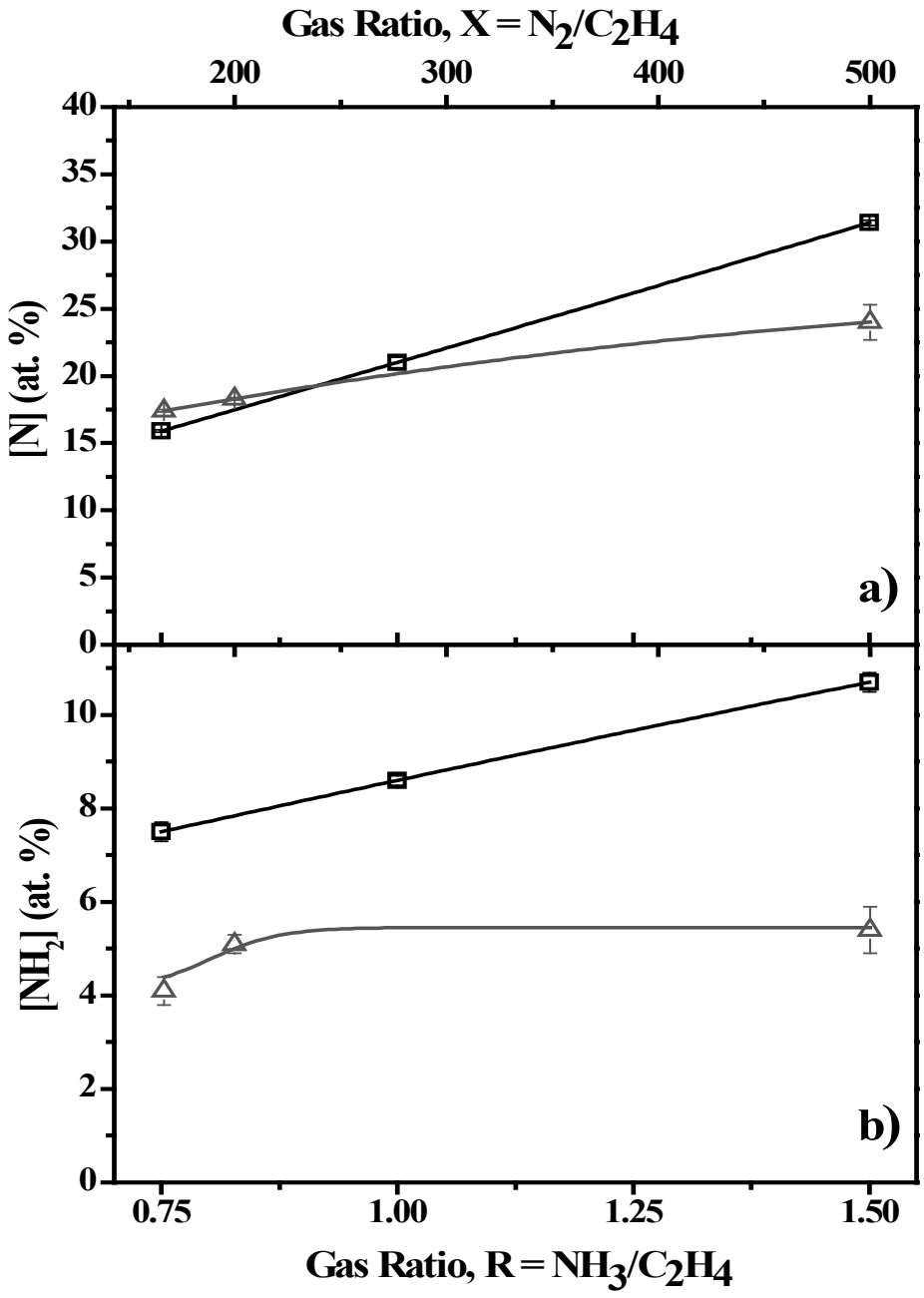


Figure 2: (a) Concentrations (in atomic %) of nitrogen, [N], and (b) of primary amines, [NH_2], in L-PPE:N (□) and H-PPE:N (Δ) deposits, as functions of the gas mixture ratios, $R \equiv F_{NH3}/F_{C2H4}$ (for L-PPE:N), or $X \equiv F_{N2}/F_{C2H4}$ (for H-PPE:N, see text). (From ref. [15], with permission).

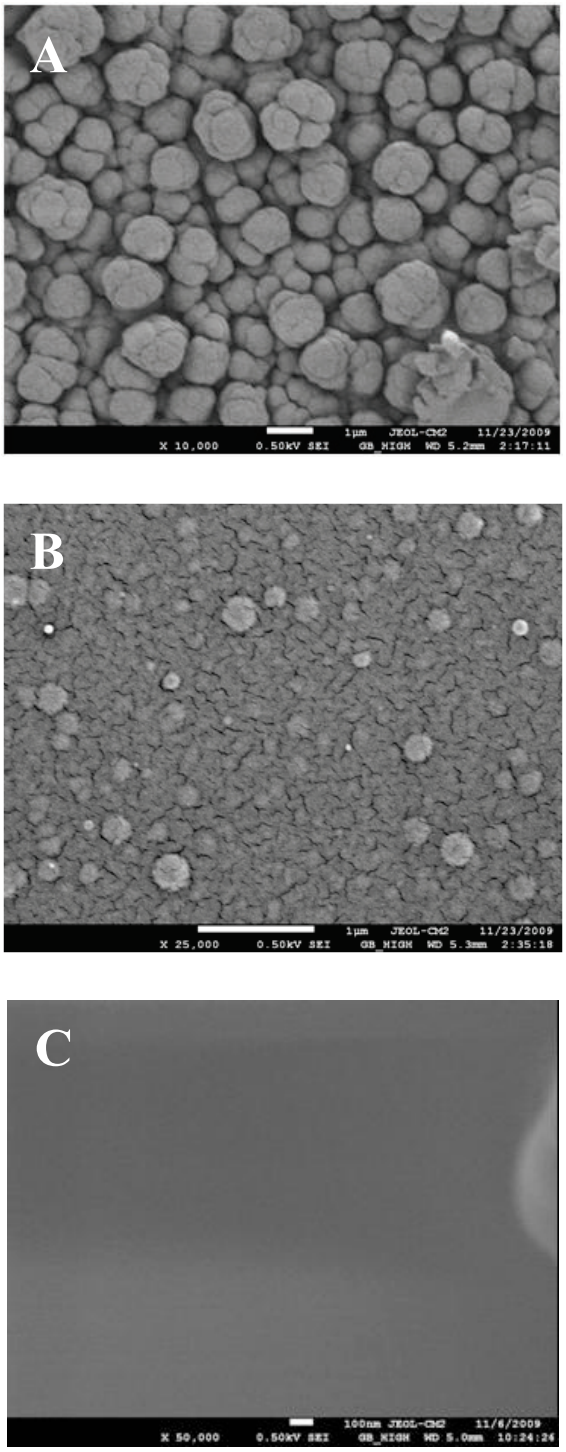
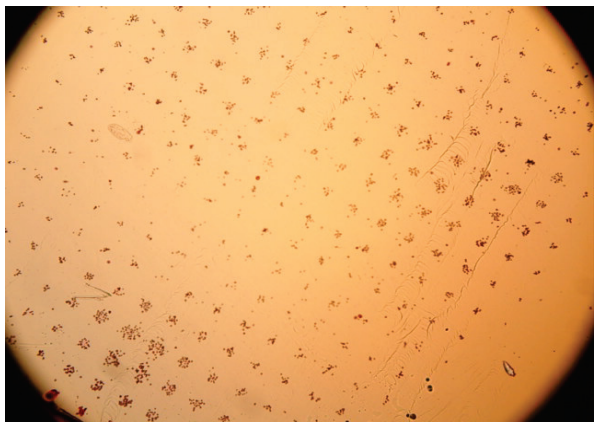


Figure 3: Surface morphologies of PPE:N coatings. Images of H20-PPE:N (A), H50-PPE:N (B), and L0.75-PPE:N (C) surfaces were acquired by field-emission scanning electron microscopy (FE-SEM). (After ref. [28], modified, with permission).

(A)



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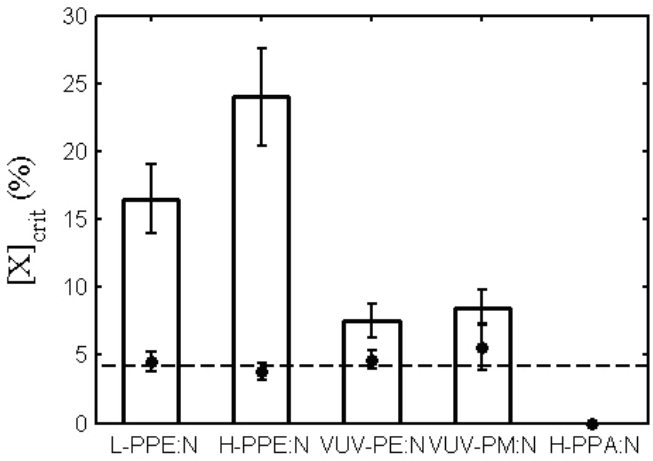

Figure 4: (A) Adherence of U937 cells on a micro-patterned BOPP surface comprising 50 μm islands of H-PPE:N in a square array with 200 μm pitch. (After ref. [7], with permission). (B) Quantification of $[N]_{crit}$ in the adhesion experiments involving U937 cells. The "critical" nitrogen content, $[N]_{crit}$, required to stimulate adhesion of U937 monocytes to various coatings, namely: L-PPE:N, H-PPE:N, VUV-PE:N, VUV-PM:N and H-PPA:N, are shown in histogram-form. For each one of the materials, the corresponding critical amino-group content, $[NH_2]_{crit}$ (●), is superimposed. The average value of $[NH_2]_{crit}$ is presented as a dashed, horizontal line. In the case of H-PPA:N, no adhesion of U937 was observed under any circumstances, in spite of high [N] values. Note that the symbol [X] on the ordinate axis refers to both [N] and $[NH_2]$, on the same scale. (After ref. [13], with permission).

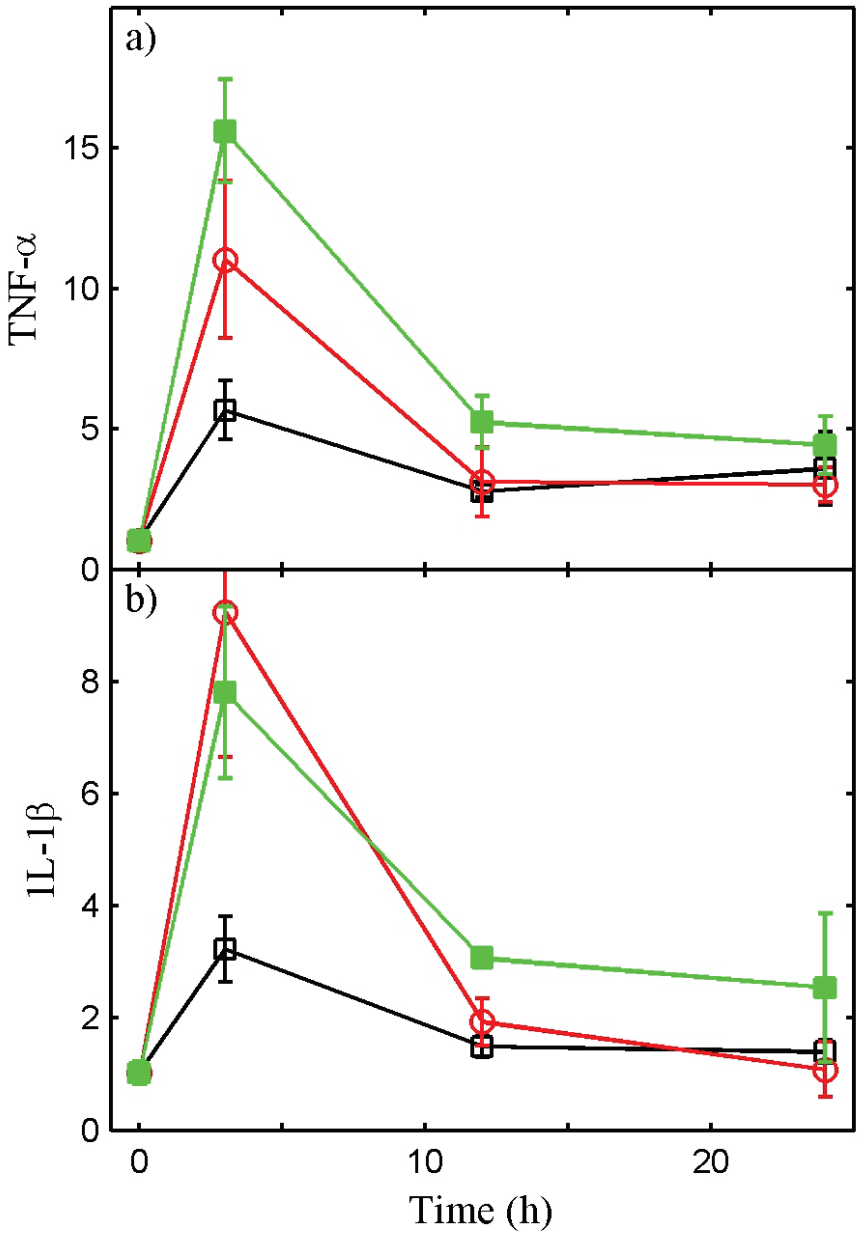

Figure 5: Expression of cytokines in U937 monocytes: U937 cells were cultured for up to 24 hours in suspension (□), and adhering on H -PPE:N (○) and L-PPE:N (■) surfaces with $[NH_2] \geq [NH_2]_{crit}$. Results shown are the mean values ± standard error of three experiments. * $p < 0.05$ vs. suspension control. (After ref. [13], with permission).

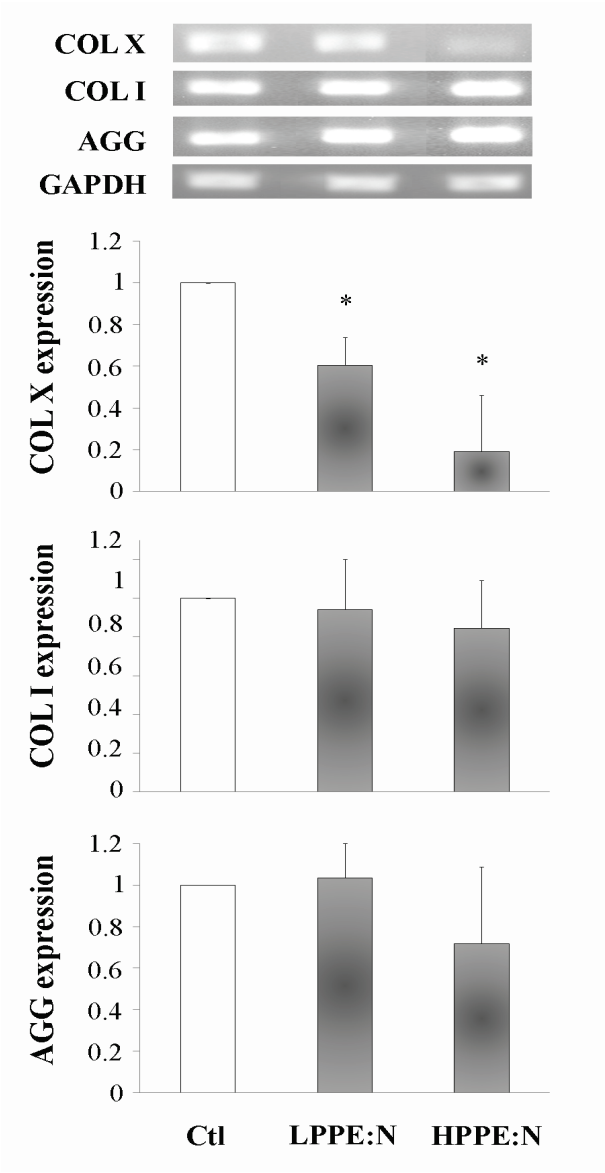

Figure 6: Expression of COL X on PS and PPE:N surfaces. MSCs from OA patients were cultured on PS (Control, Ctl), (H50) H-PPE:N, and (L0.75) L-PPE:N for 7 days. Total RNA was extracted and mRNA levels measured by RT-PCR as described in Materials and Methods. GAPDH was used as housekeeping gene and served to normalize the results. Quantitative results are the mean ± standard deviation of 4 experiments. * $p < 0.05$ vs. PS control. (From ref. [15], with permission).

B) Stem Cells, Nitrogen-Rich Plasma-Polymerized Culture Surfaces and Type X Collagen Suppression

We had previously described the ability of nitrogen-rich plasma polymerized ethylene surfaces (PPE:N) to decrease the expression of type X collagen in mesenchymal stem cells from osteoarthritic patients. However; it remained unclear as to whether this suppression can be maintained when cells are removed from the surfaces and grown in pellet cultures, a physiologically relevant culture condition necessary for the differentiation into chondrocytes. In the present manuscript, we are able to show that a particular PPE:N surface, High Pressure polymerized ethylene (H-PPE:N) serves as a better surface for suppressing type X collagen gene expression in MSCs from OA patients. Furthermore, we also show that the suppression of type X collagen induced by these surfaces, is in fact maintained when cells are removed from the surface and placed in pellet cultures in the presence of serum-free media.

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Stem Cells, Nitrogen-Rich Plasma-Polymerized Culture Surfaces and Type X Collagen Suppression

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Abstract

Mesenchymal stem cells (MSCs) are multipotent cells that can differentiate into chondrocytes, osteoblasts, myocytes, adipocytes, and a variety of other cell types. Several studies have been directed toward using MSCs from osteoarthritic (OA) patients for cartilage repair, not only because these are the ones that will require a source of autologous stem cells if biological repair of cartilage lesions is to be a therapeutic option, but also to further an understanding of stem cell differentiation. Previous studies have shown that a major drawback of current cartilage- and intervertebral disc tissue repair is that human MSCs from OA patients express type X collagen (COL X). COL X, a marker of late-stage chondrocyte hypertrophy, is implicated in endochondral ossification. However, those studies also revealed that a novel plasma-polymerized thin film material, named nitrogen-rich plasma-polymerized ethylene (PPE:N), was able to inhibit COL X expression in committed MSCs. The specific aim of this present study was to determine if the suppression of COL X by PPE:N is maintained when MSCs are transferred to pellet cultures in serum-free media. Our results confirmed the potential of two different types of PPE:N surfaces ("L"- and "H"-PPE:N) in suppressing COL X expression, more so on the latter. Interestingly, when MSCs were transferred to pellet cultures, the expression of COL X was further decreased by pre-incubation on H-PPE:N, suggesting that these kinds of coatings show promise for tissue engineering of cartilage and disc tissues. Further studies are needed to assess the relative importance of surface-chemistry versus surface-morphology in the mechanism of COL X suppression.

Introduction

Adult human bone marrow, as well as muscle [1,2] adipose [3] trabecular bone [4] and a host of other tissues, contain a population of mesenchymal stem cells (MSCs) with the ability to differentiate into a variety of connective tissue lineages, including bone and cartilage [5,6]. MSCs have been shown to undergo chondrogenesis *in vitro* when using a high cell density pellet culture system, [7-9] which mimics the cellular condensation requirements for embryonic mesenchymal chondrogenesis, and which provides the physical and biochemical environmental factors conducive to cartilage formation [8].

Treatments with transforming growth factor-beta (TGF-ß) superfamily members, TGF- ß [10-14] or TGF- ß3, [9,15] and with dexamethasone (DEX), [8,9,14] are key requirements for the *in vitro* chondrogenic differentiation of MSCs. Other growth factors, such as fibroblast growth factor (FGF), and insulin-like growth factor-1 (IGF-1), have been found to enhance chondrogenesis [16]. Several studies have been directed toward using MSCs from osteoarthritic (OA) patients for cartilage repair, not only because these are the patients that will require a source of autologous stem cells if biological repair of cartilage lesions is to be a therapeutic option, but also to help further understanding of stem cell differentiation. However, recent evidence indicates that a major drawback of current cartilage or intervertebral disc tissue-engineering is that human MSCs from these patients express type X collagen (COL X), [17,18] a marker of chondrocyte hypertrophy associated with endochondral ossification) [19,20].

Some studies have attempted to use growth factors to inhibit the expression of COL X and have suggested that BMP-4 was a promising candidate for suppressing chondrogenic hypertrophy, while simultaneously enhancing the production of chondrogenic markers [21]. We also showed recently that parathyroid hormone (PTH) can simultaneously inhibit expression of COL X and stimulate expression of type II collagen, a marker of chondrogenic differentiation [22].

However, little is presently known on the effects of cell culture surface-chemistry and surface-morphology on stem cell differentiation. We recently showed that extremely nitrogen (N)-rich plasma polymer layers, which we called "PPE:N" (N-doped plasma-polymerized ethylene) that can contain up to 36% N, suppressed the expression of COL X in monolayer cultures [18]. Since pellet cultures are commonly used to promote *in vitro* chondrogenesis, the purpose of the present study was to determine whether the suppression of COL X by two different types of PPE:N is maintained when the MSCs are transferred to this kind of 3D culture model.

Materials and Methods

PPE:N coatings on PET surfaces

Two types of thin PPE:N coatings were deposited on the surface of poly(ethylene terephthalate) (PET) film in two different reactor systems, namely (i) a conventional low-pressure ("L") capacitively-coupled radio-frequency (r.f.) glow-discharge plasma reactor (coatings hereafter designated "L-PPE:N"), [23,24] and (ii) a dielectric-barrier discharge (DBD) reactor operating at atmospheric pressure (hereafter "H-PPE:N" coatings, "H" designating high pressure) [24-26]. The 50 μm thick PET film substrate was obtained from Goodfellow Corp. (Oakdale, PA). The L-PPE:N coatings were deposited in a cylindrical aluminium/steel vacuum chamber, approximately 20 cm in diameter and 20 cm in height as described before [23,24]. Flows of high-purity feed gases (ethylene 99.5% and ammonia 99.99%, Air Liquide Canada, Montreal, QC) were admitted into the chamber using electronic flow meter/controllers (Vacuum General Inc., San Diego, CA), and a "shower head" gas distributor (10 cm in diameter). While the flow rate of C_2H_4 "monomer", F_{C2H4}, was kept constant at 20 standard cubic centimeters per minute (sccm), its NH_3 counterpart, $F_{NH3,}$ could be varied between 0 and 60 sccm [23]. Based on our previous experience, we chose to deposit L-PPE:N coatings using rather mild plasma conditions (P = 10 W, resulting in a negative d.c. bias voltage, V_B =-40 V); under these conditions, polymer-like films with maximum nitrogen and amine concentrations were deposited [23,24]. Based on previous work relating to stability of the deposits in air and water, [27] we chose in the present study to prepare particular coatings with F_{NH3} = 15 and 20 sccm, that is, with ratios R= F_{NH3}/ F_{C2H4} of 0.75 and 1, respectively (hereafter identified as L0.75 and L1). Indeed, unless otherwise specified, all of the data relating to experiments with MSCs were carried out with the L0.75 coating, the less soluble (more stable) of the two. The reason for preparing, characterizing, and discussing two coating compositions here was to illustrate the importance of the feed gas composition.

For the case of H-PPE:N, deposition was carried out in a DBD reactor, as described elsewhere [24-26]. Briefly, this system comprised a cylindrical, dielectric-coated stainless steel high-voltage (HV) electrode, and a horizontal, grounded planar aluminum (Al) electrode, covered by a 2 mm-thick glass plate that served as a second dielectric layer. The precursor gas mixture, $X = F_{N2}/F_{C2H4}$, composed of pure nitrogen (N_2) and ethylene (C_2H_4), was introduced into the discharge zone, an adjustable gap (usually 1 mm) between the HV electrode and the glass plate. The N_2 flow rate was fixed at 10 standard liters per minute (slm); as mentioned above, deposits were made using C_2H_4 flow rates of 20 or 50 sccm for reasons of their good to superior physicochemical stability (hereafter identified as H20 and H50) [27]. Again, unless otherwise specified, MSC-related experiments were carried out with the more stable H50 coating.

For both coating processes, deposition durations were selected so as to create approximately 100 nm-thick films. Elemental compositions of PPE:N samples and their primary amine concentrations were determined by X-ray photoelectron spectroscopy (XPS) in a VG ESCALAB 3MkII instrument, using non-monochromatic Mg Kα radiation, as described in detail elsewhere [23-28]. The surface-near concentrations of primary amines, [-NH_2], were determined after chemical derivatization with 4-(trifluoromethyl)benzaldehyde (TFBA, Alfa Aesar, Ward Hill, MA) vapour. With this method, [–NH_2] values can be deduced accurately from the measured fluorine concentrations, [F], also by XPS, [23,27] as shown in Table 1. As reported previously, values of [N] and [-NH_2] increase significantly with increasing NH_3/C_2H_4 or N_2/C_2H_4 ratios.

Field-emission scanning electron microscopy (FE-SEM)

Selected samples were examined by field-emission scanning electron- microscopy (FE-SEM) using a JEOL model “JSM-7600 TFE” instrument (JEOL Ltd., Tokyo, Japan). For this purpose, relatively thick (≥ 0.5 μm) H -PPE:N and L-PPE:N films were deposited on glass and on silicon wafers, respectively, in order to allow

small (~1 cm^2) representative pieces to be fractured in ambient air, then introduced into the microscope's sample-holder. The purpose was to examine both top- and side-views of their micro-morphologies, [27] but only top-views are presented and discussed here.

Source of MSCs

Human MSCs were obtained from 10 to 25 ml bone marrow aspirates from the femoral intramedullary canal of donors undergoing total hip replacement using a protocol approved by the Research Ethics Committee of the Jewish General Hospital [17,18]. The marrow donors included both males and females ranging in age from 52 to 88 years (mean, 65 years).

Isolation of MSCs

Isolation of MSCs was carried out using methods previously described [18,29]. Briefly, each aspirate was diluted 1:1 with Dulbecco's Modified Eagle Medium (DMEM; Wisent, St-Bruno, QC) and gently layered 1:1 over Ficoll (1.073 g/ml Ficoll-Plaque; GE Healthcare, Baie d'Urfé, QC) and centrifuged at 900 x g for 30 min. After centrifugation, the low-density MSC-enriched fraction was collected from the interface, supplemented to 40 ml with DMEM, and centrifuged at 600 x g for 10 minutes. After two washes, the cells were re-suspended in 20 ml of DMEM supplemented with 10% fetal bovine serum (FBS; Wisent), 100 U/ml penicillin, 100 µg/ml streptomycin and cultured under pre-confluency in 150-mm culture dishes at 37°C with 5% humidified CO_2. After 72 h, the non-adherent cells were discarded when changing the medium.

Characterization of MSCs using flow cytometry

Ten million expanded MSCs at $1x10^6$ cells/ml were incubated for 30 min at 4ºC with the following non-conjugated primary mouse antibodies: CD34-PE, CD44-FITC, CD45-FITC, CD90-PE (Beckman Coulter, Mississauga, ON), and CD73-PE (BD Biosciences Pharmingen, San Diego, CA). Cells were then washed and incubated with the secondary fluorescent-conjugated antibody (goat anti-mouse

IgG-FITC (1:100; Beckman Coulter) for 30 min at 4°C. Cells were analyzed on a Beckman Coulter EPICS-XL flow cytometer (Beckman Coulter). Cells were gated on forward and side scatter to exclude debris and cell aggregates and settings were adjusted to exclude cells stained with isotype control antibodies IgG1-PE or IgG1-FITC (Beckman Coulter). The analysis was repeated in cells from three (3) OA donors.

Cell culture on PPE:N surfaces

4 x 10^5 of passage 3 or 4 MSCs were cultured for up to 7 days on either H-PPE:N or L-PPE:N coatings in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were seeded at a density of 5.1x10^3 cells per cm^2 in 5 ml of media. Commercial polystyrene (PS) tissue culture dishes (Sarstedt, Montreal, QC) were used as controls. The medium was changed every 2 days.

Culture in serum-free media and in pellet

After 7 days of incubation on PPE:N surfaces, cells were detached by trypsination (0.25% trypsin/EDTA, Wisent) and centrifuged at 600 x *g* for 10 min. Cells were then resuspended in serum-free medium consisting of DMEM, 5 µg/ml insulin, 5 ng/ml transferrin, 5 ng/ml sodium selenite, 1 mg/ml bovine serum albumin (all from Sigma-Aldrich, Oakville, ON), 100 U/ml penicillin, and 100 µg/ml streptomycin in DMEM high glucose. Freshly prepared ascorbic acid (50 µg/ml, Sigma-Aldrich) was added to the media. Cells were then incubated for an additional 7-days period at a concentration of 4 x 10^5 cells/ml in 35x10 mm PS culture dishes, or placed in 15 ml conical polypropylene tubes, centrifuged at 600 x g for 6 min to form aggregates. The pelleted cells were cultured for up to 7 days, as previously described [30]. Medium was changed every 2 days and pellets were re-centrifuged with every medium change.

Total RNA isolation

At the end of incubations, cells were washed with phosphate-buffered saline (PBS) and total RNA was isolated by a modification of the method of Chomczynski and Sacchi [31] using TRIzol reagent (Invitrogen, Burlington ON). After centrifugation for 15 min at 12,000 x g at 4°C, RNA in the aqueous phase was precipitated with isopropanol and recovered by centrifugation for 15 min at 4°C. The resulting RNA pellet was air dried, re-suspended in 40 µl diethypyrocarbonate-treated water, and the purity of the RNA was assessed by measuring the A_{260}/A_{280} ratio.

Reverse transcription (RT) and polymerase chain reaction (PCR)

The RT reaction was performed using 1 µg total RNA and 200 units Superscript II RNAse H^- reverse transcriptase (Invitrogen) as previously described [18,19]. PCR was performed in a total volume of 25 µl with 2.5U Taq polymerase (Invitrogen), also as previously described [17,18]. Primers used in the study are described in Table 2. Amplified products were analyzed by electrophoresis on 1% agarose gels and visualized by ethidium bromide staining. Quantification was carried out using Quantity One software on a VersaDoc image analysis system (Bio-Rad Laboratories, Mississauga, ON) equipped with a cooled CCD 12 bit camera.

Statistical analysis

Statistical analysis was performed using Analysis of Variance (ANOVA) followed by Fisher's Protected Least Significant Difference (PLSD) post-hoc test using Statview (SAS Institute Inc., Cary, NC). Results are presented as the mean ± standard deviation of 3-5 experiments. Differences were considered statistically significant at $p < 0.05$.

Results

Characteristics of PS control and of PPE:N surfaces

The surfaces of commercial polystyrene (PS) tissue culture dishes are modified by plasma treatment at the manufacturer, and they typically comprise about 18 atomic % (at. %) of bound oxygen, so as to enhance wettability and cell-adhesion. Figure 1 shows the compositions of the types of PPE:N deposits used in this work, namely L-PPE:N and H-PPE:N. The total concentrations of nitrogen, [N] (in at. %) increased as a function of the gas mixture ratios, X and R, for both H- and L-PPE:N (Figure 1a). Primary amine [$-NH_2$] content also increased in function of the gas mixture ratio for L-PPE:N, but remained roughly constant for H-PPE:N (Figure 1b). Results are summarized in Table 1. The reader is reminded that for reasons of their superior chemical stability (low solubility in aqueous cell-culture media), [27] the following two compositions were used in this present work:

L-PPE:N: R = 0.75 (L0.75);

H-PPE:N: X = 200 (H50).

The other two coatings (L1 and H20) served primarily to illustrate the importance of the fabrication parameters R and X on the films' properties, as will now be discussed in more detail. From Table 1, we note that although the [N] values ranged from about 16 to about 24 at. % among the four different materials, their primary amine concentrations, [NH_2], varied from 5.1 to 8.6 at. %, being near-constant for the case of H-PPE:N, but significantly higher for the two L-PPE:N coatings. Figure 2 shows that L- and H-PPE:N coatings differed not only in their compositions, but also in their surface-morphologies. Indeed, scanning electron microscopy images show that H-PPE:N coatings (Figure 2A and 2B) possess rough surface-morphologies, while their L-PPE:N counterparts, in sharp contrast, are extremely smooth (Figure 2C).

Characteristics of expanded MSCs control

We next examined the characteristics of expanded MSCs from patients with osteoarthritis in order to determine the patterns of expression of markers most commonly associated with MSCs [32-34]. Except for OA, patients did not suffer from other diseases that may exclude them from the study as less suitable for use of their MSCs. The expression levels of the selected antigens are summarized in Table 3. Cells stained strongly for CD90 (Thy-1), and they were mildly- to highly positive for CD73 and CD44 (HCAM), three markers of MSCs. The observed wide range depended upon the particular donors. The expression range for CD73 extended from 52% to 100%, with the median at 94%. CD44 expression range was from 11% to 100%, with the median at 65%, while CD105 had an expression range from 6% to 100%, with the median at 38%. Cells stained predominantly negatively for CD34 and CD45, two markers of hematopoietic stem cells.

PPE:N induced suppression of COL X expression

Results showed that MSCs proliferated and reached confluence similarly and had comparable aspects on the three different types of surfaces, namely polystyrene (PS) control (Figure 3A), L-PPE:N (Figure 3B), and H-PPE:N (Figure 3C). After 7 days, an average of 6.50×10^5 cells were observed on the control surfaces while the number of cells reached 6.35×10^5 on L-PPE:N and 6.00×10^5 on H-PPE:N surfaces (Figure 4). The results of RT-PCR analyses of mRNA expression of type X collagen (COL X) on the different surfaces are shown in Figure 5. Expression of COL X, a definitive marker for the hypertrophic chondrocyte phenotype, [18,19] was consistently detectable in MSCs cultured on control (PS) culture dishes (Figure 5, Ctl). The expression of COL X did not change significantly throughout the 7-days culture period on PS control (results not shown). In contrast, its expression was decreased when cultured on L-PPE:N (61 ± 19% of control, $p = 0.02$) and on H-PPE:N (19 ± 27% of control, $p = 0.001$) coatings. However, L- and H-PPE:N coatings had no significant effect on the expression of

type I collagen (COL I; p = 0.57 and 0.60 for L- and H-PPE:N, respectively) and aggrecan (AGG; p = 0.86 and 0.14 for L- and H-PPE:N, respectively). As previously reported, [17] type II collagen was not expressed in MSCs from OA patients cultured on the different surfaces.

COL X expression in serum-free media

TGF-beta and dexamethasone are generally added to serum-free media to induce chondrogenesis in stem cells [7-9,30]. We then next explored the effect of serum-free media in the absence of TGF-beta and dexamethasone on the expression of COL X after pre-culture on PPE:N surfaces. Results show that when cells were cultured for 7 days on PPE:N surfaces and then transferred to PS culture dishes for an additional 7 days in culture in the presence of serum-free media, the suppression of COL X observed after 7 days on N-rich surfaces was partly maintained, with levels of expression reaching 67% (p = 0.33) and 47% (p = 0.01) of control cells at day 7, for L- and H-PPE:N, respectively (Figure 6). Serum-free media had no additional effect on the expression of COL X, down-regulation being observed only in cells pre-cultured on PPE:N surfaces, but not on PS control. Serum-free media also had no further significant effect on the expression of COL I (p = 0.50), whereas AGG expression was decreased after the cells were pre-incubated for 7 days on PS control (39% of control; p= 0.01), L-PPE:N (30% of control; p= 0.01), and H-PPE:N (30% of control; p= 0.01) and were transferred to PS culture dishes for an additional 7 days presumably because this media does not support chondrogenic differentiation. Similarly, it is unlikely that the MSCs differentiated towards the osteogenic lineage due to the absence of osteogenic factors.

Suppression of COL X using H-PPE:N in pellet cultures

Pellet culture is also used to induce chondrogenesis [7-9,30]. We next explored whether the pre-culture of MSCs on PPE:N surfaces could maintain the suppression of COL X when transferred to this kind of 3D culture. Figure 7 shows that H-PPE:N (5% of control, p < 0.0001) seemed more effective at suppressing

COL X than L-PPE:N (20% of control, $p < 0.0001$) when pre-cultured MSCs were transferred to pellet culture. However, there were no statistical differences between the two surfaces ($p = 0.21$). Importantly, pre-culture of MSCs on H-PPE:N tended to be more efficient in reducing COL X expression than pellet culture alone ($p = 0.14$) and L-PPE:N ($p = 0.21$). Pellet culture also decreased significantly the expression of COL I after pre-incubation on PS control (35% of control; $p= 0.02$), but not after pre-incubation on L-PPE:N (60% of control; $p = 0.10$) or H-PPE:N (63% of control; $p = 0.11$). The expression of AGG was similarly decreased when MSCs were cultured in pellets after pre-incubation on the 3 surfaces we studied (9 to 20% of control at day 7).

Discussion

A current drawback of cartilage- and disc-tissue engineering is that human MSCs from OA patients express COL X, [17,18] a marker of chondrocyte hypertrophy associated with endochondral ossification [19,20] . Until very recently, no study had addressed the possible effect of the culture-substratum on the expression of genes related to hypertrophy. The present data indicate that N-rich PPE:N surfaces can also down-regulate COL X expression *in vitro* when MSCs obtained from OA patients, are pre-cultured on these surfaces and transferred to pellet cultures. This is a feature needed for any agent designed to suppress hypertrophy and promote disc and cartilage repair.

The technique of depositing nitrogen (N)-based organic thin film materials by plasma for biomedical applications has been known for a long time, and this field was recently the object of a detailed review [35]. However, very amine-rich films prepared from mixtures of ethylene and nitrogen (or NH_3) have only recently been applied to tissue engineering of the kind reported here [18,26]. These types of coatings, which we previously designated “PVP:N” (for “Plasma- or VUV-Polymerised N-rich” materials), [27] were indeed specifically designed to contain high concentrations of N-bearing functionalities, mainly primary amines, [$-NH_2$],

and they have in many separate instances been shown to influence cell-adhesion and other types of cell-behaviour. We were the first to show that a particular sub-group of "PVP:N", namely H-PPE:N, were capable of selective inhibition of COL X expression in human MSCs, [18] of enabling the adhesion of human U937 monocytes, [36] of regulating the phenotype of disc (nucleus pulposus) cells, [37] of maintaining the phenotypic profile of notochordal cells, [38] of enhancing adhesion and growth of vascular smooth muscle cells, [39] and of influencing the differentiation of MSCs *in vitro* [18]. In all cases, as in the literature, [35] those abilities of the coatings were largely attributed to the films' primary amine constituents. More recently, we showed that another sub-group of "PVP:N", namely N-rich low-pressure (or "L") plasma polymerized ethylene films, L-PPE:N, are in principle even more promising than their "H" counterparts, on account of their significantly higher amine concentrations, [NH_2]; [24,27] this is clearly illustrated in Figure 1 and in Table 1. These differences were deemed to be highly significant at the outset of this research because we had observed that [NH_2] can have a major influence upon cell-adhesion, among other characteristics [36]. As we reported elsewhere, [27] higher values of [N] than those presented here inevitably gave rise to coatings that were quite soluble in aqueous media, rendering them unattractive for purposes of cell-culture. In that same article, and as reported here (Figure 2), we had shown that the L- and H-PPE:N coatings differed not only in their compositions, but also in their surface-morphologies. It is well-known from the literature that the behaviour of cells adhering to solid surfaces can be strongly affected both by the surfaces' chemistries as well as their topological characteristics, such as micro-roughness [40]. Surface roughness-related effects are encountered not only in the case of plasma polymers, but they were, for example, also suggested as a key factor for inducing a favourable osteoblastic behaviour of MSCs cultured on different hydroxyapatite deposits on titania (TiO_2) powder [41]. Since the chemical compositions, mainly in terms of [NH_2], of both L- and H-PPE:N are not very dissimilar, and since H-PPEN manifested an enhanced capability than L-PPE:N to reduce COL X expression, it is conceivable that the morphological differences observed (Figure 2) also play a

significant role in the control of COL X expression. The exact nature of the effect of surface morphology on MSC regulation remains to be elucidated and this is now the object of further in-depth investigation.

In one of our previous studies, we demonstrated that COL X was expressed when MSCs from OA patients were incubated in pellet culture in chondrogenic defined media [30]. Increased COL X expression was also observed in normal rabbit [8] and normal human [7] MSCs in pellet culture in chondrogenic defined media. In the present study, we showed for the first time that the inhibition of COL X can be maintained in pellet culture after culturing MSCs on PPE:N surfaces. Importantly, culturing on PPE:N surfaces was found to have little or no effect on COL I suggesting that these kinds of coatings show promise for tissue engineering of cartilage and disc tissues. However, the observed decrease of AGG expression remains to be addressed and ongoing investigation is presently looking at the addition of specific growth factors to stimulate AGG expression without increasing COL X expression. However, it is important to note that 7 days of culture is not sufficient to induce chondrogenic differentiation of MSCs. A minimum of 14 days is generally required to detect significant chondrogenic changes at the gene and protein expression levels [42]. Furthermore, the standard chondrogenic supplements TGF-beta and dexamethasone were not added to the medium.

Finally, the present results strongly suggest, for the first time, that not only the chemical composition but also the surface morphology of plasma-deposited coatings, more specifically of PPE:N films, affect the behaviour of MSCs; they also suggest that these surfaces offer promising opportunities for tissue engineering of cartilage and disc.

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Table 1. Plasma Deposition Parameters and Chemical Characteristics of the Various Nitrogen-Rich Plasma-Polymerized Ethylene Coatings

	H-PPE:N (X=500, H20)	*H-PPE:N (X=200, H50)*	*L-PPE:N (R=0.75, L0.75)*	*L-PPE:N (R=1, L1)*
Deposition parameters				
Gas flow rates	C_2H_4: 20 sccm N_2: 10 slm	C_2H_4: 50 sccm N_2: 10 slm	C_2H_4: 20 sccm NH_3: 15 sccm	C_2H_4: 20 sccm NH_3: 20 sccm
Pressure (Torr)	760	760	0.6	0.6
Coating characteristics				
[N] (at.%)	24±1.3	18.3±0.1	15.9±0.1	21.0±0.4
[NH_2] (at.%) ±Standard error	5.4±0.5	5.1±0.2	7.5±0.2	8.6±0.1

H-PPE:N, high-pressure nitrogen-rich plasma-polymerized ethylene; L-PPE:N, low-pressure nitrogen-rich plasma-polymerized ethylene; sccm, standard cubic centimeters per minute; slm, standard liters per minute; [N], nitrogen elemental compositions; [NH_2], primary amine concentrations on the surfaces, as measured by X-ray photoelectron spectroscopy, the latter after chemical derivatization with 4-(trifluoromethyl)benzaldehyde.

Table 2. Primer Sequences Used for Polymerase Chain Reaction

Gene	*Primer*	*Fragment size (bp)*
Aggrecan	Forward: 5′-CTACGACGCCATCTGCTACA-3′ (1089–1108) Reverse: 5′-ACGAGGTCCTCACTGGTGAA-3′ (1426–1445)	357
Col X α1 chain	Forward: 5′-ACAGGAATGCCTGTGTCTGCTTTTACT-3′ (1735–1761) Reverse: 5′-CATTGGGAAGCTGGAGCCACACCTGGTC-3′ (2038–2066)	331
Col I α2 chain	Forward: 5′-CGGTTACCCTGGCAATATTG-3′ (2979–2998) Reverse: 5′-TCCAGTGCGACCATCTTTT-3′ (3324–3342)	364
Col II α1 chain	Forward: 5′-CTGGCTCCCAACACTGCCAACGTC-3′ (574–587) Reverse: 5′-TCCTTTGGGTTTGCAACGGATTGT-3′ (964–987)	414
GAPDH	Forward: 5′-GCTCTCCAGAACATCATCCCTGCC-3′ (326–349) Reverse: 5′-CGTTGTCATACCAGGAAATGAGCTT-3′ (646–671)	346

Table 3. Characteristics of Expanded Mesenchymal Stem Cells

Markers	*% of Positive cells median (interval)*
Marker of MSCs	
CD 44	65 (11%–100%)
CD 73	94 (52%–100%)
CD 90	99 (87%–100%)
CD 105	38 (6%–100%)
Markers of hematopoietic cells	
CD 34	0.08 (0%–6%)
CD 45	0.04 (0%–9%)

MSCs, mesenchymal stem cells.

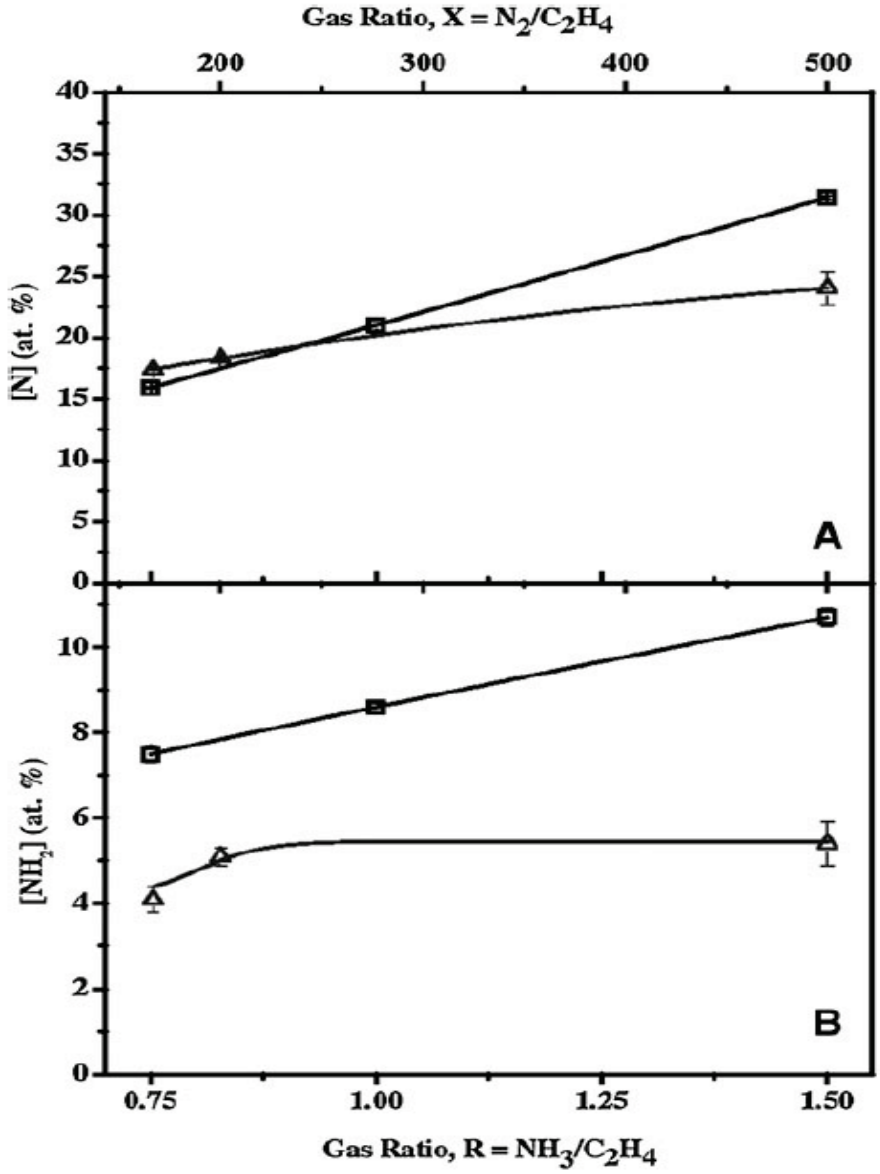

Figure 1. (A) Concentrations (in atomic %) of nitrogen, [N], and (B) of primary amines, [NH_2], in L-PPE:N (□) and H-PPE:N (Δ) deposits, as functions of the gas mixture ratios, R≡ F_{NH3}/ F_{C2H4} (for L-PPE:N), or X≡ F_{N2}/ F_{C2H4} (for H-PPE:N, see text).

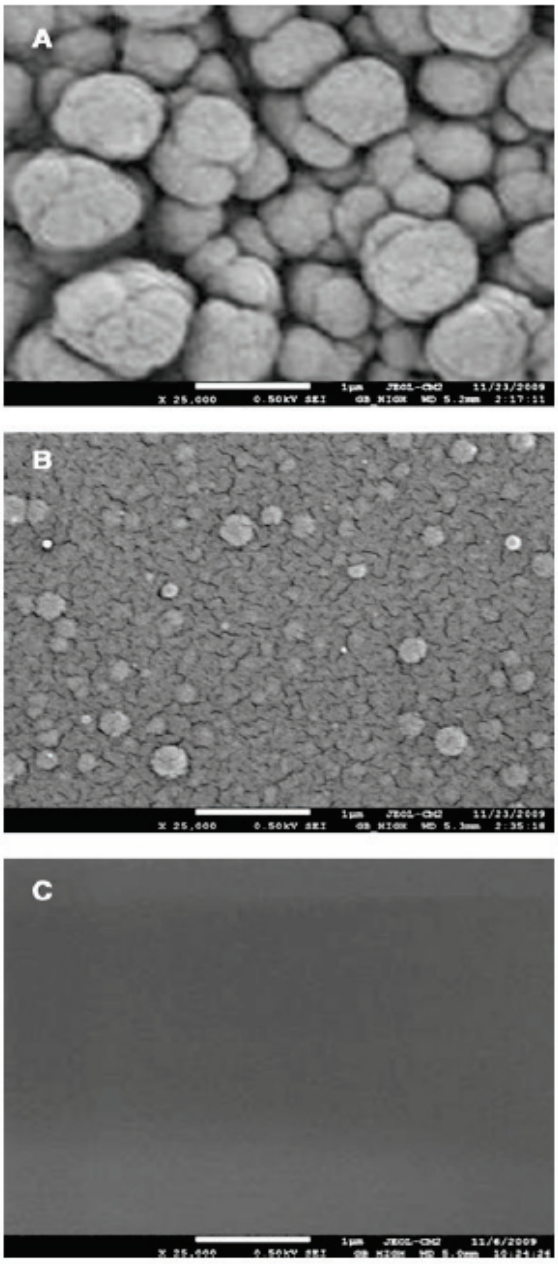

Figure 2. Surface morphologies of PPE:N coatings. Images of H20-PPE:N (A), H50-PPE:N (B), and L0.75-PPE:N (C) surfaces were acquired by field-emission scanning electron microscopy (FE-SEM).

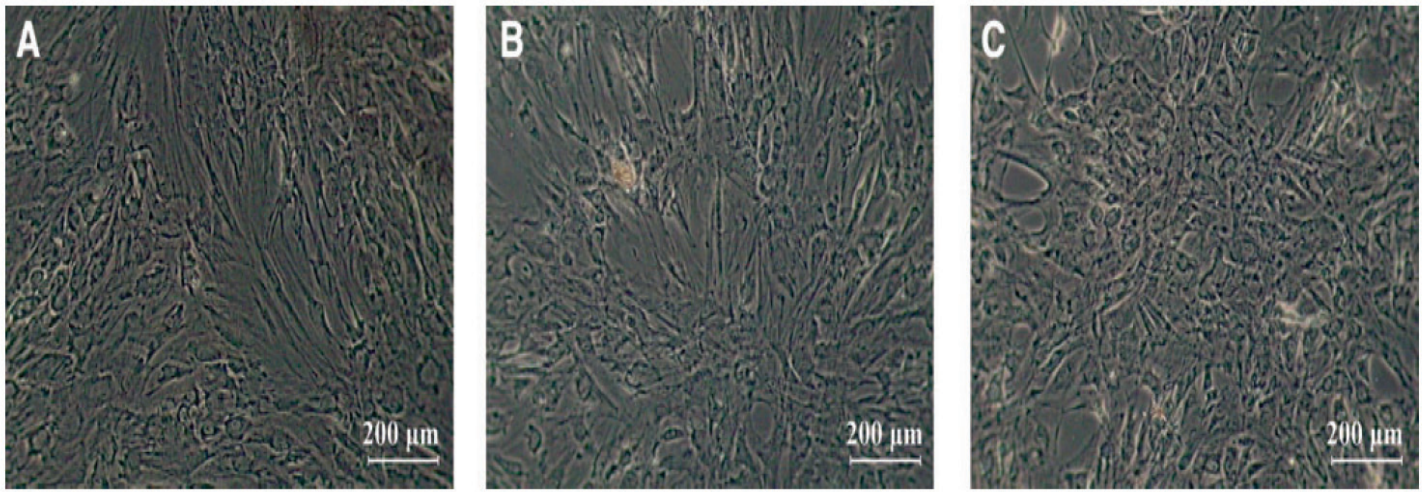

FIG. 3. Growth of MSCs from OA patients on PS and PPE:N surfaces. MSCs were grown for 7 days on PS control **(A)**, L-PPE:N **(B)**, and H-PPE:N **(C)**. Pictures were taken using an Axiovert 25 phase-contrast microscope (Carl Zeiss Canada, Toronto, ON). Magnification: 100×. MSCs, mesenchymal stem cells; PS, polystyrene; OA, osteoarthritic. Color images available online at www.liebertonline.com/tea

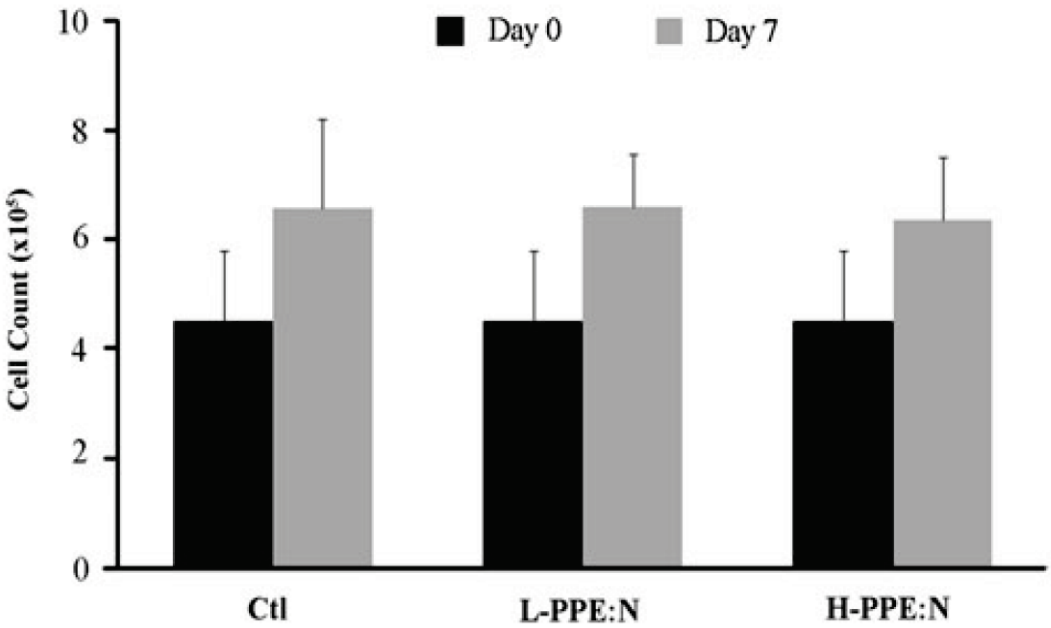

Figure 4. MSCs cell proliferation on PS (Control; Ctl), L-PPE:N, and H-PPE:N surfaces. Cell count was determined using an hemacytometer after 7 days in culture.

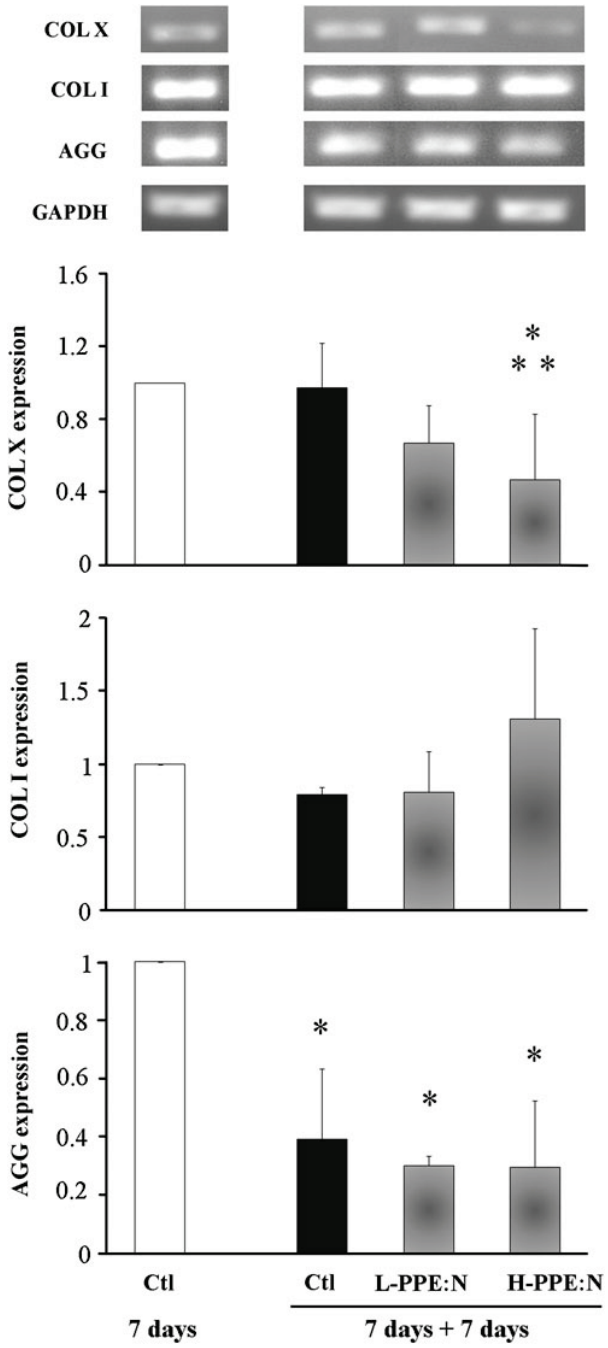

Figure 5. Expression of COL X on PS and PPE:N surfaces. MSCs from OA patients were cultured on PS (Control; Ctl), L-PPE:N, H-PPE:N and for 7 days. Total RNA was extracted and mRNA levels measured by RT-PCR as described in Materials and Methods. GAPDH was used as housekeeping gene and served to normalize the results. Quantitative results are the mean ± standard deviation of 4 experiments. * $p < 0.05$ vs. PS control at 7 days.

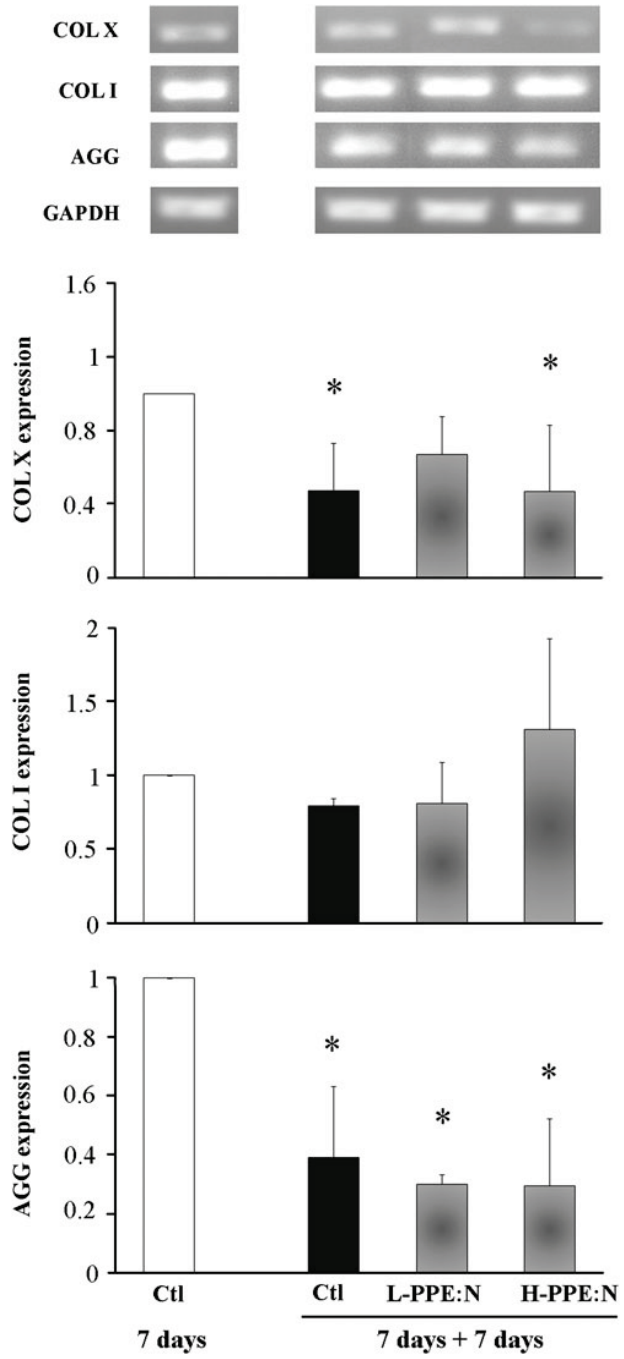

Figure 6. Expression of COL X in serum-free media. MSCs from OA patients were cultured on PS (Control; Ctl, 7 days), L-PPE:N and H-PPE:N for 7 days. Cells were then cultured on PS surfaces for an additional 7-days period in serum-free media. Total RNA was extracted and mRNA levels measured by RT-PCR as described in Materials and Methods. GAPDH was used as housekeeping gene and served to normalize the results. Quantitative results are the mean ± standard deviation of 4 experiments. * $p < 0.05$ vs. PS control at 7 days

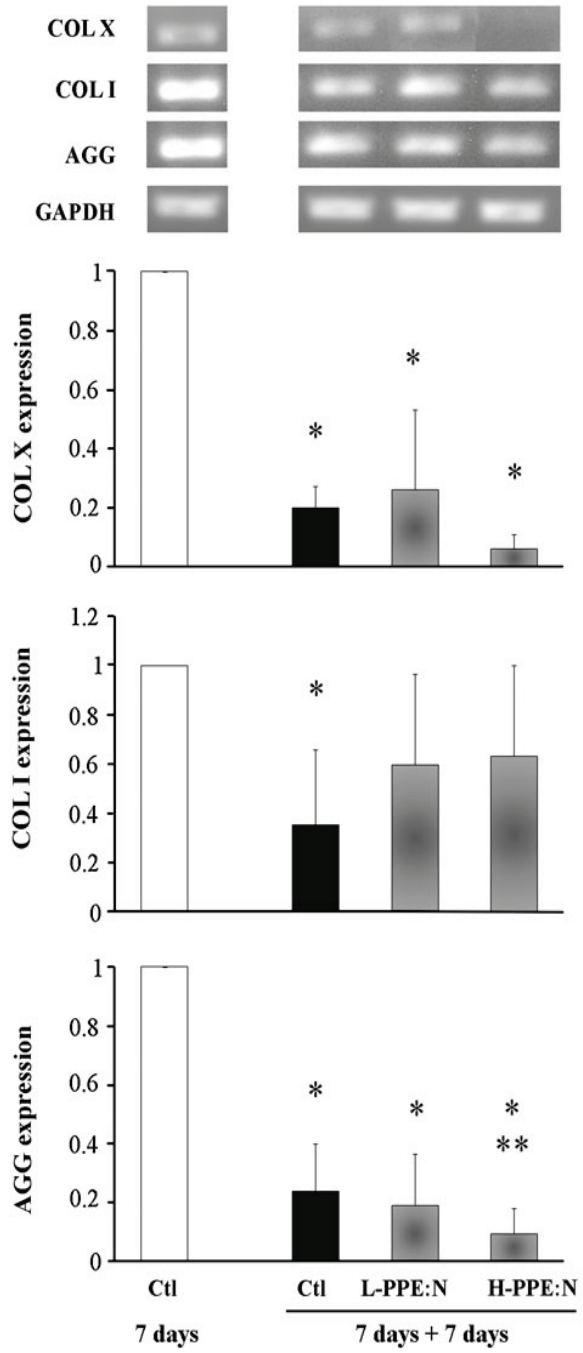

Figure 7. Expression of COL X in pellet cultures. MSCs from OA patients were cultured on PS (Control; Ctl, 7 days), L-PPE:N and H-PPE:N for 7 days. Cells were then cultured in pellet for an additional 7-days period in chondrogenic defined media. Total RNA was extracted and mRNA levels measured by RT-PCR as described in Materials and Methods. GAPDH was used as housekeeping gene and served to normalize the results. Quantitative results are the mean ± standard deviation of 4 experiments. * $p < 0.05$ vs. PS control at 7 days ** $p < 0.05$ vs. Control in pellet (7 days + 7 days).

SECTION 3: SUMMARY AND CONCLUSIONS

A) Summary

It has been previously shown that H-PPE:N, had an influence on the phenotype of cells of the intervertebral disc, more specifically, the nucleus pulposis cells [83]. This surface showed potential to influence the phenotype of notocordal cells [84] and to promote the healing around a newly implanted stent graft, after endovascular aneurysm repair [85], only to name a few of its applications. The primary amine concentration [NH_2] of these films is to be credited for the success of this surface in terms of cell adhesion and tissue engineering [86]. With this knowledge, we employed the use of another PPE:N surface, L-PPE:N. We speculated that this surface would be more promising than H-PPE:N, because it contains a significantly higher concentration of primary amines [NH_2], [87].

For all surfaces, the $[N]_{crit}$ corresponded to a constant value of 4.2 ± 0.5 (per 100 atoms measured by XPS). This confirmed the critical role of this (-NH_2) group in cell-surface interactions, particularly involving U937 monocytes, which had previously been under much speculation [86,88]. It is still necessary to investigate the critical role of (-NH_2) and its affect on the adhesion of other non-adherent cells.

The adhesion of U937 monocytes to PPE:N surfaces with $[NH_2] \geq [NH_2]_{crit}$ induced a transient expression of TNF-α and IL-1β, two cytokines related to immediate early response genes of these cells, suggesting that major inflammatory reactions were not induced. However, PPARγ, which is involved in the adhesion and retention of monocytes, had a more continuous expression.

Furthermore, we cultured MSCs on these surfaces and determined that COL X suppression was less effective when cultured on [NH_2]-richer (L0.75) L-PPE:N coating than on (H50) H-PPE:N. The L- and H-PPE:N coatings differ significantly in their surface morphologies. The ability of cells to adhere to solid surfaces can be strongly influenced by both by the surfaces' chemistries as well as their physical characteristics, particularly by micro-roughness [89]. The chemical

composition of [NH_2] in both L0.75 and H50 coatings do not differ drastically. HPPE:N however did show a significant capability to suppress COL X expression. Therefore, it can be considered that the morphological differences between these two surfaces may play a significant role in COL X suppression. The likely synergy between surface composition as well as surface morphology on MSC gene expression remains to be elucidated. This is now the object of future investigation.

A drawback of cartilage- and disc-tissue engineering is that human MSCs obtained from OA patients express high levels of COL X, [81,90] which is a marker of chondrocyte hypertrophy associated with endochondral ossification [91]. MSCs obtained from OA patients, a clinically relevant source of stem cells, when pre-cultured on N-rich PPE: N surfaces and transferred to pellet cultures can be used to engineer cartilage and disc *in vitro*, since these cells seem to be sensitive to their microenvironment, namely PPE:N pre-culture.

The technique of depositing nitrogen (N)-based organic thin film materials by plasma for biomedical applications, as well as the purposes of tissue engineering has been known for a long time, however, very amine-rich films prepared from composites of ethylene and nitrogen gas have only recently been applied to the specific tissue engineering reported here [81,92]. These surfaces were designed specifically to contain high primary amines [-NH_2] concentrations. They have in many instances been shown to influence cell-adhesion and other types of cell-behaviour.

We were the first to show that a particular type of PPE:N surface, H-PPE:N, was capable suppressing COL X expression in human MSCs, and of influencing the differentiation of MSCs *in vitro* [81]. The abilities of the coatings were largely attributed to the films' primary amine concentration. More recently, we showed that another type of PPE:N surface, L-PPE:N can be produced with higher concentrations of primary amines [NH_2], seeming more promising for the purposes of tissue engineering. The differences in primary amine concentration were considered to be noteworthy because we were aware that [NH_2] can have a

major influence upon cell-adhesion, among other characteristics [75]. We observed that the L- and H-PPE:N coatings differed not only in their compositions, but also in their surface-morphologies. It is well-known from the literature that the behaviour of cells adhering to solid surfaces can be strongly affected both by the surfaces' chemistries as well as their topological characteristics, such as micro-roughness [89].

In a previous study, we demonstrated that COL X was expressed when MSCs from OA patients were incubated in pellet culture in chondrogenic defined media [29]. Increased COL X expression was also observed in normal rabbit [53] and normal human [93] MSCs in pellet culture in chondrogenic defined media. We showed for the first time that the suppression of COL X can be maintained in pellet culture after culturing MSCs on PPE:N surfaces. Importantly, culturing on PPE:N surfaces was found to have little or no effect on COL I expression, suggesting that these coatings show promise for tissue engineering of cartilage and intervertebral discs. The observed decrease in AGG expression remains to be addressed. Future investigations will focus on looking at the addition of specific growth factors to stimulate AGG expression without increasing COL X expression.

B) Conclusions

The results presented in both manuscripts strongly suggest that the chemical composition, in this particular case the concentration of primary amines [NH_2], as well as the surface morphology of plasma-deposited coatings, more specifically of PPE:N films, affect the gene expression of MSCs. In addition, these cells, once reprogrammed on the PPE:N surface, are able to maintain their newly programmed phenotype when removed from the surface and transferred to other culture conditions. In addition, these surfaces seem to promote the adhesion of cells, which suggest that these surfaces offer promising opportunities for tissue engineering of cartilage and disc.

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APPENDIX

Abstracts

1. Rampersad S, Petit A, Ruiz JC, Wertheimer M, Antoniou J, Mwale Fackson. Using a Novel Nitrogen Rich Plasma Polymer, The Suppression of Type X Collagen in Mesenchymal Stem Cells from Osteoarthritic Patients is Maintained in Pellet Cultures. *17th Canadian Connective Tissue Conference CCTC, Montreal, Quebec (2011)*

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