[Frontiers in Bioscience 16, 458-471, January 1, 2011]

Crystalline calcium carbonate and hydrogels as microenvironment for stem cells

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1. ABSTRACT

Stem cell development and fate decisions are dictated by the microenvironment in which the stem cell is embedded. Among the advanced goals of tissue engineering is the creation of a microenvironment that will support the maintenance and differentiation of the stem cell – based on embryonic and adult stem cells as potent, cellular sources – for a variety of clinical applications. This review discusses some of the approaches used to create regulatory and instructive microenvironments for the directed differentiation of mesenchymal stem cells (MSCs) using three-dimensional crystalline calcium carbonate biomaterials of marine origin combined with a hydrated gel based on hyaluronan.

2. INTRODUCTION

It is a well-known fact that the cellular microenvironment contributes significantly to the complex of spatial and temporal signaling that directs the stem cell's developmental fate. But the regulatory mechanisms of the specific microenvironmental context – the extracellular matrix (ECM), soluble growth factors, hormones, and other small molecules – in which the cell develops and functions is poorly understood. The extracellular environment within the complex regulates an intracellular signaling cascade that influences cellular fate by activating or inhibiting the expression of proteins and genes. Three-dimensional (3D) hydrogels that can mimic the native microenvironment of

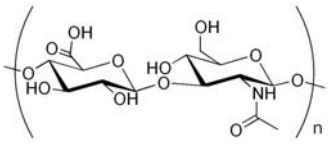

Figure 1. Chemical structure of hyaluronan. Repeating disaccharide units of hyaluronan comprise D-glucoronic acid and D-N-acetylglucosamine linked together via alternating beta-1,4 and beta-1,3 glycosidic bonds.

the cells *in vivo* provide a basis for investigations into hydrogels as tissue engineered constructs.

Recent advances in the hydrogel field provided cell research with a broad range of milieus – from pure natural to pure synthetic and including natural/synthetic hybrids which combine to make hydrogel systems dependable, malleable platforms that promote initial cell adherence and high level of biocompatibility. However, hydrogels lack sufficient mechanical strength to structurally support fully developed tissue formations. To overcome this limitation, the hydrogel can be combined with a solid, 3D scaffold that mechanically and functionally supports the tissue. The resultant biohybrid will comprise hydrogel and solid scaffold and will combine the growth-promoting properties of bioactive, ECM–mimicking hydrogels with the structural support of the bioactive scaffold material.

The ECM system is the dynamic complex within which cells grow; it fulfills multiple functions, from mechanical support to the direct regulation of gene expression and of cellular differentiation mechanisms. A cell-based tissue engineered strategy is therefore inextricably tied with the provision of a local microenvironment of signaling molecules that supports cells maintenance and differentiation. The bioengineering of functional tissues necessitates the presence of special supporting materials that resemble as closely as possible the tissue's natural milieu, which promotes self-renewal by insuring that appropriate conditions exist for controlling cell differentiation and tissue remodeling.

Recently, natural crystalline structural materials were found to incorporate a variety of chemical and architectural properties ideal for biomedical applications (1-4). At the foundation of these findings is evolution: driven by natural selection, evolution optimized the support and maintenance functions of vertebrate and invertebrate skeletal materials. A series of studies on the skeletons of corals and other marine invertebrates found that some were applicable as instructive scaffolding materials for a variety of both cell-based and acellular applications (1-4).

The present work describes a biocomposite (biohybrid) comprising a natural calcium carbonate scaffold combined with hyaluronic acid (hyaluronan) based hydrogel. Some *in vitro* models that have demonstrated the potential are reviewed as are some possible strategies for the biomedical utilization of these biomaterials. The cellular response to the instructive microenvironment that was created and possible mechanisms behind the action of the calcium carbonate-hyaluronan composite are discussed.

3. HYDRATED GEL CONSTRUCTS

Naturally occurring hydrogels (highly hydrated gels), considered the optimal environmental milieu for supporting the *in vitro* growth of cells and tissue explants, are thought to mimic the continuum of the cell cytoplasm. The latter comprises an aqueous, cohesive gel suspension of solutes that retains its integrity even in the absence of plasma membranes, thus allowing free diffusion. Their hydrophilicity and intrinsic similarity to the ECM framework make hydrogels, which are created from a large variety of constituents, highly customizable, 3D networks. Hydrogels can also form molecularly permeable network, as they contain various fluids and immobilized enzymes that effectively control slow-drug release. Hyaluronan in solutions occupies large hydrated volumes at unusually low concentrations. Because its hydrodynamic properties are markedly affected by solution ionic strength, even a small environmental change in hydration will lead to a major change in physical structure that can effect an increase of as much as 1000 fold in hyaluronan hydrated volume.

It is believed that the first cells on earth were assembled in a hydrogel environment, which retains water, oily hydrocarbons, solutes and gas bubbles, and which has the ability to function without particulate membranes. Indeed, the hydrogel provides a stable, adjusted microenvironment for cell macromolecules and for cell division, differentiation and development. Thus, hydrated gel systems with specific growth requirements are the ideal environment for cell evolvement. Specifically, hydrated gels are attractive systems for cell cultures for the production of composite implants, constructs, and tissue engineered products for regenerative medicine. Furthermore, the combination of hydrogels with mineralized scaffolds made of marine derived matrixes has a synergistic effect (5, 6).

4. HYALURONAN

Hyaluronic acid (HA) or hyaluronan is a very large, high-molecular-weight, linear glycosaminoglycan (GAG) comprising 2,000 to 25,000 disaccharide units of glucoronic acid and N-acetylglucosamine (beta1,4-GlcUA-beta1,3-GlcNAc-)$_n$ (Figure 1). Unique among the GAGs, this versatile polysaccharide has an extracellular biosynthesis site (on the plasma-cellular membrane) that lacks a core protein acceptor, its chemical composition is devoid of sulphated esters, and it has several distinct and diverse biological functions. For example, particularly, during early development, hyaluronan serves not only as a passive space filler, it also activates and regulates essential biological events by mediating cell behavior via its effects on cellular migration, proliferation, and differentiation. Furthermore, hyaluronan is a major component of tissues and is ubiquitously distributed in the ECM where cells migrate, divide, and differentiate – of invertebrate and vertebrate organisms. In addition, hyaluronan plays a

pivotal role in the homeostasis of physiological events such as tissue regeneration, wound healing and tumorigenesis.

In the tissues hyaluronan plays a passive structural role based on its unique biomechanical and hydrodynamic properties (7, 8). Hyaluronan expands the extracellular space by binding salt and water, and it promotes a highly hydrated ECM that facilitates cell movement (reviewed in Toole, 2004 (9)). In tissues such as cartilage, vitreous humour of the eye and skin hyaluronan is a supporting element in supermolecular aggregates that are created by specific interactions of hyaluronan with other macromolecules (10-13). On the other hand, hyaluronan is involved in intracellular signal transduction and in the activation of signaling pathways.

Hyaluronan-mediated signals are transmitted through membrane-localized receptors (hyaluronan receptors), many of which have been identified, including CD44 (14, 15), RHAMM (receptor for hyaluronic acid mediated motility) (16, 17), lymphatic endothelial hyaluronan receptor (LYVE-1) (18), toll-like receptor-4 (TLR4) (19, 20) and others (for reviews see (21, 22). Many hyaluronan-binding proteins contain a common domain, termed a Link module, which is involved in hyaluronan binding.

4.1. Link module

Hyaluronan-binding proteins (often termed hyaladherins) typically contain a common domain known as the Link module, which mediates the interaction of hyaladherins with hyaluronan. Most members of the hyaladherin superfamily, such as cartilage-link proteins and chondroitin sulfate proteoglycans, have large hyaluronan-binding domains that contain two tandem Link modules (23). The Link module of the hyaluronan receptor CD44, however, requires N- and C- terminal extensions to ensure the proper folding and correct functional activity of its binding domain. In TSG-6 (the protein product of the tumor necrosis factor-stimulated gene-6), which is composed mainly of contiguous Link and CUB modules, the Link module is sufficient to mediate a high affinity interaction with hyaluronan (24).

To ensure that optimal hyaluronan-Link module binding is achieved, the hyaluronan should be at least of octasaccharide (HA_8) length (24). Hardingham and Muir (25) reported that decasaccharides were the smallest HA fragments able to bind strongly to CD44, while Lesley *et al.* showed that only oligosaccharides larger than 20 residues could interact simultaneously with the two hyaluronan receptors (26). Actually, the longer the sugar chain, the more linked binding sites are present; alternatively, the higher the overall receptor density, the more binding activity is achieved. In both cases, signal strength increases.

The affinity of CD44 to hyaluronan is thought to be regulated from inside the cell (27) and can be modulated by cytokines (28) and by the hyaluronan-binding protein TSG-6 (the secreted product of tumor necrosis factor-stimulated gene-6) (29). It has been shown that CD44 affinity to hyaluronan requires both a very specific glycosylation pattern (26, 30) and also that the hyaluronan-binding domain of CD44 exhibit proper helical folding (31, 32). The participation of hyaluronan in highly-selective interactions with proteins is possible via the precise spatial folding of the hyaluronan chains; as such, hyaluronan provides a multivalent scaffold on which other bioactive molecules are assembled in multiple arrays.

4.2. Biological properties of hyaluronan as a result of conformation

A function of environment, the molecular conformation of hyaluronan in aqueous solutions significantly affects the biological functions of the biopolymer. Hyaluronan structural properties, such as the hydrophilic character of its backbone, its extended chain configuration, and its ionizability confer upon it a unique behavior pattern that explains its abundance and multiple functions. An efficient, network-forming polymer, hyaluronan forms secondary and tertiary structures by adopting a highly organized packing arrangement for its polysaccharide chains. The biological functions of hyaluronan, such as activation of its receptors and formation of macromolecular aggregates within the extracellular matrix, are also affected by the molecule's spatial conformations. A key determinant of the molecular basis of hyaluronan recognition by proteins is hyaluronan's molecular conformation, which is affected by the microenvironment. The biological functions of hyaluronan vary markedly depending on whether it is in specific binding interaction with the tertiary or quaternary structures of proteins and the level of water and ions present in its microenvironment. Hyaluronan conformation, therefore, is affected by specific ion interaction, ionic strength, and the hydrated properties of the ions in the local environment.

4.3. Clinical applications of the hyaluronan-based constructs

Biological hygrogels are produced from agarose, alginate, chitosan, hyaluronan, collagen fibrils, glucosaminoglycans and self-assembled peptides cross linked to achieve the desired form. All of these polymers make attractive scaffolding materials, as they can be synthetically tailored to simulate or mimic natural tissues. The tissue fabrication process is further augmented by combining the polymer base with selected additives.

Adhesive peptides, such as RGD (arginine-glycine-aspartic acid), can be added to the hydrogel mixture to promote the binding of many types of cells and to improve hydrogel functionality. Such additives can enhance cell migration, proliferation, growth and differentiation and improve tissue organization. In addition, they also act as encapsulation barriers and confer drug delivery capabilities of the tissue.

Hyaluronan-based biomaterials were initially introduced as cross-linked scaffolds to promote neurite outgrowth in patients with spinal cord injuries and to support spinal cord regeneration (33-35). Additionally, the presence of hyaluronan, especially during the early developmental stages of axon decussation in the chiasm,

affects optic nerve interaction with CD-44 and the routing of axons in the optic nerve chiasm (36-38). Hyaluronan-based gels have also been used to facilitate peripheral nerve reconstruction following microsurgical procedures (39-41). Multilayered, hyaluronan-based hydrogels support a variety of differentiated cells, including those of cartilage and bone, and embryonic stem cells (42).

In addition, a depolymerized, degraded hyaluronan based material was sprayed on metal stents to prevent the formation of neointima (43). Finally, hyaluronan- based hybrids are in use with collagen (44-46), with chitosan and gelatin (47), with polyelectrolyte PLL (48), and with cellulose (49), and they have also been cross-linked with 2-chloro-1-methylpyridinium iodide (50).

5. MESENCHYMAL STEM CELLs (MSCs)

Multilineage progenitor cells, MSCs can be induced to differentiate, after expansion *in vitro*, into several cell lineages, including osteogenic and chondrogenic lineages (particularly the former). Due to their differential abilities, MSCs are ideal candidate cells for bone and cartilage tissue engineering (51-53). In adults, MSCs contribute to the maintenance of various tissues. Adult MSCs can be harvested from bone marrow or other tissues of mesenchymal origin, and they are able to expand in culture for a number of passages (54-56). Their multipotency encourages the utilization of MSCs in regenerative medicine, but it also emphasizes the importance of the microenvironment, which plays a pivotal role in regulating the differential fates of MSCs. It is now evident that the MSCs, which are regulated extracellularly, have the capacity to sense changes in the local milieu (57-59).

5.1. The importance of microenvironment in MSCs growth and development

In mammalian tissues, the ECM comprises fibrillar and non-fibrillar matrix proteins, glycoproteins, and GAGs that provide the cells with structural support. A variety of other molecules, such as enzymes, cytokines, hormones, ions, and vitamins embedded in the matrix, participate in tightly regulated, dynamic cooperation with the cells. Receptors expressed on MSC membranes act as sensors that transfer the ligand-initiated molecular signals into the cell. As such, the ECM activates signaling pathways that dictate the fate of the entire cell. Changes in the structure and composition of the cellular environment mediate MSC signaling, showing how the microenvironment regulates events of cellular fate, including proliferation, migration and differentiation. Cell surface receptors on MSCs act as important sensors of microenvironmental changes, thereby regulating signal transduction in response to the spatial behavior of their major ligands. The large number and variety of extracellular stimuli that initiate and regulate cellular events suggest that multiple signaling pathways are involved in these complex processes, which function according to precisely orchestrated scenarios. Although a number of key signaling pathways have been identified, our understanding of these pathways is far from complete.

Among the most important signaling molecules is hyaluronan, which, via its specific receptors, initializes and mediates basic cellular events during development and growth. The initiation of morphological events, including cellular reorganization and phenotypic transitions during the epithelial-mesenchymal transition, joint cavity formation, and endochondral ossification, is preceded by the temporal and spatial up-regulation of hyaluronan. The accumulation of hyaluronan at sites where these processes are occurring preserves the cells in an undifferentiated state while concomitantly stimulating their proliferation. Once cell and matrix reorganization have been accomplished, hyaluronan is down regulated and degraded via several well-established mechanisms. Hyaluronan functions similarly at sites of bone fractures, callus formation, and wound healing, for which it assists mesenchymal cells in migrating to the regions requiring regeneration (60, 61).

To successfully employ MSCs in the regenerative treatments of bone and cartilage defects, the most efficient 3D scaffold must first be selected. Among the biomaterials with the greatest potential to simultaneously support cell growth and direct cell differentiation, are the mineralized, marine-origin scaffolds.

6. CRYSTALLINE CALCIUM CARBONATE OF MARINE ORIGIN

It was first proposed in the 1970s that the skeletons of different corals could be exploited as biomaterials. Marine skeletal material usually comprises the crystalline polymorphs of calcium carbonate ($CaCO_3$), i.e., either in calcite or aragonite. Magnesium and strontium are also part of the physicochemical biomineralization process, though in small ratios. The biocomposite is highly biocompatible, facilitates the rapid adhesion of most mammalian cell types, and promotes the proliferation of those cells. Later it was suggested that the porous structure and characteristically bioactive nature of the mineralized skeleton promote osteogenesis *in vitro* and *in vivo*, such that the skeletons of corals and other marine invertebrates are considered instructive scaffolding materials for a variety of tissue engineering applications. Among the marine biomaterials that have been the most intensively studied for their biomedical utilization are those based on aragonite and coralline derived biomaterials. A recently reported study on the exoskeleton of the sea barnacle *Tetraclita rufotincta*, which incorporates a calcite polymorph of calcium carbonate, showed that it has scaffolding properties – biocompatible, rapid cell adherence and growth – similar to those of aragonite (1). Although calcite's biocompatibility features are parallel to those of aragonite, cells cultured on the two polymorphs exhibit distinctly different behaviors, an outcome due possibly to the distinct microenvironments created by the two biomaterials.

6.1. Aragonite-based materials

Corals are the most thoroughly studied marine invertebrates in terms of both basic and applied biomaterials. Sessile, long-living colonial organisms that populate the luminous oceans of the tropics, corals are the

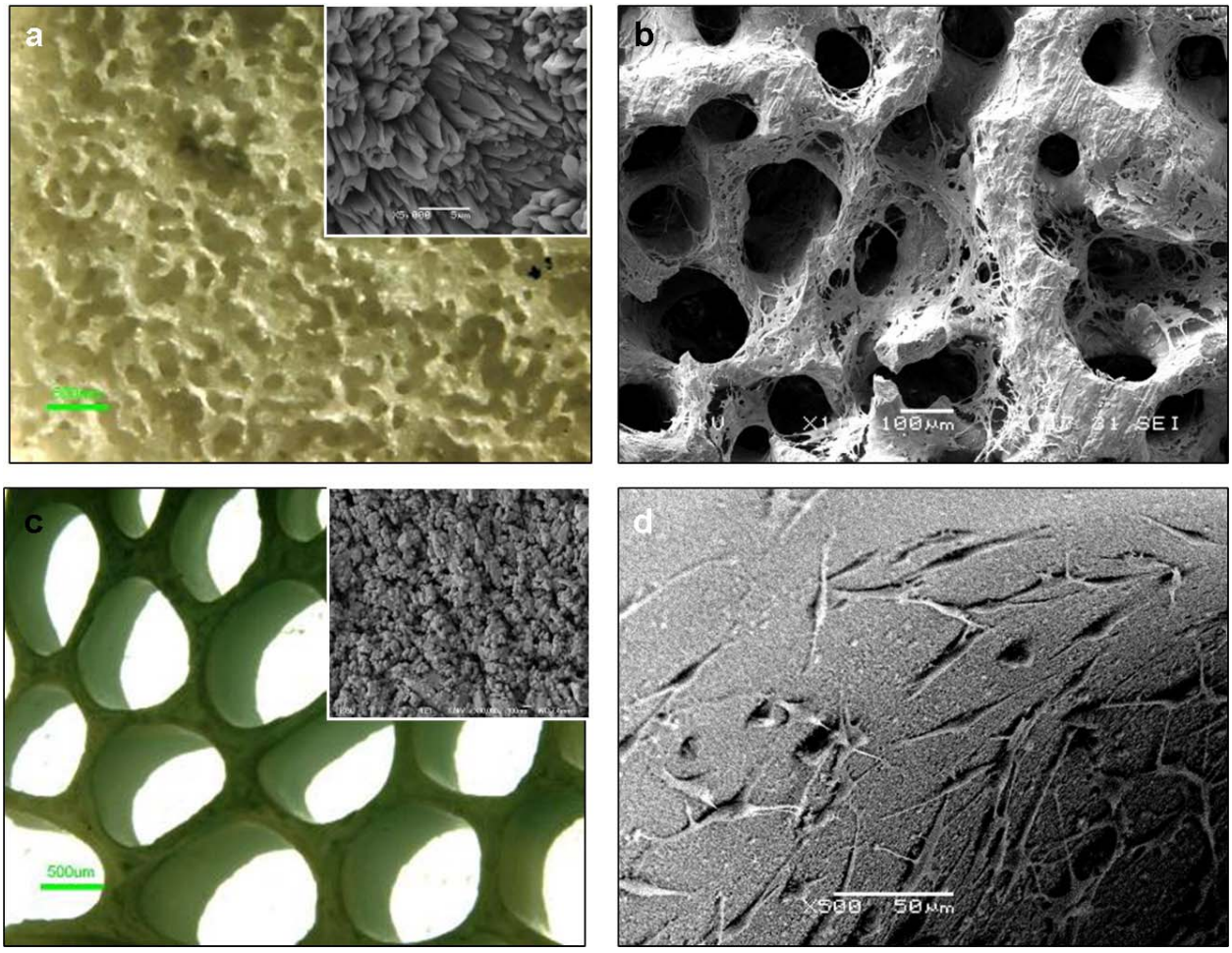

Figure 2. Three-dimensional matrixes of marine origin. Light microscope images of *Porites lutea* (a) and *Tetraclita rufotincta* (c). Bar = 500 um. Insets in the upper right corner are scanning electron microscope (SEM) micrographs of aragonite (a) and calcite (c) revealing the complex topography of the crystals. SEM micrographs show MSCs 72 h post-seeding adhering to the aragonite (b) and calcite (d) biomaterials.

main tropical reef builders. The sole constituent of coral skeletal material is aragonite (2, 62).

Coral species from the genera *Porites*, *Acropora* and *Gondiopora*, and the fire coral *Millepora dichtoma*, are the species most often tapped for scaffolding materials due to their homogenous architectures, which resemble that of trabecular bone. These materials have long histories as biocompatible grafting materials that support *in vitro* osteogenesis and *in vivo* bone tissue remodeling (63-67). Their high level of porosity and interconnecting pores promote the ingrowth of fibrovascular vessels (68, 69) and neoformed tissue (70-72), and their transient mechanical properties facilitate hard tissue remodeling after implantation of the biomaterial (73). A series of recent studies showed that seeding natural coralline lattices with MSCs resulted in fast adhesion, proliferation, and osteogenesis and, as a result, in the formation of mineralized tissue (74, 75). It was observed in those studies that MSCs underwent osteogenic differentiation without the need to add any bone-promoting factors to the growth medium. It was therefore suggested that the 3D structure and the surface topography of the porous aragonite lattice play important roles in determining MSC fates and that calcium availability may be a causative factor contributing to the highly osteogenic microenvironment.

6.2. Calcite-based biomaterials

Another crystal phase of calcium carbonate, calcite is also present in the exoskeletons of marine invertebrates, but in terms of its use as a biomaterial, it has received much less basic and applied scientific attention than aragonite. Our recent study reported on the possible application, as a cell-supporting lattice, of a barnacle exoskeleton composed solely of calcite (1). The potential of calcite to serve in cell supporting biolattices is based on its distinct architectural morphology relative to that of coralline aragonite (Figure 2) and on the differences in its porosity and pore design. Although distinctly different from aragonite, calcite displays the same level of biocompatibility as aragonite, promoting the cell's initial attachment, growth, and proliferation. Surprisingly, when

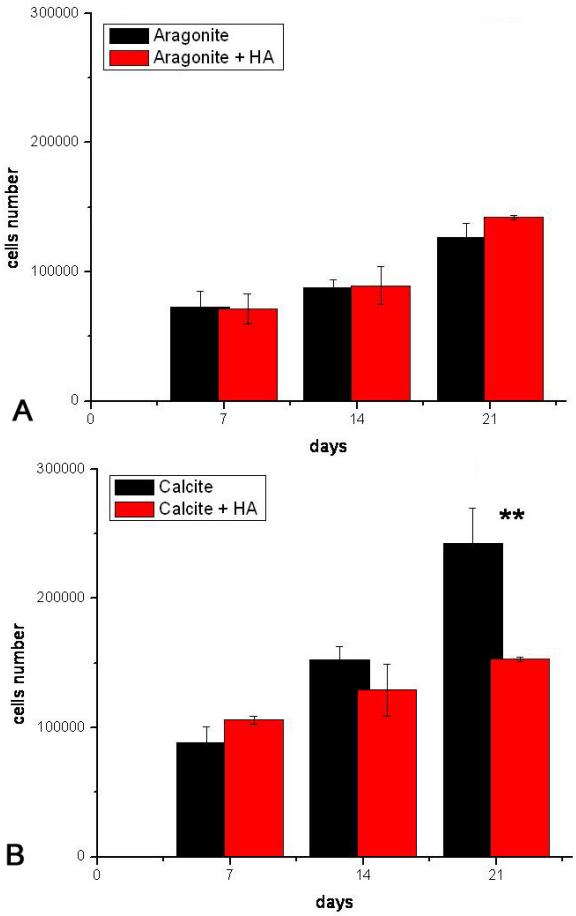

Figure 3. Cell proliferation chart of MSCs cultured on aragonite (a) and calcite (b) in the presence or the absence of hyaluronan for 21d, evaluated using the Alamar Blue™ Cell Proliferation Indicator. Initial cell density at day 0 was 10,000 cells per matrix. Cell numbers were calculated at 7, 14, and 21d. Cell proliferation rates on the aragonite did not exhibit statistically significant differences in the presence or the absence of hyaluronan (a). In contrast, cell proliferation rates on the calcite-hyaluronan biocomplexes were significantly different from those on the calcite matrixes without hyaluronan at 14 and 21d, but not at 7d (b). In the absence of hyaluronan, cells on the calcite matrixes showed linear proliferation. The experiment was performed in six duplicates, and the statistical analyses was performed with a two-tailed Student's t test (** $P < 0.005$). Data are represented as the mean. The error bars represent the standard deviations.

seeded with MSCs, the calcite lattice exhibits a more chondrogenic than osteogenic character. Histological staining and gene expression patterns showed that MSCs cultured on calcite lattices appeared to differentiate toward the chondrogenic phenotype (1). However, in long-term cultures (up to six weeks), the cells lost their chondrogenic phenotypes and demonstrated matrix mineralization and other features characteristic of the osteogenic phenotype. A possible explanation for the initial chondrogenecity of the calcite biolattice may lie in the greater stability of the calcite crystals, which release calcium more slowly than do the crystals of aragonite, thus providing the preferred microenvironment, albeit insufficient for permanent phenotype maintenance, for chondrogenic differentiation.

7. HYALURONAN-CALCIUM CARBONATE BIOHYBRIDS

The exoskeletons of some sessile marine organisms are highly bioactive, and as a result, they promote the adhesion of a variety of cells and trigger stem cell differentiation (for a review see 3, 4). The combination of a calcium carbonate-based bioactive scaffold with a biological polymeric hydrogel is designed to mimic the organic-mineral composite of developing bone by providing a fine-tuned microenvironment. The calcium carbonate scaffold triggers initial cell interactions and MSC differentiation, and we hypothesize that the presence of hyaluronan promotes chondrogenic differentiation of the cells. The efficacy of using hyaluronan to promote cell growth has been tested by creating a hybrid via the self-arrangement of high molecular-weight chains of hyaluronan dissolved in 0.05%-PBS on the surface of an aragonite or calcite bio-lattice. The hybrid composite shows promise as a material for connective tissue engineering as it combines a bioactive hydrogel, which mimics the ECM, with a bioactive supporting material.

Indeed, MSCs seeded onto the hybrid composite demonstrated rapid adhesion, proliferation, and differentiation. The kinetics of cell proliferation in the presence and the absence of hyaluronan were evaluated using the Alamar Blue™ Cell Proliferation Indicator. Measurements were taken at 7, 14, and 21 d (Figure 3). The graphs show how cell numbers changed from an initial (at day 0) density of 10,000 cells per matrix on both the aragonite and calcite matrixes with and without hyaluronan, thus revealing the effect of hyaluronan on cell proliferation rates. For cells cultured on aragonite, there was no statistically significant difference in the cell numbers between those cultured with or without hyaluronan, and in both cases (with or without the hyaluronan), the cells showed linear proliferation between time intervals (Figure 3a). Conversely, the proliferation kinetics of the cells cultured on calcite were significantly affected by the addition of hyaluronan (Figure 3b). The number of the cells cultured on the calcite-hyaluronan biohybrid reached a maximum at 7 d, showing no statistical difference at either 14 or 21 d. The cell count for cultures on calcite in the absence of hyaluronan, however, increased linearly during the entire experiment, ultimately exceeding, at 21d, the cell count on aragonite culture. The difference in the number of the cells cultured on calcite and on aragonite may be the result of the dissimilar matrix architectures, which translates into correspondingly different matrix surface areas. The slower proliferation kinetics of the cells grown on the calcite-hyaluronan biocomplex could indicate that the differentiation processes prevailed over the proliferation processes.

To evaluate MSC differentiation on the biohybrids we performed several tests. Sulfated GAGs

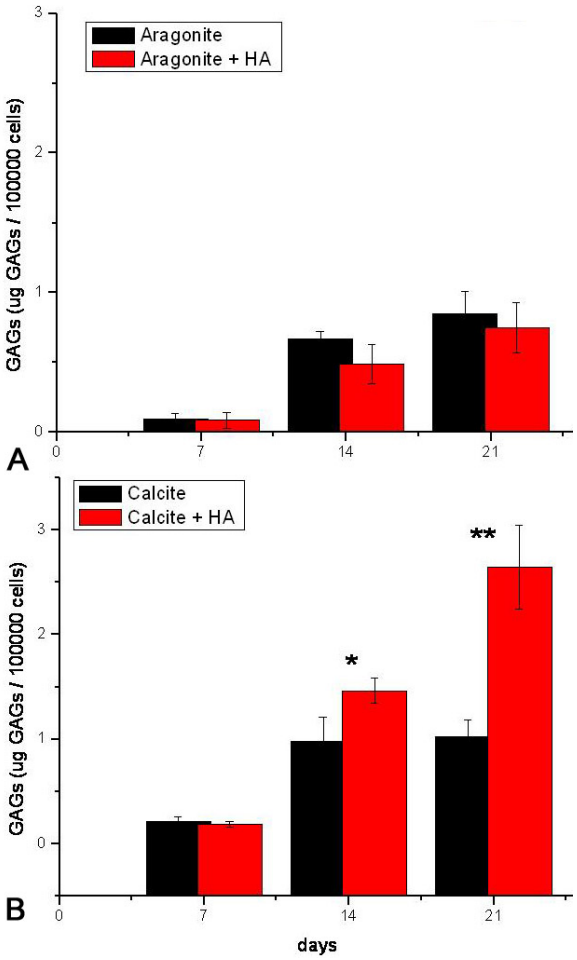

Figure 4. Sulfated GAG accumulation in tissues evaluated by DMB assay. GAGs amounts were evaluated at 7, 14, and 21 d and represented in µg GAGs per 100,000 cells. GAG release was evaluated after papain digestion of the tissues for aragonite versus aragonite-hyaluronan (**a**) and for calcite versus calcite-hyaluronan (b). The experiment was performed in six duplicates. Statistical analyses were performed with a two-tailed Student's t test (* $P < 0.05$; ** $P < 0.005$). Data are represented as the mean. The error bars represent the standard deviations.

quantification by 1,9-dimethymethylen blue assay was performed after digestion with papain (Figure 4). GAGs accumulation in the ECM of the tissue culture indicates chondrogenic differentiation of the tissue. The amount of GAG was evaluated at 7, 14, and 21 d and normalized to the cell numbers. We found that the addition of hyaluronan did not affect GAG accumulation by the cells cultured on the aragonite. Although the total GAG accumulation increased over the time of the experiment, it was low compared to that measured for the calcite and calcite-hyaluronan cultures (Figure 4a). For the cells cultured on the calcite and calcite-hyaluronan biocomplex, however, GAG accumulation substantially increased in the presence of hyaluronan compared with the cells cultured on calcite alone (Figure 4b). One possible explanation for the large difference is that the calcite-hyaluronan biohybrid stimulated GAG accumulation and initiated chondrogenic differentiation of the MSCs.

To further investigate MCS differentiation, we ran immunohistochemistry analyses of collagen I and II (Figure 5). Collagen I is a metabolic marker of osteogenesis. As one of the most important components of the ECM of native articular cartilage, collagen II is expressed in ECM tissue cultures during chondrogenic differentiation of the cells. The results of immunohistochemistry staining with monoclonal antibodies against collagen I and collagen II of MCSs show that the cells cultured on the aragonite matrixes express both collagen I and collagen II, indicating the heterogenic character of the tissue formed (Figs. 5a-c and m-o). Collagen I expression on aragonite tended to increase during the culture period, however, and on day 21 it exceeded the expression of collagen II (Figure 5c), indicating that osteogenesis predominated. The addition of hyaluronan to the aragonite did not significantly change cell differentiation fate (Figs 5d-f and p-s). When the MCSs were cultured on the calcite, however, collagen I expression increased while that of collagen II decreased over the experimental period (Figs. 5j-i and t-v). In contrast, cells cultured on the calcite-hyaluronan hybrid showed much stronger collagen II than collagen I expression, a trend that increased over time (Figs. 5 j-l and w-y), providing evidence that the calcite-hyaluronan biohybrid promotes MSC chrondrogenic differentiation.

The differential fates of the cells depended on which scaffold – aragonite or calcite – was used in their culture. On the aragonite-hyaluronan complex, the tissue formed had a heterogenic character that tended slightly toward osteogenic development. But when cultured with the calcite-hyaluronan complex, the cells displayed a strong chondrogenic tendency as they underwent chondrogenic differentiation and maintained the chondrogenic phenotype in long-term cultures. Thus, in the case of calcite, the addition of hyaluronan supported and promoted the chondrogenic potential of the calcite biomaterial. The cells cultured on the calcite-hyaluronan complex had the spherical morphologies characteristic of chondrocytes (76) and well– developed extracellular matrix (Figure 6). In contrast, cells cultured on the aragonite-hyaluronan complex exhibited fibroblast-like morphologies.

The divergent results of the experiments to test calcite or aragonite complexes with hyaluronan suggest that the microenvironment created at the calcium carbonate - hyaluronan interface is a causative factor. The interactions of the cells with their microenvironment trigger cellular responses that activate the cells' fate mechanisms. The character of that microenvironment varies depending on which crystal lattice, aragonite or calcite, is used, as the two surfaces arrange the hyaluronan chains differently (Figure 7). Therefore, we hypothesize that the essence of hyaluronan receptor activation depends on the hyaluronan molecule conformation. The evidence suggests that the calcite-hyaluronan interface creates a more suitable microenvironment for hyaluronan receptor activation than does the aragonite-hyaluronan interface, in which the

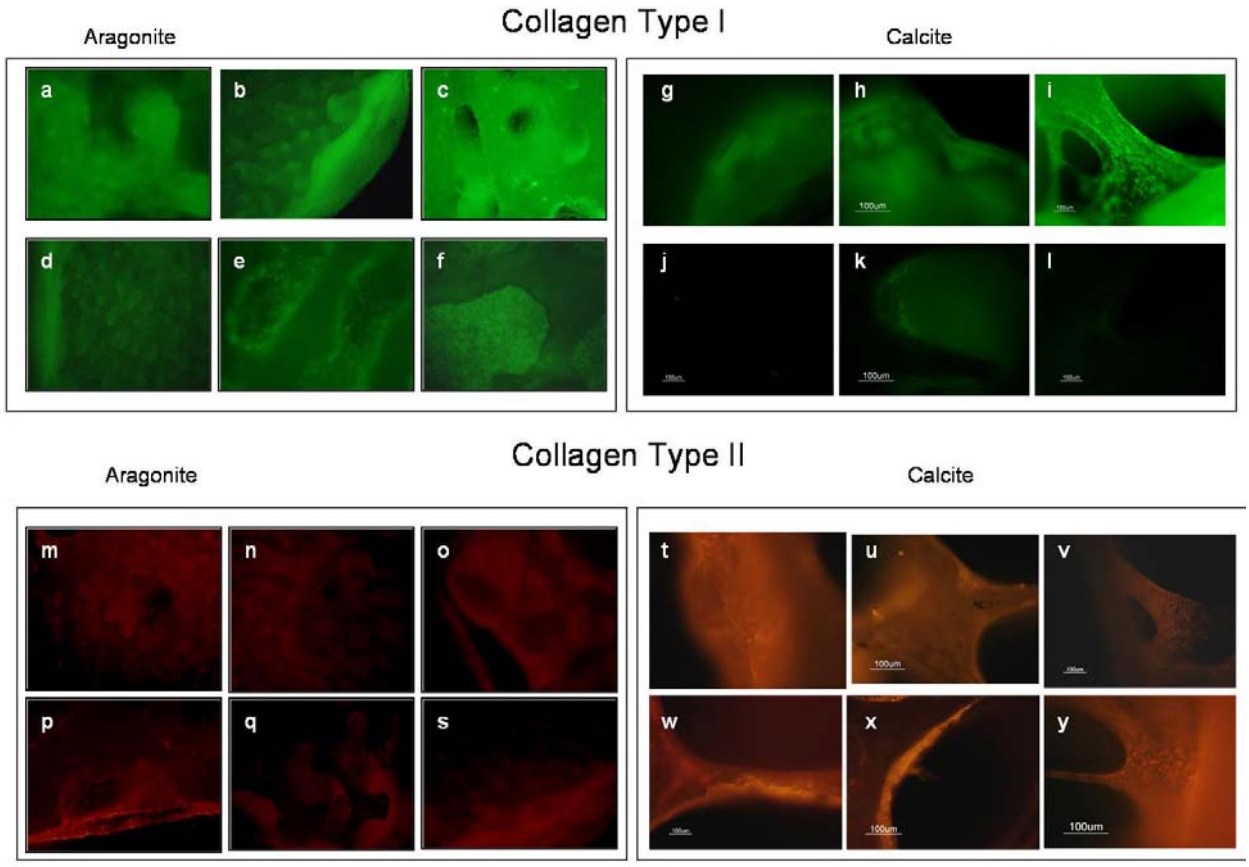

Figure 5. Immunohistochemical staining with monoclonal antibodies for the detection of collagen type I and collagen type II of MSCs cultured on aragonite (a-c for collagen I and m-o for collagen II) and on calcite (g-i for collagen I and t-v for collagen II) matrixes without hyaluronan and with hyaluronan (aragonite-hyaluronan: d-f for collagen I and p-s for collagen II; calcite-hyaluronan: j-l for collagen I and w-y for collagen II) at 7 (a, d, g, j, m, p, t, v), 14 (b, e, h, k, n, q, u, x), and 21 (c, f, i, l, o, s, v, y) d. Both the aragonite and aragonite-hyaluronan constructs exhibit heterogeneous tissues in which both collagen I and collagen II are expressed, while an osteogenic trend prevailed in the tissue formed. In the calcite, collagen I expression increased while that of collagen II decreased over the experimental period. The tissue formed on the calcite-hyaluronan biohybrid exhibited strong collagen II and very weak collagen I expression, a result indicates that this biohybrid is better suited to the chondrogenic differentiation of MSCs.

conformation of aragonite-hyaluronan does not promote molecular interaction with the hyaluronan receptors.

8. SUMMARY AND PERSPECTIVE

An eventual goal in the design of tissue engineered scaffolds is a biologically active, 3D hybrid system that promotes specific cellular attachment and proliferation to ultimately produce functional tissue. Understanding the regulatory cues of tissue remodeling remains a challenge for basic science, which will bridge the gap between developmental research and applicative engineering. Investigations into the approaches to stimulate cells to organize into tissue can provide better insight into the role matrix interactions will have on cellular function.

Hyaluronan molecules are commonly found in the typically long, linear, stretched chains of sodium hyaluronates. Under such conditions, the negative charges of the carboxyls are poorly compensated – only a limited number of negative charges are neutralized by the sodium ions. Because they are hydrated, the sodium ions occupy a relative large volume, such that they can occupy the positions between the chains of hyaluronate simultaneously only in limited numbers. These conditions lead, in turn, to maximum repulsion by, and distance between, the negative charges of the carboxylic groups. When hyaluronan molecules are exposed to calcium carbonate scaffolds, however, the sodium ions are replaced by calcium ions that succeed in compensating most of the negative – carboxylic charges (due to the small hydrated volumes of the calcium ions). The resultant product, calcium hyaluronate, is characterized by low solubility, low hydration, and a folded, compact conformation that increases hyaluronan molecule packing density, thereby reducing the availability of the hyaluronan molecules. This phenomenon of changes in volume within the polymer network and the chemically driven contractile forces was first described by Kuhn *et al.*

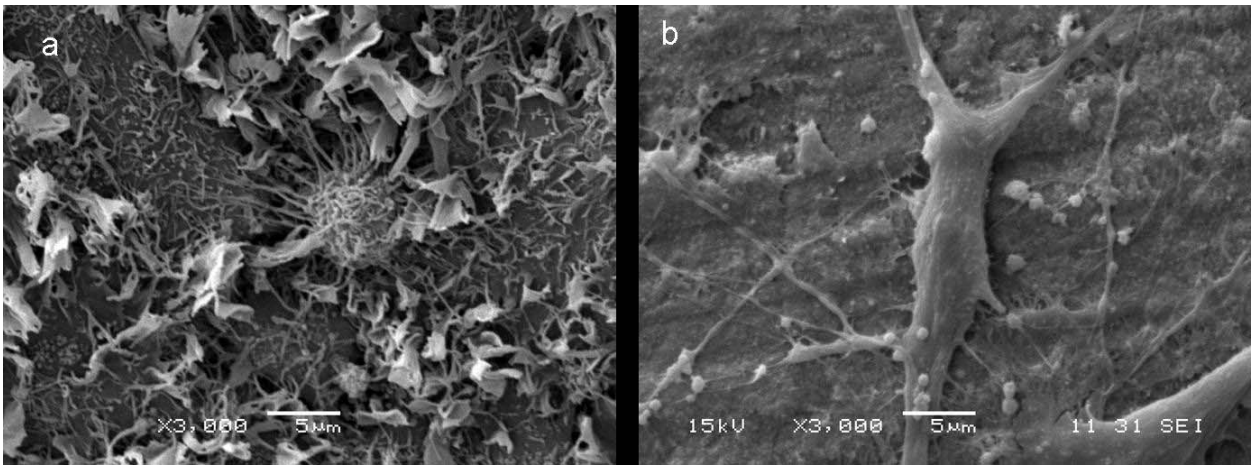

Figure 6. SEM micrographs of MSCs 14 d post-seeding on calcite-hyaluronan (a) and on aragonite-hyaluronan (b) biocomplexes. Note the spherical morphology of the cell on the calcite matrix (a) and fibroblast-like morphology of the cell on the aragonite matrix (b).

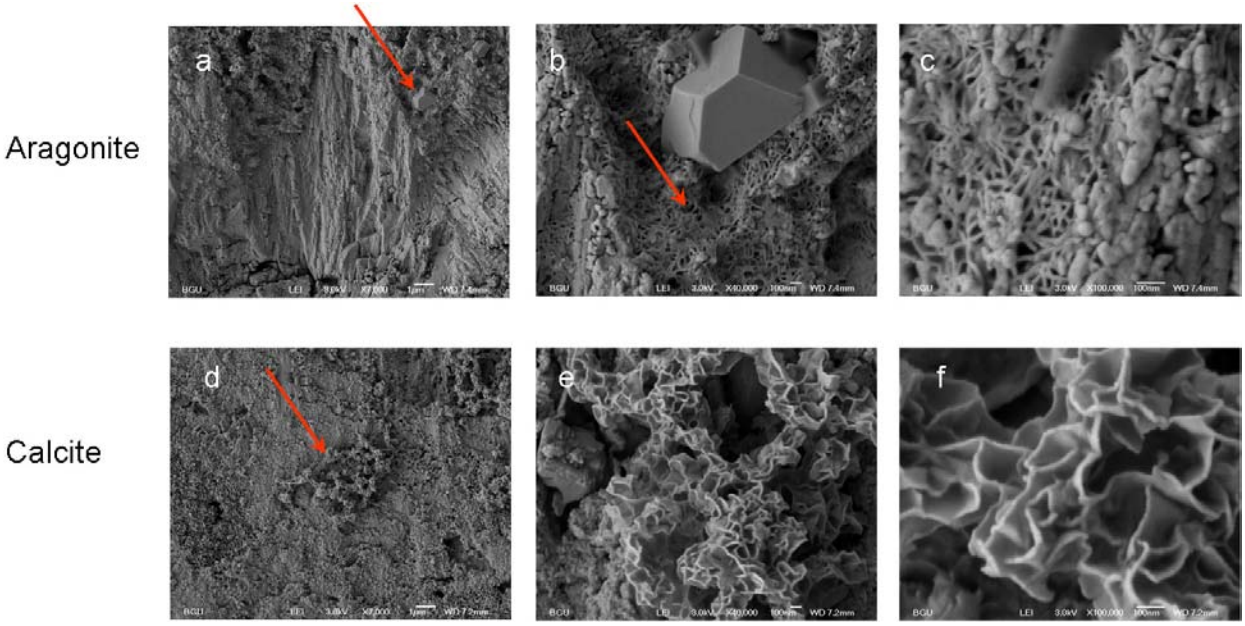

Figure 7. High resolution (HR) SEM micrographs of hyaluronan chains on the surface of aragonite (a, b, c) and calcite (d, e, f). Arrows indicate hyaluronan insets on the mineral surfaces. Magnifications: a, d – × 7,000; b, e – × 40,000; and c, f – × 100,000.

(1950) (77). The calcium ions seem to bind, from two opposing chain sections, the acetamide group of the aminoglycane unit and the carboxylate group of the glucoronic acid to produce a binding complex that involves two disaccharide units (78). As a result, both the hydrodynamic radius and the volume of the hyaluronate molecule are considerably reduced, and the molecules become compact, globular, and folded (see Figure 8).

The structural differences between calcite and aragonite include the arrangements of calcium ions on the surface of each calcium carbonate polymorph. On calcite, an octahedral structure is formed as each calcium ion is surrounded by its six nearest neighboring oxygen atoms. In contrast, each calcium ion on aragonite is surrounded by its nine nearest neighboring oxygen atoms. The ionic potential of the calcium cation is a measure of the ion's charge density and of its hydrated volume (charge/radius). Therefore, the ionic potential of aragonite is higher than that of calcite, a fact that affects their overall biocomposite stabilities and their physical properties. Non-directional electrostatic parameters, such as the average charge density or the mean dipole moment in the crystal plane, determine the spatial orientation of the hyaluronan chains. Further research should investigate more rigorously the mechanisms of MSC differentiation in calcium

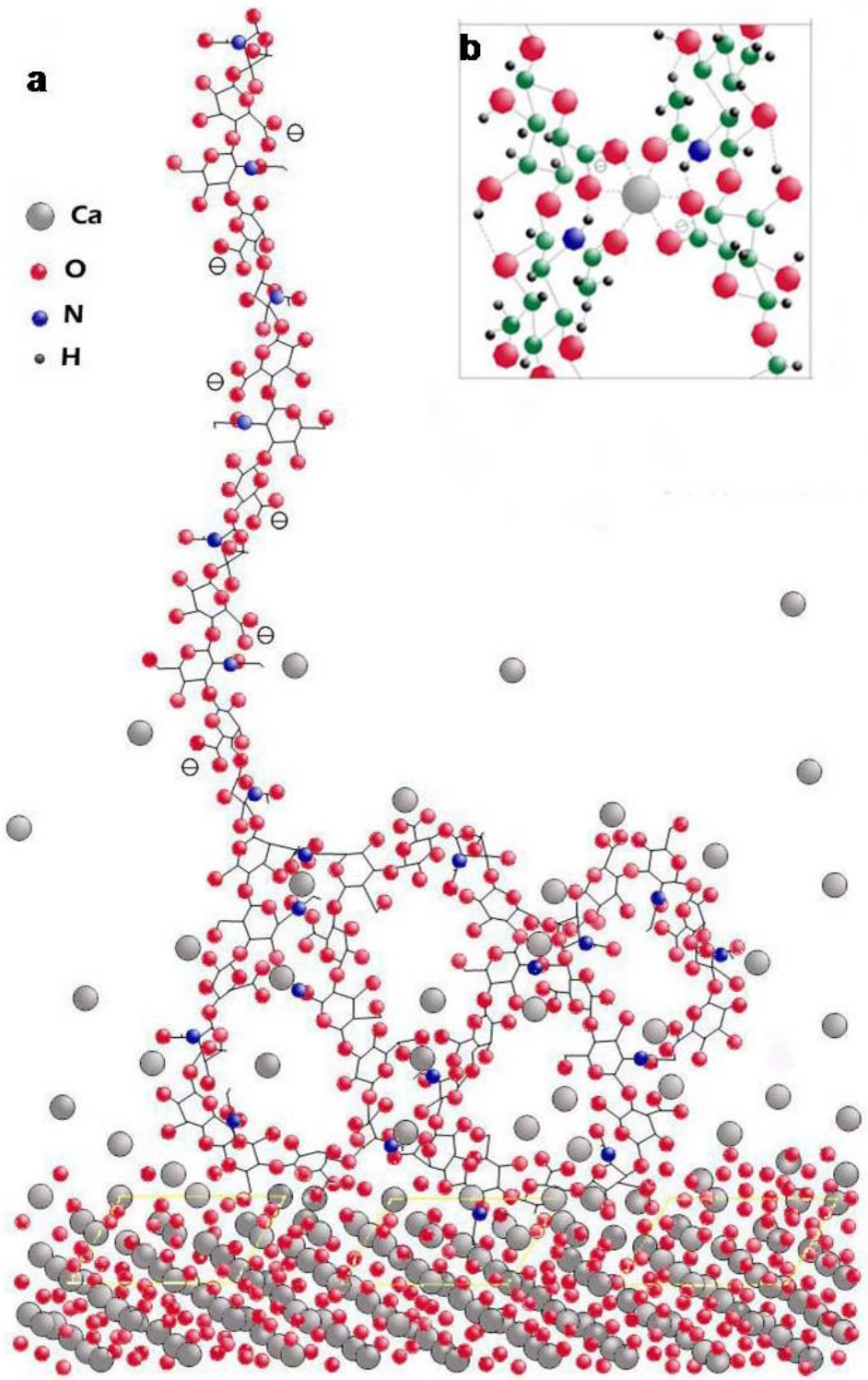

Figure 8. Proposed model of hyaluronan chains arrangement on the calcium carbonate surface. a: Hyaluronan molecule in an environment dominated by sodium ions assumes a stretched, linear conformation. Near the calcium carbonate surface, where the concentration of the calcium ions rises, they replace the sodium ions, and as a result, the hyaluronate molecule adopts a dense, globular-chain conformation. b: The calcium ion interacts with oxygen atoms of two N-acetylamino groups and two carboxylate groups to form a chelate-like complex involving two dissacharide units of the opposing hyaluronate chains. As a result, the opposing chains of the hyaluronan molecules move closer to one another.

carbonate-hyaluronan biocomplexes and the response of MSCs in animal models for medical applications.

9. ACKNOWLEDGEMENTS

All authors contributed equally to this article.

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Abbreviations: ECM: extracellular matrix; GAGs: glycosaminoglycans; LYVE-1: lymphatic endothelial hyaluronan receptor; MCSs: mesenchymal stem cells; RHAMM: receptor for hyaluronic acid mediated motility; TLR4: toll-like receptor-4; TSG-6: the protein product of the tumor necrosis factor-stimulated gene-6

Key Words: Hydrogel, hyaluronan, MSCs, Aragonite, Calcite, Review

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