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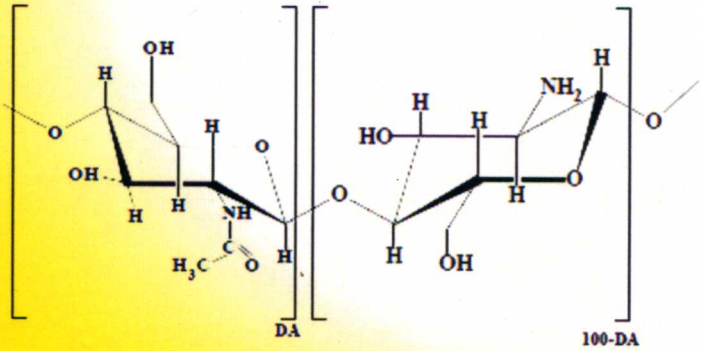
Chitosan-Based Hydrogels for Tissue Engineering Applications



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BIOTECHNOLOGY IN AGRICULTURE, INDUSTRY AND MEDICINE

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Nova Biomedical Books
New York

Library of Congress Cataloging-in-Publication Data

Chitosan-based hydrogels for tissue engineering applications / G. Luna-Barcenas ... [et al.].
p. ; cm.
Includes bibliographical references and index.
ISBN 978-1-61761-312-8 (softcover)
1. Chitosan--Biotechnology. 2. Tissue engineering. I. Luna-Barcenas, G.
[DNLM: 1. Chitosan--therapeutic use. 2. Hydrogels--therapeutic use. 3. Prostheses and Implants. 4. Tissue Engineering. QT 37.5.P7]
TP248.65.C55C545 2010 660.6'3--dc22 2010031177

Published by Nova Science Publishers, Inc. † New York

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Preface

The interest for finding better materials with the objective to use them as implants has led to a search in the mixture of natural polymers for a source to satisfy this necessity. This chapter presents the information about the synthesis, characterization, and some applications of hydrogels based on the chemical reactions between the hybrid, natural-synthetic, Chitosan-g-Glycidyl Methacrylate (CTS-g-GMA), of cationic nature, with water-soluble anionic polymers, such as Xanthan gum (X) and Hyaluronic acid (HA). The polyelectrolyte complexes formed due to electrostatic attraction between the polymers have improved properties when compared to hybrid CTS-g-GMA, which provides a wide range of applications in the biomedical field. All materials have been characterized by different analytical techniques such as infrared spectroscopy (FTIR), X-ray diffraction (XRD), or thermal analysis (DSC and TGA), and the results were compared to the precursor materials (chitosan, X, HA, and CTS-g-GMA). Due to the HA nature, the film obtained from this reaction has been assessed for use as a patch for wound healing; whereas the properties showed by the hydrogels obtained from the reaction with X make them very promising for applications in the treatment of recovery of spinal cord injuries. Cell culture was performed in all materials; different cell types were seeded and the viability has been quantified by the DNA (proliferation) assay, over several time intervals. The analysis showed satisfactory results of the (CTS-g-GMA)-X when compared to pure chitosan. Peroxide and interleukin-1β (IL-1β) assays have been performed to analyze the inflammatory response caused by biomaterials. Results show a moderated inflammatory response of our hydrogels when compared to raw chitosan. The implant of the hydrogels [(CTS-g-GMA)-X] in Wistar rats was performed after injuring the spinal cord by a laminectomy. The somatosensory evoked potentials (SEP) obtained by electric stimulation

onto peripheral nerves were registered in the corresponding central nervous system areas, showing a successful recovery after 30 days of the implant. The results are promising and strongly support the future use of these hydrogels as scaffolds for tissue engineering and recovery.

Contents

Chapter I

Introduction

Currently. natural polymeric materials have received much attention due to their potential use in vivo as implants. Biomaterials are defined as materials which are designed to restore, augment, or replace the natural functions of the living tissues or organs in the body. In simple words, a biomaterial is a material which becomes part of the body either temporally or permanently. Biomaterials should perform with an appropriate host response in a specific application without toxic, inflammatory, carcinogenic or immunogenic response [1, 2].

The first generation of biomaterials has been developed with the goal of combining physical and chemical properties to match those of the replaced tissue with a minimal toxic response in the host. Due the exigency of the new materials, a second generation was developed to increase bioactivity and the resorbable capacity. The third generation would effectively combine the properties of both generations to help the human body heal in a short time [1].

Polysaccharides form a class of materials which have generally been underutilized in the biomaterials field. Recognition of the potential utility of this class of materials is however, growing, and the field of polysaccharide biomaterials is poised to experience rapid growth. Three factors have specifically contributed to this growth. First, the large and growing body of information points to the critical role of saccharide moieties in cell signaling schemes and in the area of immune recognition. Secondly, the recent development of powerful new synthetic techniques with the potential for automated synthesis of biologically active oligosaccharides. These techniques may allow us to finally decode and exploit the language of oligosaccharide signaling. The third factor is the explosion in tissue engineering research and the associated need for new materials with specific, controllable biological activity and biodegradability [3].

An alternative to these materials have been the hydrogels, which are used as biomaterials such as soft contact lenses, artificial corneas, and artificial skins. Hydrogels are usually made of hydrophilic polymer molecules which are crosslinked by different chemical interactions. Hydrogels are elastic solids in the sense that there exists a remembered reference configuration to which the system returns to even after being deformed for a very long time. They can be of either chemical or physical nature. In the first case, the three-dimensional gel network is formed by the covalent crosslinking of the polymer chains, and as a consequence, these gels are not reversible. In the second case, the junctions between the chains are due to low energy interactions such as hydrogen bonding as well as Van der Waals or hydrophobic interactions. These gels are generally solvo- and thermo-reversible [2 - 5].

Some disadvantages of the hydrogels are their low hardness and low mechanical resistance after swelling in water. The mechanical properties of these materials can be substantially improved by adding a synthetic material with good mechanical properties. The implant of hydrogels over the biomaterial surfaces changes only their surface properties while the properties in the rest of the bulk remains unchanged. These implants can be made by physical absorption, couple insert, or polymerization [6 - 14].

Due to their size and surface properties, the hydrogels have been analyzed in biomedical studies, for therapeutic applications and clinical diagnosis. Hydrogels with high water content make it difficult for cells to adhere. The development of hydrogels compatible with cell attachment and growth is a major goal of biomaterial research [4, 6].

Success in the application of biomaterials relies heavily on the biocompatibility of biomaterials. Biocompatibility is the appropriate biological performance, both local and systemic, of a given polymer in a specific application [2].

Numerous strategies currently used to engineer tissues depend on employing a material scaffold. These scaffolds serve as a synthetic extracellular matrix (ECM) to organize cells into a 3D architecture and to present stimuli, which direct the growth and formation of the desired tissue. Depending on the tissue of interest and the specific application, the required scaffold material and its properties will be quite different [15, 16].

Natural and synthetic biomaterials serve as fundamental research and therapeutic tools to investigate and facilitate the repair of damaged or dysfunctional tissues, both in cell-based and acellular therapies [17].

The successful large-scale production of engineered tissues requires an adequate source of healthy expandable cells, the optimization of scaffolds, and the creation of bioreactors, which mimic the environment of the body and that are amenable to scale-up. Additional challenges include the preservation of the

product so that it has a long self-life and the successful use of various approaches to prevent tissue rejection [16].

The potential of chitosan as a biomaterial stems from its cationic nature and high charge density in solution. The charge density allows chitosan to form insoluble ionic complexes or complex coacervates with a wide variety of water-soluble anionic polymers [3, 16].

Chitosan ([β-(1→4)-2- amine-2-desoxy-D-glucose]) is a natural polysaccharide that is formed by altering the N-deacetylation of its precursor, chitin. Chitin and chitosan can be represented by a unique structure, show in figure 1. They can be considered as belonging to the family of glycosaminoglycans (GAG). GAGs are particularly interesting since they seem to be the fewest in number among the polysaccharides that express the property of bioactivity [5].[5]

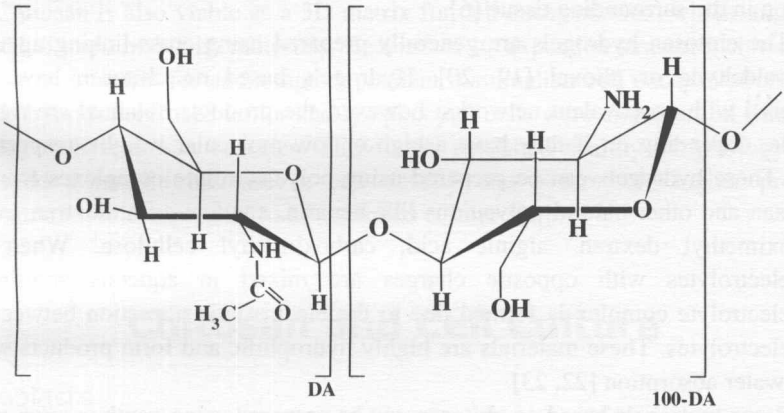

Figure 1. Chemical structure of chitin and chitosan. Note the similarity of both structures [5, 18].

The difference between chitin and chitosan is essentially related to the possibility to solubilize the polymer in dilute acidic media. Therefore, the degree of acetylation (DA), which is related to the population balance of acetylated and deacetylated (100-DA) groups (left and right chemical structures in figure 1) is essential to define these two terms. When chitin is deacetyled in heterogeneous conditions, the solubility in aqueous acidic media is achieved for DA, generally below 30%. Nevertheless, on reacetylating chitosan it is possible to observe a solubilization up to DA close to 60%. As a consequence, the frontier between chitin/chitosan can be located at a DA of 60%. An effect of the DA can be appreciated in the chitosan, which is a polymer semi-crystalline and the degree of crystallinity is a function of the degree of deacetylation [5 - 7].

Considering the chemical structure schematized in figure 1, the chitosan has a primary amino group and a hydroxyl group. Chitosan undergoes a host of chemical reactions under mild conditions and can be functionalized with a great variety of atoms [5 - 7]. On the other hand, the absence of chitosan in much living media must be regarded as an interesting opportunity for numerous possible applications, especially if we consider that it corresponds to a more or less charged polycation, a chemical structure not very common in nature [5].

Recent researches show that chitosan hydrogels have some properties that made them biocompatible materials, among those are: 1) they have low interfacial tension in the presence of the biological fluids of live tissue. Due to its large water content, the surface of the hydrogel is recognized as a diffuse super hydrophilic surface, 2) the hydrogels mimic some properties of the natural gels, cells and tissue, and 3) the soft and elastic nature of the hydrogels minimizes the mechanical tension in the surrounding tissue [6].

The chitosan hydrogels are generally prepared using cross-linking agents as glutaraldehyde or glioxal [19, 20]. Hydrogels based on chitosan have been prepared with no-covalent networks; however, the products obtained are rigid or fragile, depending on if they have a high or low molecular weight, respectively [21]. These hydrogels can be prepared using polyelectrolyte complexes from the chitosan and other natural polyanions like heparin, xanthan gum, dextran sulfate, carboximethyl dextran, alginic acid, carboximethyl cellulose. When two polyelectrolytes with opposite charges are mixed in aqueous solution, a polyelectrolyte complex is formed due to the electrostatic attraction between the polyelectrolytes. These materials are highly hydrophilic and form products with a high water absorption [22, 23].

Some hydrogels based on chitosan can be prepared using xanthan gum, which has carboxyl groups like in the acidic polysaccharide, and the chitosan that behaves as a basic polysaccharide with amine groups. These hydrogels do not dissolve in alkaline solutions and are very sensitive to swelling in solutions with a pH in the range of 9 and 12 [1, 22].

These hydrogels in a solution with a pH around of 8 and in presence of some salt could have favorable characteristics to drug liberation in applications via an oral route, because the hydrogels can react with the pH variation in the gastrointestinal tract [24 - 26].

Chitosan has been investigated for a variety of tissue engineering applications because it is structurally similar to naturally occurring glycosaminolglycans and is degradable by enzymes in humans, such as lysozyme, in which the kinetics of degradations are inversely related to the degree of crystallinity [27, 28]. In this topic, some works have reported the use of lysozyme to determine the biodegradation of some chitosan derivates which the modification of the degree of acetylation provides a powerful means for controlling biodegradation and

biocompatibility and can be optimized for tissue engineering applications, increasing the importance of this enzyme [9, 29].

Although considered as nontoxic, chitosan is often shown as being a strong elicitor of biological activity whether in plants or in animals. In both cases, the consequences of the contact between chitosan and living media have been extensively studied during the last 20 years. Some results in the case of plants could be used to understand some behaviors observed in animals [5].

The cytocompatibility of chitosan films at physiological pH, toward keratinocytes, fibroblast or chondrocytes have recently been studied in vitro [5]. Due to the chitosan properties, this contributes to wound healing through interaction with various cell types. Chitosan macromolecules can strengthen and accelerate cell proliferation and tissue organization of connective tissue comprising the supportive framework of an animal organ [30, 31].

Chitosan is also viable as a 3D matrix for cell encapsulation, which might be applied to implantable biomaterials. Because of its expanded structure, chitosan is suitable as a matrix for anchorage-dependent mammalian cell encapsulation [30]. Viable hybridoma (fused tumor and lymphocyte) cells were entrapped in chitosan-carboxymethylchitosan capsules. These cells exhibited healthy morphology, and displayed a tenfold increase in cell density and a threefold higher product concentration in comparison to a suspended culture [32].

Chitosan and Cell Culture

Fibroblasts

A fibroblast is a type of cell that synthesizes and maintains the extracellular matrix (ECM) of many animal tissues. Fibroblasts provide a structural framework (stroma) for many tissues, and play a critical role in wound healing. They are the most common cells of connective tissue in animals. Their main function is to maintain the structural integrity of connective tissue by continuously secreting precursors of the ECM. Fibroblasts secrete the precursors of all the components of ECM. The composition of ECM determines the physical properties of connective tissues [33].

Fibroblasts can migrate slowly over substratum as individual cells, in contrast to epithelial cells. While epithelial cells form the lining of body structures, it is fibroblasts and related connective tissues which sculpt the "bulk" of an organism [33].

One very important tissue engineering approach was developing skin substitutes with chitosan-based materials. The success of these applications lies in

part due to chitosan's favorable interactions with fibroblasts and bioactive molecules intimately related to fibroblasts and wound healing [30].

Chitosan was successfully utilized for the regeneration of skin tissue when it was inserted into a cut on the back of rats. A normal inflammatory reaction was observed after 2 days, followed by cell colonization after 7 days. A dermal equivalent with an average pore size of 100 pm provided an excellent environment for fibroblast growth and proliferation *in vitro*. This substrate was a mixture of bovine collagen types I and III, 85% weight per volume (w/v) chitosan extracted from shrimp shells, and GAGs of chondroitin-4 and chondroitin-6 sulfate, with a final composition of 72% (w/v) collagen, 20% (w/v) chitosan and 8% (w/v) GAGs. This study indicates that a minimum pore size is required as not to inhibit fibroblast migration, growth and metabolic activity, as well as the diffusion of nutrients in and out of the matrix [34].

Keratinocytes

The keratinocyte is the major cell type of the epidermis, making up about 90% of epidermal cells. The epidermis is divided into four or five layers (depending on the type of skin) based on keratinocyte morphology [35 - 36].

Keratinocytes originate in the basal layer from the division of keratinocyte stem cells. They are pushed up through the layers of the epidermis, undergoing gradual differentiation until they reach the stratum corneum where they form a layer of enucleated, flattened, highly keratinized cells called *squamous cells*. This layer forms an effective barrier which prevents entry of foreign matter and infectious agents into the body and also minimizes moisture loss. Keratinocytes are shed and replaced continuously from the stratum corneum. The time of transit from the basal layer to shedding is approximately one month, although this can be accelerated in conditions of keratinocyte hyperproliferation such as psoriasis [35 - 36].

For the attachment in chitosan films of keratinocytes, the results revealed that no matter what the DA is, all chitosan films are compatible for these types of cells. The adhesion tested showed that cell adhesion increases considerably on decreasing DA. The increase is important as long as the DA is low. In addition, for a given DA, fibroblasts appeared to adhere twice as much as keratinocytes on these films, on the contrary, the proliferation of keratinocytes is quite good. This proliferation increases when the DA of chitosan decreases [37].

Nerve Cells

Neurons (also known as neurones and nerve cells) possess electrical excitability: the ability to respond to a stimulus and convert it into an action potential. Once this process begins, a nerve impulse travels rapidly and at a constant strength. Most notably, vertebrate electrical synapses are bidirectional. With their speed, simplicity, and reciprocity, electrical synapses are a unique feature of neuronal circuits in the mammalian brain. In vertebrate animals, neurons are the core components of the brain, spinal cord and peripheral nerves [38, 39].

The cell line NG108-15 was selected for this work due to the fact that it presents many aspects similar to a motor neuron. The NG108-15 cell line is a neuroblastoma x glioma hybrid, which was derived by somatic cell hybridization [40 - 42].

Nerve cells are influenced by the presence of chitosan. Studies indicate that neurons cultured on a chitosan membrane can grow well and that the chitosan conduit can greatly promote the repair of the peripheral nervous system. Studies considered the attachment, spreading and growth of gliosarcoma cells as a model of affinity of nerve cells to chitosan membranes. Chitosan coated with polylysine and a chitosan-polylysine mixture are even better materials than chitosan itself in nerve cell affinity, and are promising materials for nerve repair. Pre-coating materials with ECM molecules, especially laminin, can greatly improve their nerve cell affinity [30, 43].

Chitosan and Inflammatory Response

The inflammatory response is triggered whenever body tissues are injured by physical trauma (a blow), intense heat, irritant chemicals or infection by viruses, fungi, or bacteria. The inflammatory response enlists macrophages, mast cells, all type of white blood cells, and dozens of chemicals that kill pathogens and help repair tissue [33].

Macrophages are the chiefs of phagocytes, which derive from white blood cells called monocytes that leave the bloodstream, enter the tissues, and develop into macrophages. These cells belong to the body defense and they are known to be important mediators of inflammation and play an important role in immune regulation [33, 44].

Chitosan exhibits a positive effect on wound healing through its interaction with macrophages and leukocytes. Moreover, chitosan enhances the immune response, which is desirable for the application of drug carriers to tumor-bearing hosts, whose immunities are depressed [30].

Chitosan's positive effect on wound healing is also displayed through its interaction with macrophages. The activation of normal macrophages for the destruction of tumor cells occurs when they interact with activating agents, such as microorganisms, and with substances secreted by T cells in response to antigen stimulation. Upon activation, these macrophages can lyse tumor cells either by direct contact or through the release of diffusible cytotoxic molecules. Nitric oxide, Interleukin -1β (IL-1β), tumor necrosis α-factor and reactive oxygen intermediates are among the major cytotoxic molecules produced by activated macrophages for the lysis of tumor cells. Chitosan shows a biological aptitude for activating macrophages to destroy tumor cells and to produce IL-1β. IL-1β is a compound that regulates cell-mediated immune responses and other biological functions. These facts demonstrated that macrophages are activated as a consequence of direct interaction with polymer materials and produce IL-1β [44 - 46].

Spinal Cord and Biomaterials

The spinal cord is elastic, but it is exquisitely sensitive to direct pressure. Any localized damage to the spinal cord or its roots leads to some functional loss, either paralysis (loss of motor function) or sensory loss. Severe damage to ventral root or ventral horn cells results in a flaccid paralysis of reach within these muscles, which consequently cannot move either voluntary or involuntary [33]. The result of an incomplete or complete spinal cord lesion is either paraplegia (paralysis of lower body) or quadriplegia (paralysis of the body from the neck down), depending on whether the injury was sustained in the thoracic/lumbar region or neck region of the spinal column, respectively [47]. Anyone with traumatic spinal cord injury must be watched for symptoms of spinal shock, a transient period of functional loss that follows the injury. Spinal shock results in immediate depression of all reflex stop, blood pressure falls, and all muscles below the injury are paralyzed and insensitive. Neural function usually returns within a few hours injury. If function does not resume within 48 hours, paralysis is permanent in most cases [33].

Destruction of the spinal cord can be compared to a bomb exploding in a computer centre, and repairing the spinal cord is as complicated as trying to rebuild all of the computer connections. In the last years, there has been encouraging progress in animal models, with sufficient regeneration of the damaged spinal cord to enable some recovery of motor ability. When the spinal cord is injured, the first phase of injury involves mechanical tissue destruction. It is followed by a second phase of tissue loss, which is principally caused by several local disturbances of the blood supply [47 - 49]. There have been attempts to

minimize this secondary damage with neuroprotective agents, but, so far, only high-doses of a synthetic corticosteroid given within the first hours after injury is in use clinically [49, 50].

It is estimated that the annual incidence of spinal cord injury (SCI), not including those who die at the scene of the accident, is approximately 40 cases per million of the population in the U. S. or approximately 11,000 new cases each year. SCI primarily affects young adults. However, the age of the general population of the United States has increased by approximately 8 years since the mid-1970's, and so, the average age at injury has also steadily increased over time. Since 2000, the average age at the time of injury has been 38.0 years [51].

Injury to the spinal cord may involve the destruction of a substantial amount of tissue, including the white and gray matter, and blood vessels. This occurs following trauma, degenerative process or stroke, where the amount of tissue damage may increase with secondary pathophysiological changes, or following surgery, where sectioning neural tissue is unavoidable during elective oncological surgery which necessitates the removal of a rim of vital tissue from around a tumor [51].

Whilst the functional deficit is related to the amount of tissue damage and to the interruption of the associated axonal pathways, it is also related to the inability of the adult mammalian central nervous system (CNS) to repair its own structures and to restore the tissue defect, as this occurs in the CNS of adult inframammalian vertebrates. Tissue repair does in fact result in scarring, despite evidence that the CNS has significant repair potential following injury, and would be capable of restoring a cellular terrain for axonal growth [52, 53]..

For the spinal cord, the recovery is complicated, because the spine receives sensory information from almost tissues of the body. It transmits this information in the form of electrical impulses to the brain, along millions of nerve fibers that are grouped together in bundles. A sharp blow to the spinal column can cause dislocation of individual vertebrae and severe damage to the spinal cord, including its complete severance [47].

Life expectancy is the average remaining years of life for an individual. Life expectancies for people with SCI continue to increase, but are still somewhat below life expectancies for those with no spinal cord injury. Mortality rates are significantly higher during the first year after injury than during subsequent years, particularly for severely injured persons [51].

Based mainly on the advantages offered by the chemical properties of chitosan, this work describes the synthesis, characterization and applications of novel materials formed by a chemical reaction between the hybrid chitosan/glycidyl methacrylate (CTS-g-GMA) polymer with water-soluble anionic polymers, such as Hyaluronic acid (HA) and Xanthan gum (X). The resulting