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Application of Chitosan-Based Biomaterials for Blood Vessel Regeneration

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Summary: Vascular diseases are the leading cause of morbidity and mortality in the western world. Autologous vessels remain the standard for coronary grafting and peripheral bypass surgery; however, their availability in patients can be limited. Therapeutic angiogenesis using growth factors, genes, or progenitor cells has been given considerable scientific attention over the last decade, but has not yet provided a definitive clinical benefit. Biomaterials could be developed to protect protein, DNA and cells against hostile conditions. Chitosan, a natural polymer of glucosamine and N-acetyl glucosamine, has been widely studied in tissue engineering due to its biocompatibility, biodegradability, and muco-adhesive and antimicrobial properties. Notably, the application of chitosan has been gaining attention in the vascular field due to its structural similarity to glycosaminoglycans, which are components of a tissue's extracellular matrix. In this review, chitosan-based materials, and their use in tissue engineered blood vessels, and as protein, gene and cell vectors for angiogenic therapy are discussed.

Keywords: blood vessel; cardiovascular disease; chitosan; drug release; tissue engineering

Introduction

According to the American Heart Association, 16.7 million people worldwide die of cardiovascular diseases (CVD) each year, which represents one third of all deaths around the globe.[1] CVD is predicted to surpass infectious diseases and become the leading cause of mortality on the planet by the year 2020. In 2009, the direct and indirect cost of CVD was estimated in the United States to be about 475.3 billion dollars.[2] Among the pathologies associated with the cardiovascular system, atherosclerosis, a disease of the arteries characterized by a thickening of the artery wall, participates in nearly three-quarters of all deaths attributed to CVD. The principal therapies used for the treatment of CVD patients range from anti-coagulant therapy to angioplasty to bypass surgery, depending on the severity. When vessel substitutions are required, then autologous or artificial blood vessels are used. New regenerative approaches for the revascularization of affected tissues are under development such as angiogenic therapy, which includes gene-, protein- and cell-based therapies. Although the use of autologous vascular substitutes has had a major impact in advancing the field of reconstructive arterial surgery, the availability of healthy autologous vessels for bypass grafting procedures may be limited and has necessitated the fabrication of prosthetic vascular conduits (artificial blood vessels) using biomaterials. Alternatively, therapeutic ''angiogenesis'' (a misnomer commonly used to designate therapeutic arterio- or vasculo-genesis), is promising for the treatment of patients with end-stage, diffuse CVD, who have failed medical therapy and

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are not amenable to further catheter-based or surgical interventions.[3] It is also constitutes a plausible future first-line therapy for stable myocardial ischemia. The goal is to develop regenerative therapies that will allow vasculogenic processes, extremely active during growth and development, to be selectively and non-invasively recreated in adult tissues. However, although various angiogenic therapies have been given considerable scientific attention over the last decade, none has been shown to provide a definitive clinical benefit due to short half-life of protein, low efficiency of gene transfection, and low implant capability of transplanted cells. Biomaterials scaffold could be developed to protect protein, DNA and cells against hostile environment by providing a microenvironment that promotes the sustained release or retention of protein, DNA and cells locally.

Blood Vessel Substitutes

Over the past half century, several strategies have been explored for arterial reconstruction. These include xenografts, allografts, autografts and synthetic grafts. In 1966, using bovine sources, Rosenberg was the first to create an artery substitute.[4] However, over the long-term, adverse outcomes included aneurysms, infections, and a high incidence of thrombosis were observed with their use .[5] To avoid the issues surrounding xenotransplantation, allografts have been developed to replace diseased blood vessels.[6] However, due to calcification, these have limited durability and the potential to develop aneurysms and ruptures.[5] More favourable outcomes have come from the use of autologous vascular grafts. The five-year patency rate for autologous vein grafts varies between 60 and 80%.[7] Although the use of autologous vascular substitutes has had a major impact on advancing the field of reconstructive arterial surgery, these tissue sources may be inadequate or unavailable because of venous abnormality and/or poor quality due to previous damage. Thus, there is an increasing need of vascular grafts for coronary and peripheral artery bypass applications.

Vinyon-N fibre[8] was used to create the first synthetic arterial substitute, and the intent was to provide a scaffold for tissue ingrowth, and ultimately mimic the anatomic structure of the vascular wall. However, insufficient long-term stability and the lack of cellular incorporation were observed when tested in patients. Currently, expanded polytetrafluoroethylene (ePTFE), and polyethylene terephthalate (Dacron), are regarded as the standard biomaterials for prosthetic vascular grafts.[9] Although they have been shown to perform well as blood vessel grafts at diameters greater than 6mm, two limitations prevent its widespread use for low-flow and small-diameter (<4mm) vessel applications.[10] First, as small-diameter grafts, they exhibit poor compliance compared to native arteries, and second, thrombogenicity occurs. Therefore, alternatives are needed to replace the saphenous vein, internal mammary or radial artery as a vascular substitute. Furthermore, PET and ePTFE lack the ability to grow, repair, or remodel. This disadvantage may limit their application in long-term treatment compared to materials based on natural substrates. Consequently, finding a solution for small-diameter bypass grafting has been a major focus of attention.

Tissue engineering is providing new possibilities in blood vessel grafting. Scaffolds have been made from a variety of different materials, such as synthetic polymers, natural materials, and decellularized xenogenic tissues. The first tissue-engineered blood vessel substitute was reported by Weinberg and Bell in 1986.[11] Bovine endothelial cells, smooth muscle cells, and fibroblasts were cultured in layers of collagen gel supported by a Dacron mesh. Although the engineered vessel was able to sustain physiologic pressures for only 3 to 6 weeks, they did demonstrate the feasibility of tissue engineering a vascular graft. Since then, strategies to create a suitable polymeric material for a blood vessel substitute have focused on three main areas: (1) coating and surface chemical modifications of synthetic materials; (2) biodegradable

scaffolds; and (3) biopolymers.[12] The development of polymers can be further categorized into strategies for in situ vascular regeneration, in which the body's natural healing response is modulated by polymer design and fabrication, or into strategies for ex vivo formation of a vascular graft, whereby the culture of human cells on polymer substrates before implantation defines their mechanical and biological properties. [12]

Chitosan

Chitosan, as a natural material, has been widely investigated in this field due to its structural similarity to glycosaminoglycans (GAGs), which are the components of the extracellular matrix (ECM). Chitosan, the partially deacetylated derivative of chitin, is a linear polysaccharide, composed of glucosamine and N-acetyl glucosamine units linked by β (1-4) glycosidic bonds. It has been widely used in tissue engineering due to its unique biological, chemical, and physical properties.[13] Chitosan is a crystalline polysaccharide and is normally insoluble in aqueous solutions above pH 7.0. Its solublility in dilute acids below pH 6.0 has been attributed to the protonated free amino groups on glucosamine. The pH-dependent solubility facilitates a convenient mechanism for processing under mild conditions.[14] The cationic nature of chitosan also allows for pH-dependent electrostatic interactions with DNA, anionic glycosaminoglycans (GAG) and proteoglycans distributed widely throughout the body and other negatively charged species.[15] Because of this pH dependency, the transfer of ionic complexes to physiological pH can result in dissociation of a portion of the immobilized polyanion group. This property can be exploited for local delivery of biologically active polyanions such as GAGs and DNA. For example, chitosan has been complexed with heparin in order to control heparin release[13], thereby enhancing the effectiveness of growth factors released by inflammatory cells in the vicinity of the chitosan-heparin implant. The combination of DNA with chitosan has been shown to protect the DNA from degradation against nucleases before it enters the cell[16]; and use of a DNA-chitosan complex can also play an important role in membrane adhesion[17] and lysosomal escape[18] of the encapsulated DNA. One of chitosan's most promising features is its excellent ability to be processed into porous structures for use in cell and protein transplantation, and tissue regeneration. Porosity and microstructure of chitosan materials can be controlled freezing and lyophilisation techniques[14] or by processes such as the ''internal bubbling process'' (IBP).[19] The advantage of this property of chitosan is that several tissue-relevant geometries can be conceptualized and fabricated.

The N-acetylglucosamine moiety in chitosan is a structural feature also found in GAGs. GAGs have many specific interactions with growth factors, receptors and adhesion proteins, and also perform many important regulatory functions in development, angiogenesis, microbial pathogenesis and anticoagulation. This suggests that the analogous structure in chitosan may also have related bioactivities, and in fact, studies have been reported to this effect. For example, chitosan oligosaccharides can have a stimulatory effect on macrophages, and this effect has been linked to the acetylated residues of chitosan.[15,20] Furthermore, chitosan and its parent molecule, chitin, have been shown to exert a chemoattractive effect on neutrophils in vitro and in vivo.[21]

In vivo, chitosan is degraded by enzymatic hydrolysis. The primary agent is believed to be lysozyme, which is present in almost all secretions and tissues, and which appears to target the acetylated residues of chitosan.[22] A number of researchers have examined the tissue response to various chitosan-based implants. In general, these materials evoke no more than a minimal foreign body reaction, and major fibrous encapsulation of the implant does not occur. Typically, the formation of normal granulation tissue is observed, often with vascularization.[23] In

the short-term (<7days), neutrophils accumulate in the vicinity of the implants, but this dissipates rapidly, and without the development of a chronic inflammatory response. Chitosan and chitosan fragments may stimulate immune cells to induce local cell proliferation, and ultimately the integration of the implanted material with the host tissue.[15] The antibacterial activity of chitosan against a broad spectrum of bacteria[24] could be another benefit from the use of chitosan for protein encapsulation and tissue engineering applications. Chitosan has three types of reactive functional groups, an amino group as well as both a primary and a secondary hydroxyl group. These groups provide many possibilities for covalent and ionic modification, giving chitosan extensive and adjustable physical and biological properties, thereby enabling the production of various useful scaffolds for drug release and tissue engineering applications.

Chitosan-Based Materials for Tissue Engineered Blood Vessels

Zhu et al. developed a poly (ethylene terephthalate) film by chitosan and O-carboxymethylchitosan surface immobilization and demonstrated that cell adhesion, morphology, and growth can be mediated by varying the functional groups, electric charge, and specific biological recognition elicited from the biomaterials.[25] In other work, mixed poly (vinyl alcohol) (PVA) and chitosan hydrogels were fabricated by freeze/thaw cycles and further crosslinking in $KOH/NaSO_4$ for vascular tissue engineering scaffolds.[26] With this strategy, the addition of chitosan into PVA hydrogels improved cell (bovine aortic vascular smooth muscle and endothelial cells) attachment without compromising the material's physical properties. Chupa et al.[27] incorporated GAGs into porous chitosan scaffolds in an attempt to overcome both incomplete endothelialization and smooth muscle cell hyperplasia, which are two problems contributing to the poor performance of existing small-diameter vascular grafts. Materials containing GAGs are promising because of their growth inhibitory effects on vascular smooth muscle cells and their angiogenic and anticoagulant activities.[28] Zhang et al. developed a chitosan-based sandwich tubular scaffold using an industrial knitting process and a thermally induced phase-separation technique.[29] The knitted structure conferred mechanical strength, enough to withstand the hemodynamic pressure and suture strength during surgery. In addition, the scaffold's porosity provided a favourable microenvironment for cell infiltration and tissue integration. In order to avoid the possible risks associated with the use of xenoproteins (immune response and rejection for example), these scaffolds were fabricated using human-like collagen and chitosan by a cross-linking and freeze-drying process.[30] Human venous fibroblasts were seeded in the scaffold and cultured for 15 days prior to implantation into rabbits' livers. In this model, the scaffolds were able to enhance cell adhesion and proliferation and ECM secretion, while maintaining adequate mechanical strength and pliability.

Recently, a strategy to coat the vessel lumen with factors that encourage endothelialisation and inhibit platelet accumulation has been reported.[31] In this approach, nanocoatings of two polyelectrolytes, chitosan and hyaluronic acid (HA), were deposited onto arteries through a layer-by-layer technique.[32] The adsorption of the polyelectrolytes occurred within a few seconds without the need for initiators or an energy supply (e.g. light). The coating significantly reduced the adhesion of blood platelets, thus demonstrating a restored patency of the denuded arteries. These multilayers have also been used for the incorporation and release of bioactive drugs, proteins (i.e., adhesion and growth factors), and nucleic acids.[33] The release of these molecules may then help to regulate the blood response and vascular repair in a desired manner. For example, incorporation and release of heparin may reduce blood clotting, enhance the re-endothelization of the luminal vessel

surface, and diminish the proliferation of smooth-muscle cells.[33]

Chitosan-Based Materials for Protein/gene-Based Therapy of Blood Vessels

Therapeutic angiogenesis using growth factors has drawn considerable attention as a treatment for myocardial ischemia. In the early 1990s, the first study of protein-based therapeutic angiogenesis was reported.[34] In this study, the intracoronary administration of basic fibroblast growth factor (bFGF) resulted in an increased number of collateral blood vessels and alleviated myocardial ischemia in a dog model of the disease. Since then, several pharmacological and molecular approaches to stimulate collateral development have been investigated in multiple animal studies.[35] This includes the direct delivery of growth factors into ischemic target tissues, or of genes that encode for synthesis of growth factors by target tissues. The delivery of vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), monocyte chemoattractant protein-1 (MCP-1) and granulocyte-macrophage colony-stimulating factor (GM-CSF), have all been shown to induce angiogenesis and arteriogenesis.[36] Both of these processes require the proliferation as well as the migration of endothelial and smooth muscle cells.

The level of uptake of growth factor protein into ischemic tissue is relatively low compared with the plasma level. For example, only 0.26% of systemically administered FGF-2 has been reported to be found in the myocardium.[37] Therefore, if angiogenic growth factors are delivered systemically, high doses are likely necessary in order to achieve effective angiogenesis.[38] However, this exposes the risk of systemic side effects such as hypotension and edema with VEGF treatment,[39] and hypotension, thrombocytopenia and renal insufficiency with FGF treatment.[40] In addition, angiogenic growth factors such as VEGF and FGF have been reported to be associated with malignant tumors[41] and retinal neovascularization.[42] Therefore, strategies for the local delivery of growth factors are needed to enable beneficial effects without the systemic side effects. Furthermore, the half-life of growth factors is very short, and in order to maintain a sufficient tissue concentration of growth factors for an extended period, a sustained release system for growth factors is required. Biodegradable materials can be used to directly control the release of drugs or growth factors, or they may be used as delivery vehicles for gene therapy products.

Chitosan is recognized as a promising delivery material for its physicochemical and biological properties. Its hydrophilicity and positively charged properties enable it to interact with negatively charged DNA, protein and other polymers in an aqueous environment. Also, chitosan is a penetration enhancer as it is capable of opening epithelial tight-junctions.[43] Fujita et al.[44] developed an FGF-2 release system using a photocrosslinkable chitosan hydrogel and found that the majority of FGF-2 that was retained within the hydrogels remained biologically active, and it was gradually released as the hydrogel was degraded in vivo. This controlled FGF-2 release system promoted angiogenesis and collateral circulation in healing-impaired diabetic (db/db) mice and in the ischemic limbs of rats.

Chitosan is one of the most reported non-viral naturally derived polymeric gene carriers due to its non-toxicity, strong electrostatic affinity for DNA and its ability to protect DNA from nuclease degradation. Despite this, few data regarding chitosan as a gene vector for the stimulation of blood vessel growth and regeneration have been reported.

Chitosan-Based Materials for Cell-based Angiogenic Therapy

More recently, cell-based therapy has emerged as a promising approach for the repair and regeneration of the damaged or diseased heart.[45] Stem cells of bone marrow, embryonic, and cardiac origins, for example, have been used in an attempt to regenerate the cardiac vasculature and myocardium. In clinical trials, the use of

mostly heterogeneous populations of marrow-derived cells has revealed a small, yet significant benefit of cell therapy on cardiac function.[46] However, the therapy remains hindered by low cell engraftment, poor survival, and inadequate phenotype and function.

Biomaterials could be designed to provide mechanical, physical, and biochemical properties needed for more effective cell therapies for cardiac regeneration, thereby improving functional outcomes. Hydrogels can provide a physical barrier to protect transplanted therapeutic secreting cells from hostile extrinsic factors. Such hydrogels also function as substrates for the cells, possibly protecting them against external mechanical loading.[47] In addition, hydrogels are structurally similar to extracellular matrices and can therefore confer many advantages compared to other biomaterial forms.[48] This includes a high permeability for oxygen and nutrients, the ability to exchange ions and metabolites with surrounding tissue fluids, and the potential for the encapsulation of growth-promoting protein. For example, we previously used a collagen-based tissue engineered matrix for improved delivery of cells and revascularization.[49] On transplantation, the retention of the cells and contribution to vasculogenesis in ischemic hindlimbs was enhanced by delivery using a collagen matrix, compared to cells delivered alone.

Chitosan is another promising material candidate for improving the effects of cell-based angiogenic therapies. It has been documented to promote vascular endothelial cell and fibroblast migration, angiogenesis, and wound-healing.[23] Generally, chitosan hydrogels can be crosslinked by ultraviolet (UV) irradiation, high temperature, high pH and chemical crosslinkers.[50] In 2000, Ono et al.[51] first published the photocrosslinkable chitosan by introducing lactose (LA) moieties into chitosan. The addition of these LA moieties produced much better water-soluble chitosan at neutral pH, and the introduction of photoreactive azide (Az) groups to react with amino groups, resulted in the formation of a Az-chitosan-LA gel. The Az-chitosan-LA hydrogel had the consistency of transparent and soft rubber.[52]

Hydrogels that are crosslinked by exposure to elevated temperatures are termed thermogels. An example of a chitosan thermogel involves neutralization of highly deacetylated chitosan solutions with glycerol phosphate (GP) to retain chitosan in solution at physiological pH.[53] The ability of the chitosan/GP solution to form a gel at physiological pH and at body temperature makes this thermogel attractive as an injectable delivery system in tissue engineering. The injectable delivery system offers some advantages over preformed scaffolds: 1) liquid gels are able to fill any space or shape within the target site; 2) various proteins, living cells and other therapeutic agents can be encapsulated into the solution prior to the injection; and 3) the injectability may allow for delivery to the site without the need for invasive surgery. To date, the chitosan/GP system has been widely used for drug delivery and tissue engineering. For example, Lu et al. recently reported a chitosan hydrogel that improved embryonic stem cell (ESC) survival and the differentiation of cardiomyocytes in a rat infarct model.[54] In their study, the function, wall thickness, and microvessel densities of infarcted myocardium improved in chitosan + ESC and chitosan-only groups.

We have developed an injectable matrix for cell delivery and vascularization by using a combination of chitosan and collagen. The structure of this gel was confirmed by ^{13}C NMR and FTIR. The peak observed in ^{13}C NMR between 97.7 and 103.2 ppm, representing α-C in the six-member ring of chitosan, indicated that chitosan had been incorporated into collagen-chitosan hydrogels (data not shown). The appearance of peak at 1081 cm^{-1} and 1385cm^{-1} in FTIR spectra (Figure 1), attributed to the N-H rocking mode of NH_3^+ and C-O stretching in chitosan, further confirmed that chitosan has been incorporated into collagen-chitosan materials. The collagen-chitosan gel showed

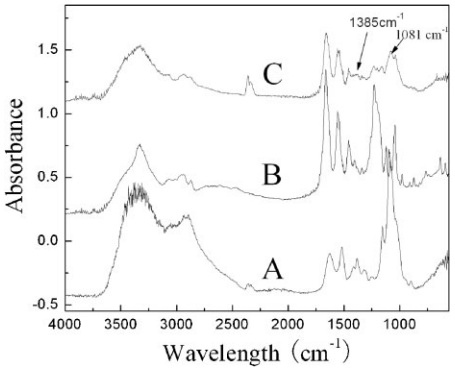

Figure 1.
FTIR spectra of chitosan (A), collagen (B) and collagen-chitosan (C) materials.

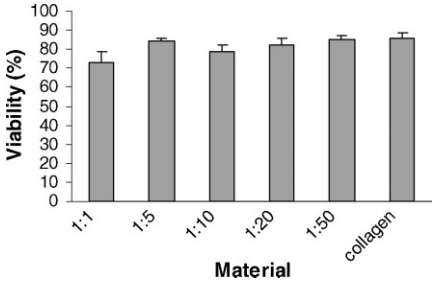

Figure 3.
Endothelial progenitor cell viability on collagen-chitosan hydrogels after four days of culture.

excellent injectability, stability and mechanical properties. Using contact angle measurement, it was found that the water contact angle was greater with increasing chitosan content (Table 1). This indicates that the addition of greater chitosan concentrations decreased the surface hydrophilicity of the hydrogel. In culture, endothelial cells formed significantly more vascular-like structures on collagen-chitosan, compared to collagen-only matrix after 45 hours (Figure 2). After four days culture, the viability of endothelial progenitor cells seeded on collagen-chitosan hydrogels was about 80%, which was not significantly different from collagen-only matrices (Figure 3). These results indicate that the incorporation of chitosan can improve the physical properties of injectable collagen matrices, and enhance their ability to support endothelial cells and angiogenesis for use in cardiovascular tissue engineering applications.

Discussion and Future Direction

New research into methods to improve the regenerative potential of genes, proteins and stem cells for treating and preventing heart failure are underway. Tissue engi-

Table 1.
Water contact angle data of collagen and collagen-chitosan hydrogels.

Samples(coll:chitosan)	1:1	5:1	10:1	20:1	50:1	collagen
Contact angle (°C)	93.63 ± 2.31	86.40 ± 4.88	76.2 ± 1.35	69.27 ± 0.35	65.9 ± 1.64	64.62 ± 2.30

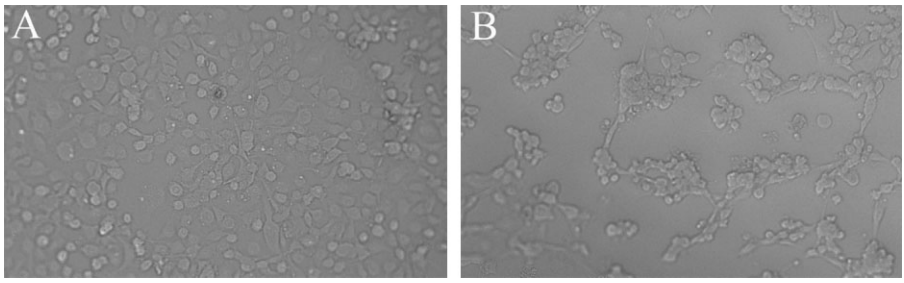

Figure 2.
Images of HUVECs cultured on collagen (A) and 5:1 (w:w) collagen-chitosan (B) hydrogels after incubation at 37 °C for 45h. Capillary–like networks were formed on collagen-chitosan hydrogels, but not on the collagen-only gels.

neering offers the opportunity to improve cell engraftment, persistence and function within the regenerating tissue, and to improve control over the release of gene and protein products. Due to its unique properties, chitosan is an excellent candidate for developing these systems. In particular, chitosan is attractive for use in tissue engineered blood vessels, and as protein, gene and cell vectors for angiogenic therapy. More research and development of chitosan materials is needed to further improve their efficacy in cardiovascular tissue engineering applications.

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