REAGENT-FREE COVALENT CROSSLINKING OF CHITOSAN-GELATIN FILMS FOR MEDICAL APPLICATIONS

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ABSTRACT

A reagent-free, inter-molecular covalent crosslinking technique was developed in order to incorporate desirable traits such as flexibility, mechanical robustness and high interconnective porosity into composite chitosan-gelatin base films. Since any form of contact between biomaterials and reactive chemicals typically raises safety concerns, the principle goal in developing a reagent-free method was to bypass all adverse physiological effects that might otherwise be induced by some reactive chemical step in the processing history of the biomaterial. Intrinsically antimicrobial and biodegradable chitosan-gelatin compositions were targeted. To being, chitosan-gelatin films were prepared in lyophilisate format and heat-treated under dehydrative vacuum conditions, yielding crosslinked porous materials. The mechanical properties, morphology, porosity and pore interconnectivity of this material were fine-tuned by varying the starting composition and experimental conditions, eventually yielding a product amenable to cell proliferation tests. Structural changes of the material were spectroscopically probed after heat application, revealing subtle but general alterations in the hydrogen-bonding of amide groups. While new covalent bonds were not established by direct observation, minor inter-molecular crosslinking could be concluded from the observable swelling of the material in PBS buffer and its inability to re-dissolve under the most stringent of non-destructive reducing conditions. In fact, dehydrothermal processing was found to

have dramatically improved the long-term stability of the film in aqueous media, even under proteolytic conditions. Processed films also retained the antimicrobial traits of chitosan, as evidenced by the lack of visible growth within the film and surrounding media. Mechanical tests implied that the dehydrothermally-crosslinked films, once re-hydrated, possessed the necessary flexibility and tensile strength to potentially assume many of the minor-load applications expected of chemically crosslinked soft biomaterials. Typical *in vitro* cell culture studies showed good adhesion of cells along the surface and within the pores of the film, with sub-surface proliferation apparently being aided by the concomitant and gradual biodegradation of the film structure. Finally, hemoglobin was dehydrothermally immobilized to these crosslinked chitosan-gelatin films, yielding a catalytically active oxygen-loading film and further attesting to the benign nature of this crosslinking technique. Based on these positive outcomes, it follows to reason that reagent-free covalent crosslinking may conveniently yield suitable and safer alternatives to many soft biomaterials commonly used in medical applications.

TIBBİ UYGULAMALAR İÇİN REAKTİFSİZ KOVALENT ÇAPRAZ BAĞLAMA TEKNİĞİ KULLANILARAK KİTOSAN-JELATİN FİLMLERİN SENTEZİ

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ÖZET

Kitosan-jelatin compozit bazlı filme; esneklik, mekanik dayanıklılık ve bağlantılı porozite gibi istenilen özellikleri katmak için reaktifsiz moleküler arası kovalent çapraz bağlama tekniği geliştirilmiştir. Biyomateryal ile kimyasal reaktifin herhangi bir teması sonucu ortaya çıkan güvenlik tehlikesinden dolayı, reaktifsiz bir metot geliştirilmesinin ana amacı; biyomateryal fabrikasyon sürecinde ki zararlı fiziyolojik etkilere yol açan kimyasal reaktif uygulaması adımını atlamaktır. Bu çalışmada, doğal olarak antimikrobiyal ve biyodegrade edilebilir özellikte kitosan-jelatin kompozisyonlar kullanılmıştır. Bu çalışmada, gözenekli yapıya sahip çapraz bağlı materyal elde etmek üzere; kitosan-jelatin filmler liyofilizat formunda hazırlanmıştır ve susuz vakum şartları altında ısı muamelesi yapılmıştır. Hücre prolifirasyon testleri için materyalin uygun mekanik özellikleri, morfolojisi, porozitesi ve bağlantılı gözenek yapısı; bileşenlerin başlangıç konsantrasyon/oranları ve deney şartları optimize edilerek sağlanmıştır. Materyalin yapısında ısı uygulaması sonrasında, spektroskopik analiz sonucu amit gruplarının hidrojen bağlarında zayıf fakat belirgin değişiklikler saptanmıştır. Kovalent bağ oluşumu doğrudan tespit edilmemesine rağmen, materyalin PBS tampon çözeltisi içerisindeki belirgin şişmesinden ve destrüktif olmayan indirgen şartlar altında

çözünmemesinden dolayı moleküler arası çapraz bağlanma oluşumu sonucuna varılabilir. Aslında bu işlemin, sulu ortamda ve proteolitik şartlar altında filmin uzun süreli stabilitesini yüksek derecede arttırdığı bulunmuştur. Film üzerinde ve çevresinde mikrobiyal büyüme gözlemlenmediğinden dolayı, işlenmiş filmin kitosana ait antimikrobiyal özellikleri koruduğu sonucuna varılabilir. Mekanik testler sentezlenen materyalin hidratasyonu sonrasında, çapraz bağlı yumuşak biyomateryalden beklenen düşük yüklenme uygulamaları için uygun esneklik ve germe direncine sahip olduğunu göstermiştir. *In vitro* hücre kültürü testleri, filmin yüzeyinde ve gözeneklerin içinde hücre adhezyonunun uygun olduğunu göstermiştir ve filmin içindeki proliferasyonun filmin biyodegredasyonuyla paralel olarak gerçekleştiği sonucuna varılmıştır. Son olarak, hemoglobin aynı yöntem kullanılarak çapraz bağlı kitosan-jelatin filmler üzerinde immobilize edilmiştir ve oluşan filmlerin oksijen bağlama aktivitesine sahip olduğu tespit edilmiştir. Immobilize hemoglobinin katalitik aktivitesini koruması, bu çapraz bağlama tekniğinin ılımlı şartlar sağladığını bir kere daha doğrular. Bu olumlu verilere dayanarak, reaktifsiz kovalent çapraz bağlama yöntemiyle elde edilen materyalin, tıbbi uygulamalarda genelde kullanılan bir çok yumuşak biyomateryale göre daha güvenli, uygun alternatif oluşturabileceği sonucuna varılır.

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1. Introduction

1.1 Biomaterials and biocompatibility

In general the term "biomaterial" can be described as a nonviable natural or synthetic material, employed in medical applications, and intended to directly contact biological systems. The main characteristic of a successful biomaterial is its biocompatibility, namely, the ability of a material to perform and to receive an appropriate response from the surrounding tissue as well as the whole organism (1).

The biomaterials considered in this thesis may be described as biodegradable soft composite biopolymeric films having potential merit as dressing material for wound healing. In view of the exceptionally large number of potential parameters to be considered in preparing and testing biomaterials, emphasis herein was only placed on promoting reagent-free crosslinking, flexibility, mechanical robustness, porosity and pore interconnectivity. The starting materials, chitosan and gelatin, were chosen for their ability to be gradually degraded *in vivo* as well as for the antimicrobial and scar-free wound-healing traits specific to chitosan. Biodegradability is extremely desirable in certain biomedical applications, primarily for two reasons. Firstly, resorption of the biomaterial by default will bypass any risk of long-term chronic foreign body reactions.

Moreover, biodegradable materials have also been found to enhance tissue regeneration. In the presence of chitosan for instance, the scar-forming attribute of type I collagen is suppressed, permitting a wound to heal with less or little scar tissue. This and similar findings have prompted extensive study of new approaches to address the problem of tissue regeneration. Even implants, for instance, traditionally prepared from non-degradable materials, have become a subject of biodegradation. In many examples, alternative implants made from biodegradable biomaterials have been used successfully as a temporary scaffold to promote healing and to bypass any need of a surgical "retraction" procedure (2).

1.2 Wound healing mechanism and scar formation

Acute cutaneous wounds, such as incisions and burns, heal in a very orderly complex fashion, while chronic wounds arousing from pathological abnormalities, such as diabetic ulcers, result in impaired healing mechanism.

Factors determining the normal wound healing process in a otherwise healthy adult individual are mainly type, size and depth of the wound, and the healing proceeds aiming the quick repair with the least energy consumption, thus leading to scar formation as opposed to extensive regeneration of the native tissue. The repair process starts immediately upon injury, is directed by cytokine signaling, and can be classified in four overlapping phases (Figure 1.1). Vaguely summarized these are (3,4):

1. Hemostassis phase: Blood components are spilled into the injury site, and the contact of platelets with elements of extracellular matrix triggers clotting factors and cytokine release. The main functions of this step are bleeding control and primary protection of the wound from the environment by forming the provisional fibrin matrix, as well as establishing essential regulatory system for further processes of healing.

2. Inflammation phase: Cytokines released during the hemostassis phase trigger chemotaxis of neutrophils and macrophages responsible for phagocytosis. Additional cytokines further secreted by macrophages are also responsible for fibroblast and smooth muscle chemotaxis and collagen and collagenase expression regulation, thus aiding the following proliferation step. Inflamation phase is characterized by successive increase in number of neutrophils, mast cells, and at the later stages wound macrophages and lymphocytes and their metabolites.

3. Proliferation phase: This phase is started by migration fibroblasts and subsequent extracellular matrix deposition, triggered by regulatory factors secreted, followed by contraction of the wound. Matrix protein, namely collagen, proteoglycan and fibronectin, transcription is enhanced, while secretion of proteases is decreased. Fibroblasts form the predominant cell population during the progress of this phase,

attaching to the previously formed fibrin matrix and producing collagen. Collagen stability is strongly dependent on the efficiency of series of posttranscriptional modifications, prior to crosslinked fiber formation of collagen released into extracellular spaces. Upon many collagen types identified collagen type I is predominant in scar tissue formation. Increased metabolic activity of the injured site, indicated by low pH, low oxygen tension and high lactate concentrations in the local environment, dictates angiogenesis.

4. Remodeling phase: Collagen matrix formed in the previous phase is croslinked and organized. Collagen fibers forming a scar tissue are smaller and much more randomly organized, compared to native dermal fibers, which leads to significant reduction in tensile strength. Some of the scar tissue formed is being digested by enzymes secreted by fibroblasts, neutrophiles and macrophages.

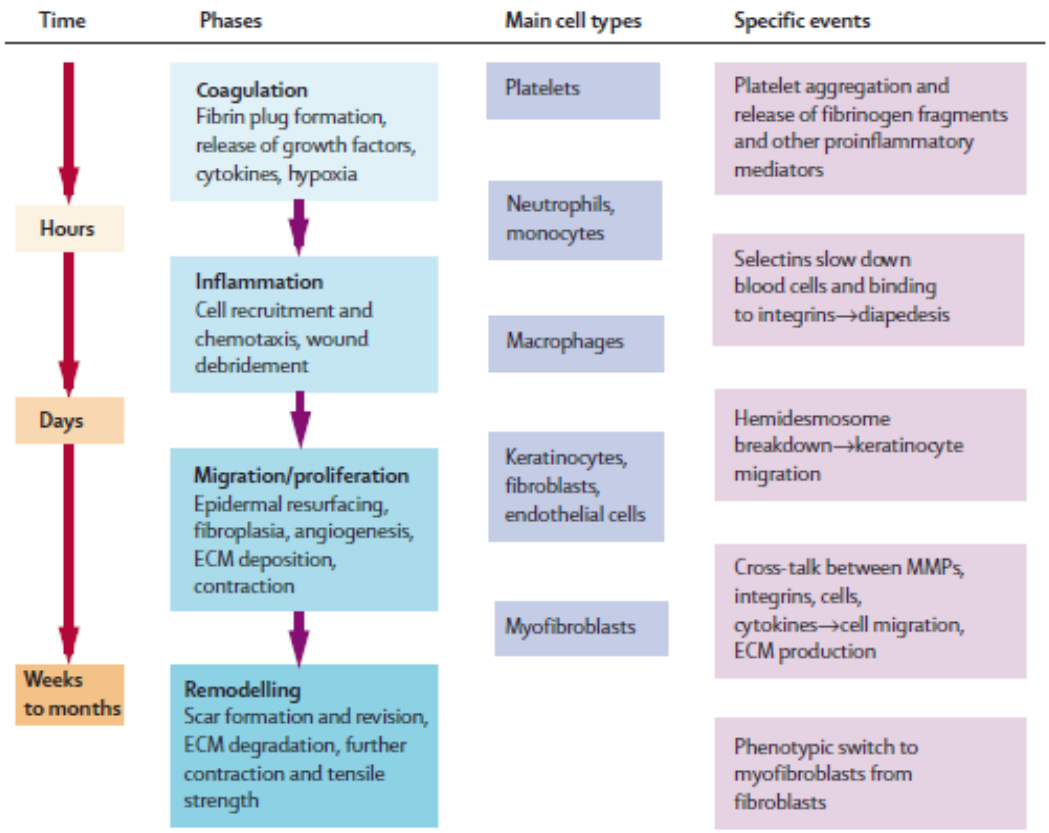

Figure 1.1: Phases of wound healing, major types of cells involved in each phase, and selected specific events (4). Abbreviations: ECM-extracellular matrix; MMP-matrix metaloproteinase

A typical wound healing period dependent on the type of the wound and environment, usually hemostassis phase being on the order of hours, inflammation phase lasts longer, while it takes proliferation and remodeling phases from weeks up to months to be completed. Among the factors that determine the healing prognosis, some determinants include age, sex, ethnicity, overall constitution, thickness of skin (i.e., in the case of skin wounds), inherent regenerative potential (e.g., epithelial cells versus mucous cells), extent of damage, and location of damage (e.g., around finger joints, groin, etc.).

Chronic wound depict even more difficult prognoses, disruption of a complex wound healing cascade occurs due to lack of vital factors or pathological conditions.

In cases of chronic inflammation (e.g., ulcers), abundance of reactive oxygen species and enzymes in the micro-environment conditions of the wound inhibits proliferation phase and leads to deficient healing, resulting in insufficient deposition of connective tissue matrix to the point where matrix can not preserve it's mechanic integrity (5).

In the opposite case of excessive healing (e.g., fibrosis), arising from elevated deposition of connective tissue matrix disrupting the equilibrium between scar formation and scar remodeling, leads to impaired structure and consequently loss of native function (5)**.**

1.3 Facilitating wound healing

Slow to regenerate, perfect tissue replacement does not typically occur in difficult medical cases, leaving areas of fibrous (scar) tissue in place of the original tissue. Apart from being non-functional for the most part, scar tissue may be also aesthetically unpleasant and psychologically harming, particularly when considering damage to visible regions of the body as well as regions that routinely play a role in the patient’s intimate life.

In the course of promoting healing, damaged skin is typically isolated from the environment in order to minimize infection and to promote unimpeded skin

regeneration (6). Traditionally prepared from non-degradable materials, most bandage dressings have provided ample protection from the environment but they have also required frequent changes. Dressing removal is not only extremely painful, but the act of removing the bandage often re-opens any raw wounds to some extent. In short, wound dressings offer minimal patient convenience (7). Ideally, the perfect wound dressing would prevent infection, pain and scarring, actively accelerate healing, retain transparency to permit visual inspection, allow gas and liquid transport, and self-disintegrate after the task is complete. Some physico-chemical and physiological response traits to optimize in envisaging such a material would include purity, biocompatibility, biodegradability, mechanical and hydrolytic stability, porosity, pore-interconnectivity, sterility and sterilizability, incipient loading potential (e.g., antibiotics, growth factors, etc.), transmembrane carrier potential (e.g., oxygen and moisture transport), electrolyte equilibration potential and microbe-killing potential (8). In view of the dramatic benefits that could be realized by such a material, the production cost would not be expected to play a decisive role in that undertaking.

1.4 Chitosan-gelatin composites as potential next-generation wound dressings

Chitosan is the deacetylation product of chitin (poly-N-acetyl-D-glucosamine), which is isolated from insect exoskeletons and crustacean shells, forming linear polysaccharide composed of β-(1-4)-linked D-glucosamine units (Figure 1.2). It is also found in the cell walls of some fungi (9). Chitosan, as opposed to insoluble chitin, dissolves in mildly acidic solutions and can be molded into different forms such as powder, film and fiber, thus lending itself suitable to a great variety of potential applications. The molecular weight of chitosan ranges between 300-1000 kDa depending on the source, and the deacetylation degree ranges between 50-95% depending on the preparative method.

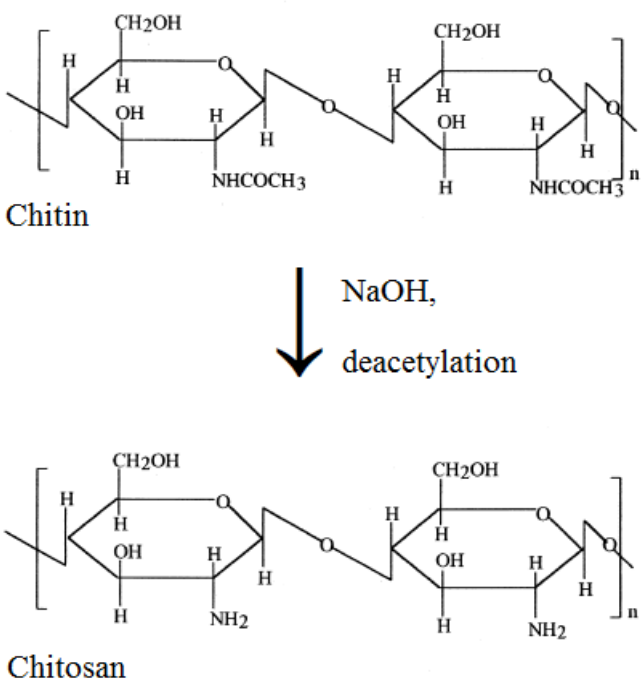

Figure 1.2: Structure of chitin and it's deacetylation product, chitosan.

Due to the abundance of chitin, chitosan is sufficiently inexpensive so as to be used in bulk quantities. For instance, chitosan continues to be applied to water treatment in view of its chelating properties (9). Chitosan as well as readily derivatizable variants such as carboxylated chitosan have drawn the attention of many biomaterials research groups. Indeed, chitosan has been extensively investigated and successfully utilized as a drug delivery or food additive platform and as various tissue regeneration matrices and coatings. Being biocompatible, biodegradable, antimicrobial and somewhat antiviral, chitosan easily meets the requirements of a base material in any potential wound dressing technology (10). The degradation rate of chitosan is inversely proportional to the extent of deacetylation. Lysozyme plays a major role in degrading chitosan-based biopolymers under *in vivo* conditions, via hydrolysis of β-(1-4)-linked D-glucosamine units (11).

Studies have indicated that chitosan possesses antimicrobial activity against bacteria, fungi and viruses (12). Different modes of action have been proposed, albeit, none are conclusively established. Efficacy has a strong dependency on intrinsic factors such as

degree of deacetylation, backbone length of the polymer, side-chain modifications (relating to different types of chitosan), and environmental factors, such as water activity and nutrient content (12).

In one study, bacteriocidal and bacteriostatic activity was proposed to proceed through the suppression of biosynthesis (13). In particular, interaction of the many cationic amino groups of chitosan was envisaged with anions positioned along the bacterial cell wall, accompanied by disruption of mass transport through the cell wall. In other studies, inhibition of microbial growth has been attributed to the chelating properties of chitosan, with an affinity towards crucial trace metals (14). Other scenarios used to rationale the antimicrobial effect have been the binding and sequestering of water as well as direct enzyme inhibition. Another mechanism has been based upon the migration of chitosan to the nucleus of microorganisms. In particular, DNA binding is thought to occur, thereby inhibiting mRNA synthesis. Interestingly, the antimicrobial performance of chitosan against fungi appears more pronounced than against bacteria (15). The antiviral activity of chitosan has been shown to be independent of the virus type. Hence, the most plausible explanation of a general response is that chitosan would interact with the pathogen much like the host cells. In the host organism, such an interaction could trigger a wide spectrum immune response, thus inducing inhibition of viral infections. While the anti-pathogenic trait of chitosan has been more extensively studied using plants, there is nonetheless enough evidence to suppose a similar cause-and-effect relation in mammalian tissue.

Apart from its excellent anti-pathogenic quality, chitosan has also been shown to possess the characteristics favorable for promoting rapid dermal regeneration and accelerated wound healing (9). In particular, chitosan was shown to elicit a stimulatory effect on macrophages and to attract neutrophils to the site of injury, which is an essential early-stage response to enhance wound healing (16). Chitosan was also found to stimulate the formation of granulation tissue and epithelialization during the proliferative phase of the wound healing process (17). Moreover, chitosan does not interfere with normal skin fibroblasts, whereas keloidal-type scar formation is blocked via the strong inhibitory interaction of chitosan upon type I collagen (18). The

combination of chitosan with incipients such as growth factors, extracellular matrix components and antibacterial agents has been reported to effectively accelerate wound healing (19).

Despite the very promising physiological traits, chitosan falls short in terms of mechanical traits, as the material alone is too brittle to serve as a biomaterial. In fact, chitosan is typically prepared as a composite biomaterial using a variety of polymers to improve the overall mechanical properties (20).

Gelatin, the second component of the film under study, is obtained by the partial acid hydrolysis of collagen, a protein typically isolated from cow skin, connective tissue and bones. Being a biological polymer, it is naturally biocompatible and its biodegradation products are non-toxic and non-pyrogenic. Also, chitosan-gelatin composites have accelerated wound repair in comparison to gelatin-free chitosan-based materials, the reason likely being due to better tissue adhesion of these readily wettable composites. (21). The compliancy of gelatin and abundance of carboxyl groups within its structure has elicited its utilization in combination with chitosan in prior biocomposite investigations.

1.5 Composite wound healing bandage fabrication

Various methods of porous scaffold and film fabrication using biocompatible soft polymer composites have been extensively investigated. Main properties of a resultant material to be considered include pore size and structure, porosity, vapor and liquid permeability, mechanical stability and biodegradation rate (22). These properties, being a function of each other, can be controlled using various factors including initial constituent concentrations in the solution or gel form and different drying modes (air drying, lyophilization etc.). In the later case pre-freezing temperature is a vital factor. Molds or containers used in preparation dictate the pore geometry of the material. Solutions used to rehydrate the fabricated material, also factor in the resultant material properties.

It has been shown that material gelled prior to freezing possesses much smaller average pore diameter as opposed to the solutions directly frozen at the same temperatures (22). Average pore size also decreases with decreasing pre-freezing temperature, resulting in a higher number of pores, which can be explained the larger number of crystal nuclei formation at lower temperatures (23). The wall thickness not being directly related to pre-freezing temperature, varies insignificantly.

Initial concentration of the constituents inversely effects the overall porosity (24). The concentration should be optimized considering increase of overall porosity at expense of decrease in mechanical strength of the material. Too dilute initial solutions result in easily irreversibly deformable material, while extensive viscosity of concentrated solutions results in poor porosity; both preparations are difficult to handle and reproducibility of it's morphology is low.

In case of chitosan-gelatin preparations, toughness of the resultant material increases with initial concentrations of constituents and chitosan-gelatin ratio has a significant influence on mechanical behavior of both dry and re-hydrated material. Increase in gelatin content of the material yields to decrease in Young's modulus (E), and increase in resulting strain (ε). Re-hydrated films posses deformation behavior of a ductile material, while tougher dry films undergo brittle fracture upon tensile loading (25)
Intermolecular crosslinking is necessary in order to establish mechanical and chemical stability vital to a given biomaterial application. Various crosslinking methods have been employed (26).

For instance, chitosan-gelatin material has yielded soft and elastic films following treatment with conventional crosslinkers such as glutaraldehyde (27, 28). While effective, reagent use carries along concerns in view of the direct toxicity of many crosslinkers as well as the potential or established threat posed during *in vivo* degradation by their breakdown products. Most recently, the use of naturally available and relatively non-toxic crosslinkers such as genipin has impacted biomaterials research (29). Being an excellent cross-linker of proteins, collagen, gelatin, chitosan and combinations thereof, the low acute toxicity of genipin potentially forecasts major

changes to materials that are normally prepared using synthetic cross-linking regents.

In keeping with these trends, the dehydrothermal reagent-free preparation of chitosan-gelatin films seemed to depict a logical next step in the development of chitosan-gelatin scaffold films for improved tissue regeneration and engineering. The fundamental processes related to *in vacuo* dehydrothermal crosslinking are described below.

1.6 Dehydrothermal crosslinking

The reagent-free dehydrothermal crosslinking approach adopted herein was an extension of examples known to organic chemists, such as the thermally-promoted transformation of ammonium carboxylate bridges into covalent amide bonds (30). In this process, carboxylic acids and primary or secondary amines are neutralized to yield powdered salts, which are dehydrated by incubation at elevated temperatures (e.g., 200°C), evolving water:

$$\text{i.e., } RCOOH + H_2N\text{-}R' \rightarrow RCOO^- {}^+NH_3\text{-}R' \rightarrow RCONH\text{-}R' + H_2O_{(\uparrow)}$$

In addition to the positive effects of heat, the thermodynamic spontaneity of the second step should be promoted in an open system placed under vacuum, as each salt molecule will generate one amide and one volatile water molecule. As heating and possibly vacuum conditions may be assumed to similarly facilitate the kinetic demands of dehydration, a convenient *in vacuo* heat-based rationale to crosslink biomaterials begins to take shape. While the notion of subjecting proteins to such unusual conditions might seem counter-intuitive at first, the theme adopted in this work has not been without precedent. For instance, the act of desolubilizing protein powders by crosslinking intermolecularly bridged ammonium carboxylate groups has been shown chemically and dehydrothermally (31).

The fact that functional groups of lyophilized proteins will reflect their general reactivity in solution has been established using various reagents, as has been the ability to conveniently apply chemical reagents directly to the dry lyophilisates in order to

modify protein groups (32, 31). The “pH memory”, a term coined to signify the retention of functional group ionization states over the course of lyophilization, has allowed one to optimize many water-free, “pH-dependent” chemical transformations of a lyophilized material by appropriately adjusting the pH of the protein solution prior to lyophilization (33, 34).

Isolated work has also shown that proteins in hydrophobic environments benefit from enhanced stability. Hence it followed to reason that dehydrothermal processing of dry biomaterials could be achieved without encountering detrimental structural changes (31, 35).

Dehydrothermally-promoted amide bond formation was directly confirmed in later work using lyophilized proteins (36). In these protein-based materials, covalent bonds were formed at relatively low temperatures (i.e., 75-100ºC) with a vacuum applied to better drive off water. The amide bonds between adjacent molecules had resulted in zero-length crosslinks and these were confirmed using chemical methods as well as mass spectrometry. Upon chemical blockage of either amine or carboxyl groups, *in vacuo* heating of lyophilized protein did not lead to desolubilization, showing the direct contribution of both components. In this work the pH optimum of crosslinking was noted as being between 7-9, suggesting an interaction between ionized states of carboxyl and amino groups (37). Replacing the aqueous medium with organic solvent prior to lyophilization decreased the number of covalent interactions formed upon *in vacuo* crosslinking, indicating the importance of first establishing direct intermolecular interactions. Crosslinked dimers, formed by the *in vacuo* thermal treatment of RNase A, had retained the activity of the monomer. In fact, more detailed recent work showed an enhancement of enzymatic activity upon dimerization.

A single zero-length crosslink was confirmed between Lys_{66} and Glu_{9} (38). In view of work similar to the above, it has been suggested that the number and location of covalent crosslinks could play a role in fine-tuning biological activity. The *in vacuo* method has been employed to crosslink horseradish peroxidase to anti-rabbit immunoglobulin G(IgG), the resultant enzyme-linked antibody professed as possessing

an improved sensitivity towards antigen detection (39). The same method has also been applied to lysozyme, horseradish peroxidase and lysozyme–peroxidase dimers (40, 41). As highlights to some of this work, all enzyme dimers were found to have retained their activity. In the case of peroxidase, a two-fold rise of activity was noted in comparison to the monomer. The activity of dimerized lysozyme remained unchanged whereas its thermal stability rose compared to the monomer. The crosslink sites of these dimers were not established, however, circular dichroism analyses indicated minor changes of tertiary structure in the dimerized lysozyme. An increased thermal stability was not observed in dimerized peroxidase, which may be related to the bulkier structure of this protein.

Dehydrothermal crosslinking of films was carried previously using albumin, in view of its ability to bind almost anything. In this study, the *in vacuo* method was used to immobilize bovine serum albumin (BSA) in film format along glass surfaces bearing the polymerized 3-aminopropylsilsequioxane structure (42). SDS-PAGE analysis showed complete insolubility of albumin, indicative of intermolecular crosslink formation between juxtaposed albumin molecules. Repeated washings could not purge the glass surfaces of albumin. It followed to reason that albumin molecules, dried at pre-set pH values, had simultaneously bonded to one another and to functional moieties distributed along the surface of the glass. Non-covalent interactions arising through dehydrothermal treatment were ruled out as treatment with salts solutions would be expected to break any ammonium carboxylate bonds, liberating the protein into solution. The fact that albumin was slightly swollen when hydrated showed that intermolecular hydrophobic bonding was not strong enough to prevent the infusion of water. Disulfide bonding was also ruled out, as the samples were boiled in SDS denaturating buffer prior to gel analysis. The results hence pointed to amide and possibly ester bond formation as the strongest possibilities.

In the case of forming chitosan-gelatin composite films, the same *in vacuo* dehydrothermal principle has been applied (Figure 1.3). Amino groups located at position 3 of each glucosamine unit of chitosan contribute to amide bond formation between juxtaposed chitosan and gelatin molecules. The reaction will result in both

gelatin-gelatin and gelatin-chitosan covalent interactions. Thus, dehydrothermal treatment of lyophilized chitosan-gelatin films should lead to the formation of highly heterogeneously crosslinked material.

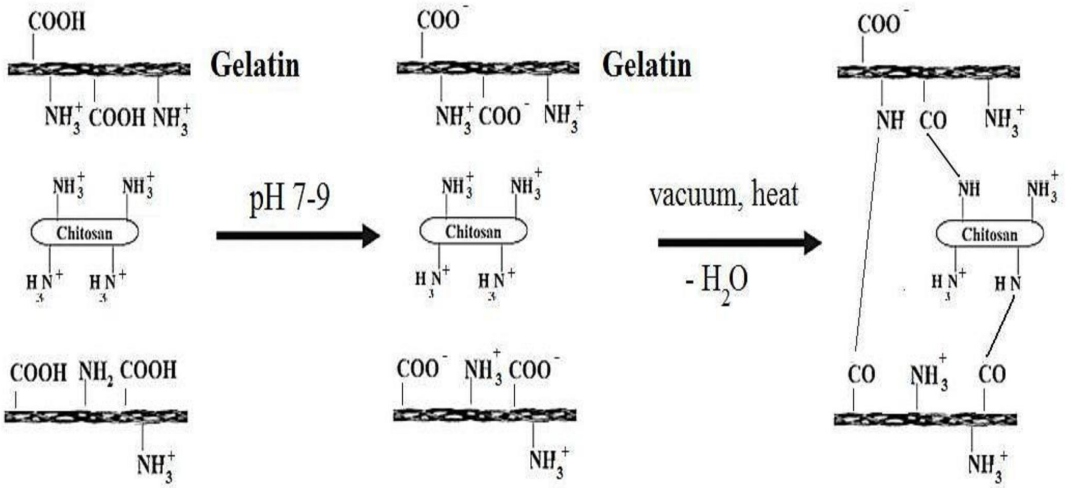

Figure 1.3: Schematic visualization of the envisaged gelatin-gelatin and chitosan-gelatin intermolecular crosslinking reaction.

In this work a number of crosslinked composite chitosan-gelatin films were fabricated with variable compositions using the *in vacuo* dehydrothermal technique. The attraction towards chitosan and gelatin has been expressed previously in the text. The technique was also employed in series to yield hemoglobin-immobilized chitosan-gelatin films (Figure 1.4).

The choice to incorporate hemoglobin was not only made in view of its readily observable catalytic properties, but also for its potential to be used as an effective biomaterial. For instance, alternative artificial blood substitutes have been extensively researched and hemoglobin-based oxygen carriers (HBOC) have formed a major subject thereof. Various methods have been employed in crosslinking and co-polymerizing the hemoglobin units, but none have addressed the possibility to use a reagent-free technique (43). A recent study related to zero-length crosslinks expressed the use of carbodiimide activation to crosslink hemoglobin units. In this work, activation of surface carboxyl groups led to the formation of an intra-molecular pseudopeptide bond

via an adjacent amino group (44). By way of this method, “polyhemoglobins” approaching molecular weights as high as 25MDa were realized, and moreover, the oxygen-carrying capacity of such material was retained. These remarkable an important results further encouraged the use of hemoglobin in this work.

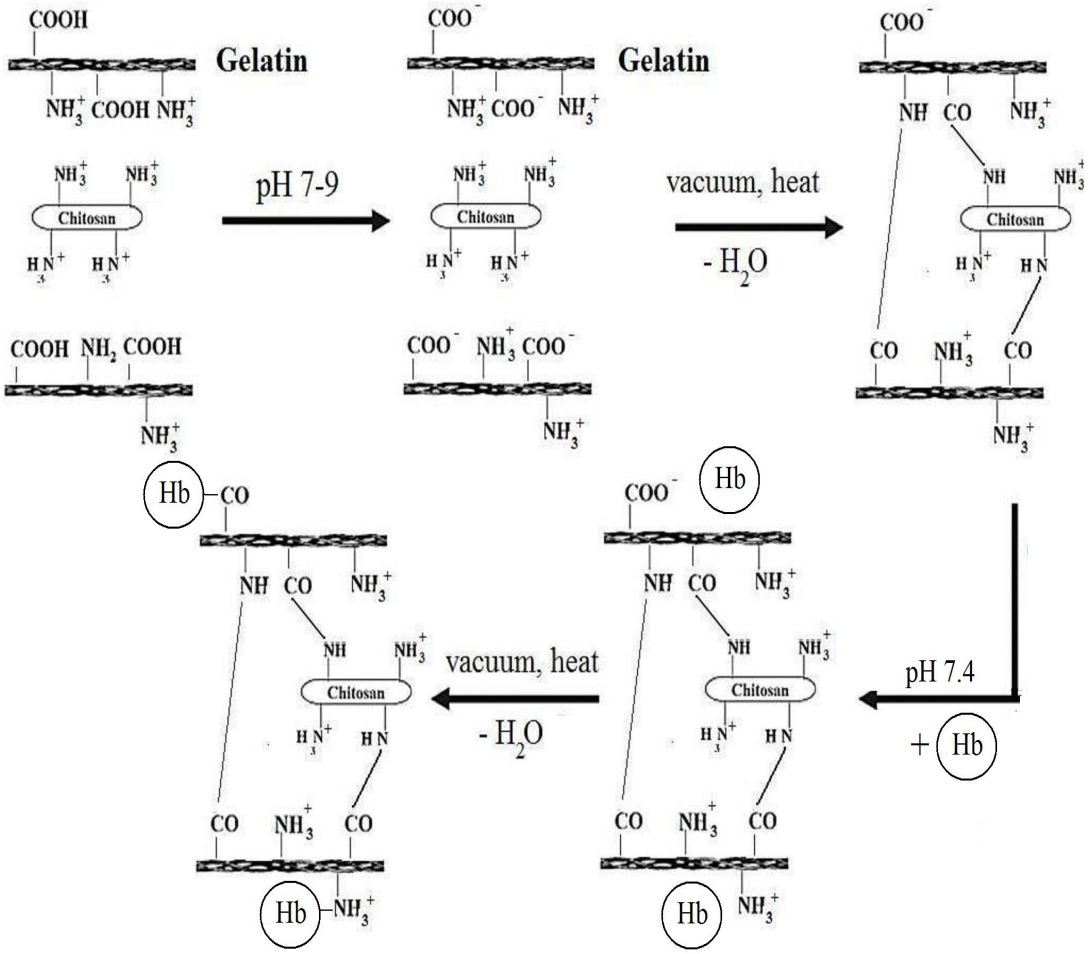

Figure 1.4: Schematic visualization of bond formation upon dehydrothermal hemoglobin immobilization on crosslinked chitosan-gelatin film.

The layout of this thesis has been heading oriented. Chapter 2 describes the materials and methods used to prepare and test each chitosan-gelatin film as specified in the abstract. Chapter 3 describes analysis of the results obtained upon chitosan-gelatin film preparation with respect to it's structure and functionality. Chapter 4 concludes the completed work.

2. Materials and Methods

2.1 Materials

Chitosan (degree of deacetylation 85%) isolated from shrimp shells (FlonacN), was purchased from Aquapremier Co., Ltd, Thailand. Gelatin Type B from bovine skin was purchased from Sigma-Aldrich, Inc. Trypsin from porcine pancreas was purchased from Biological Industries Ltd. Dulbecco's modified Eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from Pan Biotech GmbH. HCT-116 human colon carcinoma cell line was kindly donated by Batu Erman of Sabanci University, Biological Sciences and Bioengineering Program. K293-GFP human embryonic kidney cell line was kindly donated by Huveyda Basaga of Sabanci University, Biological Sciences and Bioengineering program. All other reagents were of analytical grade and were used without further purification.

2.2 Methods

2.2.1. Material preparation

2.2.1.1 Stock solutions

Chitosan (2-4wt%) was dissolved in dilute acetic acid (1-2wt%, 50-60˚C) under vigorous agitation. Once homogeneity was established, gelatin (2-8wt%) was added and dissolution was continued under mild agitation. Finally, sucrose (0-10wt%) was added and dissolved under mild agitation.

2.2.1.2 Film preparation

Warm aliquots (5ml) of stock solution were poured into polystyrene Petri dishes and allowed to cool. The Petri dish and contents were subsequently submerged in liquid

nitrogen. Frozen samples were lyophilized (0.02-0.04mbar, 12h) without applying external cooling. Prior to *in vacuo* heating, lyophilized films were incubated (RT, 8-12h) in a partially-evacuated desiccator containing solid ammonium carbonate (5g), the idea being to adjust the ionization state of the film to promote good crosslinking.

2.2.1.3 In vacuo thermal crosslinking

A make-shift apparatus consisting of an evacuatable glass vessel and variable heat source was employed to drive the *in vacuo* crosslinking reaction (Figure 2.1 (a)). Lyophilized samples were transferred into the glass vessel and incubated (100ºC, 0.2-0.6mbar, 14h). To remove porogens and/or unreacted chitosan and/or gelatin, the crosslinked films were soaked (12h) in phosphate buffered saline (PBS) buffer, pH 7.4, and washed using distilled water. Finally, the samples flash frozen again and re-dried under vacuum in a bell jar (Figure 2.1 (b)).

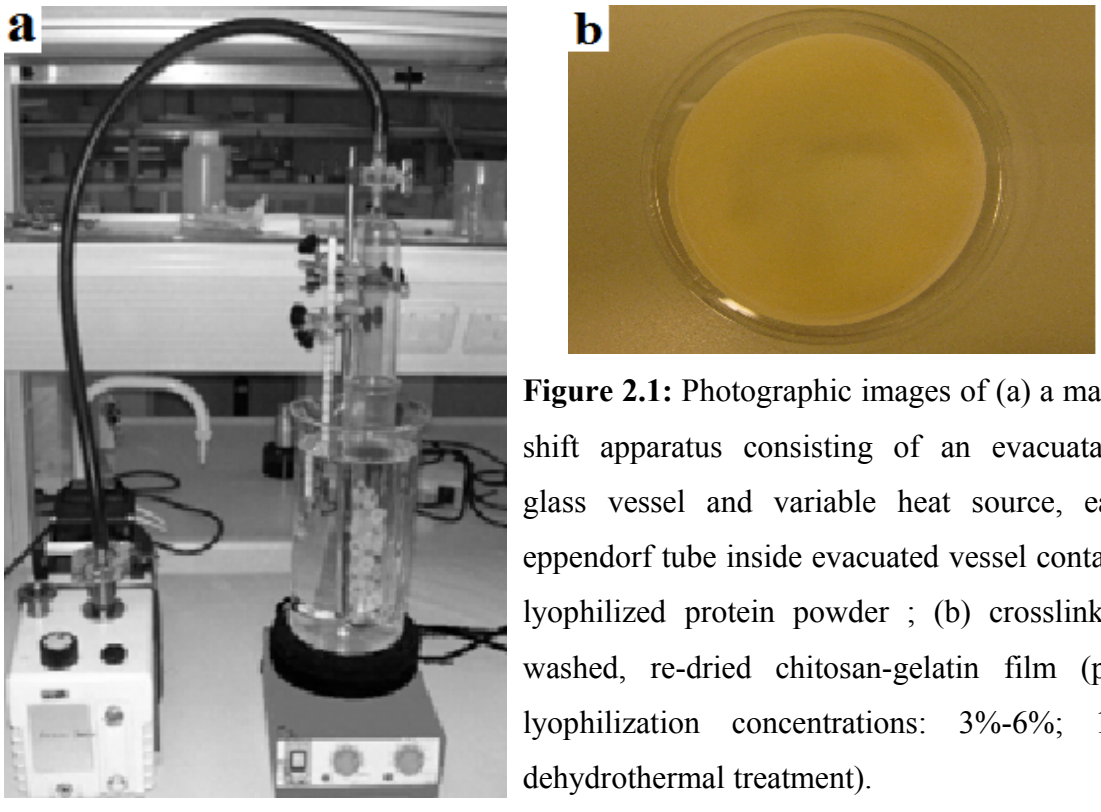

Figure 2.1: Photographic images of (a) a make-shift apparatus consisting of an evacuatable glass vessel and variable heat source, each eppendorf tube inside evacuated vessel contains lyophilized protein powder ; (b) crosslinked, washed, re-dried chitosan-gelatin film (pre-lyophilization concentrations: 3%-6%; 14h dehydrothermal treatment).

2.2.1.4 Immobilization of bovine serum hemoglobin (Hb) on crosslinked films

20mg crosslinked chitosan-gelatin films were incubated in Hb/PBS buffer solutions of different concentrations (0,05-1 mg/ml) and volumes (0,5-10 ml) at room temperature, with and without agitation or partial vacuum conditions (used to promote infusion of liquid) for a minimum of 2 hours.

Following incubation, the films were removed from the solution, flash frozen and dried under vacuum. Since the PBS buffer imparted a pH value of 7.4 to the film, the pre-crosslinking ionization state of the film was already near-ideal. Hence, the gas-phase pH adjustment step, which was normally used to crosslink lyophilized chitosan-gelatin, was skipped. A make-shift apparatus consisting of an evacuatable glass vessel and variable heat source was employed to drive the *in vacuo* crosslinking reaction. Dried samples were transferred into the glass vessel and incubated (100ºC, 0.2-0.6mbar, 8h). To remove unreacted hemoglobin and to ensure that the measured activity did in fact reflect only immobilized hemoglobin, the films were pre-incubated (RT) thrice in fresh PBS buffer (10ml; 0.5h, 1h and finally 1h). The hemoglobin activity residing in the films as well as in the wash solutions was measured as per the OPDA-hydrogen peroxide assay (see below for further details).

2.2.2. Material analysis

2.2.2.1 Scanning electron microscopic (SEM) structural analysis

Samples were frozen in liquid nitrogen and cut with a fresh razor blade before treatment with a carbon coater. A gun voltage of 2kV was employed.

2.2.2.2 Stability tests

Solubility - Crosslinked films and non-heated controls were agitated in PBS buffer (1wt %, 37°C, 450rpm, variable time) and dried under vacuum. SEM images were obtained.

Biodegradation – Samples (5mg) were cut and incubated (37°C, 450rpm, variable time) in PBS buffer (1ml) containing trypsin (310 USP/ml) before being retrieved and dried for SEM analysis. Ninhydrin analysis was performed using spent buffer/ninhydrin/isopropanol comprising 100:1:99 *[v/w/v]* (70˚C, 20min). Color yields were analyzed spectrophotometrically (570nm). All comparisons were made against the non-crosslinked control, which was arbitrarily assigned a color yield of 100%.

2.2.2.3 Porosity

Porosity was estimated from the weight and dimensions of at least 6 samples acquired from different regions of each film. Samples were incubated in absolute ethanol (30min, partial vacuum) until permeated. Excess ethanol was wiped from the surface with filter paper and the samples were re-weighed. Porosity (%) was determined according to 100% x $((W_w-W_d)/\rho_{EtOH})/V$ where W_d & W_w are dry and ethanol-perfused weights, V is film volume and ρ_{EtOH} is ethanol density (27).

2.2.2.4 Mechanical testing

Rectangular samples (1mm×5mm×10mm) were cut and strained against dynamic tension (35-37°C; 1Hz) using NETZSCH DMA 242C. Analysis was performed on dry and 96%ethanol-perfused samples (i.e., suspended in 96%ethanol under partial vacuum, 30min). NETZSCH DMA 242 software was employed to evaluate the results.

2.2.2.5 FTIR spectroscopy

FTIR spectra of the films were obtained using an attenuated total reflection (ATR) accessory. Prior to measurement, films were incubated in a desiccator with a small amount of distilled water in order to equilibrate the moisture content of the films at accessible sites. Once hydrated as such, the films were clamped directly along the ATR cell. Twenty scans were averaged and the baseline was corrected before reading were made.

2.2.2.6 Cell culture studies

Studies were conducted using films obtained from 4wt%:8wt% chitosan-gelatin starting concentrations. These films were appropriately cut to fit along the bottom of the culture plate wells. The film samples were collectively perfused first with absolute ethanol, then with 70% ethanol under partial vacuum prior to incubation using pre-autoclaved PBS (20 ml) and next with Dulbecco's modified eagle medium (DMEM) (20 ml). All manipulations were conducted under a laminar flow chamber. Finally, the sterilization process was completed by exposure to UV radiation. Sterilized films were placed along the bottom of the culture plate wells. Cells were cultured over the films in complete DMEM (i.e., containing 10% fetal bovine serum and 1% antibiotic solution). The samples were incubated at 37 ºC in a tissue culture incubator under a 5% CO_2 atmosphere. The culture medium was changed every 2 days. HCT-116 cells (human colon carcinoma cells) were cultured at a density of 10^5 cells/ml on films placed in a 6-well plate. Optical images of the film were taken following one week incubation. HEK293-GFP cells (GFP expressed, human embryonic kidney cells) were cultured at a density of 10^4 cells/ml (i.e., 200µl/well) on films placed in a 96-well plate.

The MTT test was performed following every 2 days of incubation. Absorption values were measured at 570 nm using a reference wavelength of 665nm in accordance with the Roche Applied Science Cell Proliferation Kit I (MTT) protocol (45). Films were transferred to new plate wells prior to each MTT test in order to minimize any potential errors related to residual cell growth directly on the surface of the well. Additionally, cells cultured on blank plate wells (in the absence of film) were used as non-film positive controls.

Just prior to removing film for the MTT analysis (at 0, 2, 4, 6 and 8d incubation times), fluorescent images were acquired using a phase contrast fluorescence inverse microscope (Olympus IX70).

2.2.2.7 Activity detection of free and immobilized hemoglobin

The assay used was a variant of a published procedure (46). In particular, this method was based on the ability of hemoglobin to catalyze the reaction between o-phenylenediamine (OPDA) and hydrogen peroxide to form 2,3-diaminophenazine (DAPN) (Figure 2.2).

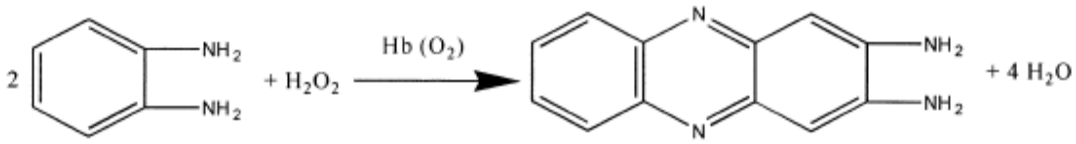

Figure 2.2: Hemoglobin catalyzed 2,3-diaminophenazine formation reaction.

In a typical run, the reaction was initiated by adding hydrogen peroxide ($2x10^{-3}$ mM) to a stirred solution containing free or immobilized Hb and OPDA ($4x10^{-3}$ mM). Product formation was spectrophotometrically quantified at 425 nm after one hour of incubation. Dilutions, whenever necessary, were made using PBS buffer. Immobilized hemoglobin films were soaked (30 min) in the reaction solution after a pre-wash in PBS, prior to initiation of the reaction with hydrogen peroxide addition.

3. Results and Discussion

3.1 Morphology and physical properties

3.1.1 Morphology and porosity

Some film properties relevant to biomaterial applications were optimized by varying the chitosan-gelatin ratio and absolute concentrations of the starting solutions. The contribution of sucrose, as an established porogen, was examined by inclusion into these solutions. The pre-lyophilization concentration of chitosan-gelatin in these solutions have been described as chitosan%:gelatin% where % signifies weight percent.

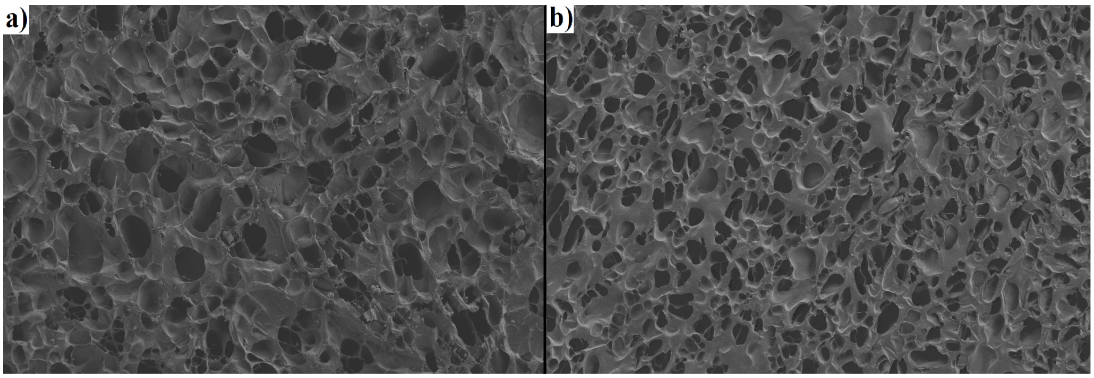

Figure 3.1.1: Surface morphology of crosslinked chitosan-gelatin film 4%:4% without sucrose (a) and containing 5% sucrose (b) (14h dehydrothermal treatment). Horizontal dimension = 330μm.

The major established factors effecting porosity and structure of similarly targeted biomaterials have included the pre-freezing temperature, the initial component concentration and the porogen type and amount. SEM analyses (Figure 3.1.1, Figure 3.1.2) showed that thermally treated films bore considerable porosity and interconnectivity in the presence or absence of sucrose as porogen. Also, it could be seen that porogen-incorporated crosslinked materials featured smaller average pore sizes, more regular pores and a denser distribution compared to porogen free material. Pore diameter of fabricated material varied between 5-50 μm according to the parameters discussed.

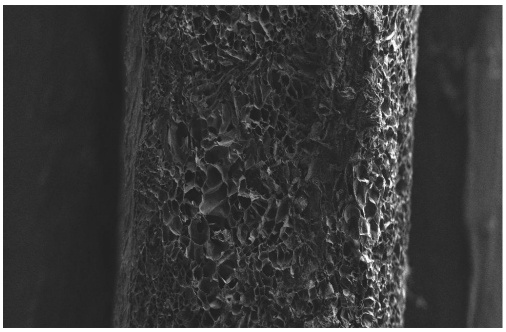

Figure 3.1.2: Cross-sectional morphology of crosslinked chitosan-gelatin film (4%:8%, 14h dehydrothermal treatment). Horizontal dimension = 660μm.

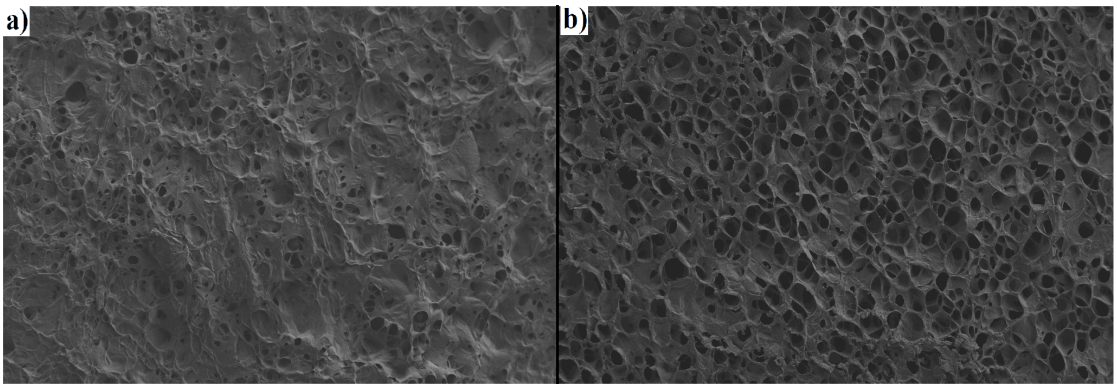

Figure 3.1.3: Surface morphology of crosslinked chitosan-gelatin film 2%:4% containing 2.5% sucrose before (a) and after(b) washing (14h dehydrothermal treatment). Horizontal dimension = 471μm.

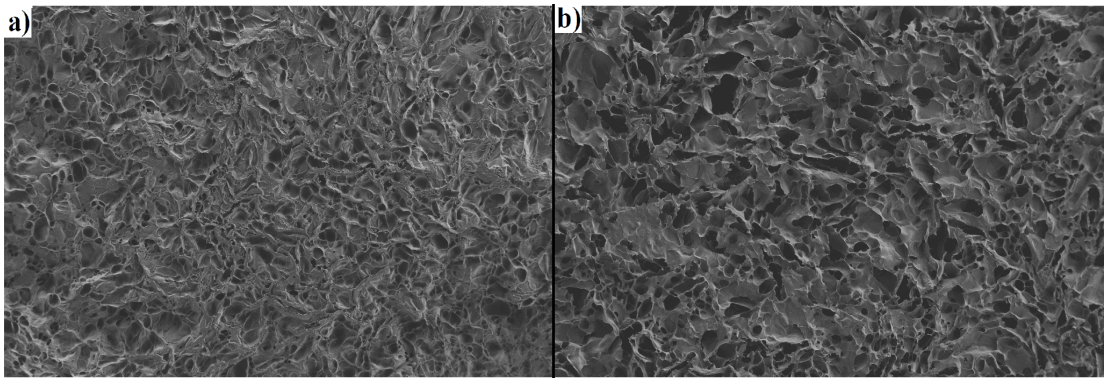

Figure 3.1.4: Surface morphology of crosslinked chitosan-gelatin film 2%:4% before (a) and after(b) washing (14h dehydrothermal treatment). Horizontal dimension = 660μm.

Porosity measurements were performed using ethanol-perfused films as opposed to hydrated films. While perhaps counter-intuitive in view of the relevance of water, the choice to use ethanol had merit in that ethanol, being less polar, was found to limit porosity calculation errors attributed to film volume changes upon re-solvation. The crosslinked films appeared denser than the non-heated blanks and initially nonporous. However, a porous morphology was observed upon washing, concomitant with a considerable weight loss. In zero-sucrose examples, such an observation led to the conclusion that non-crosslinked material in thermally treated samples served as the porogen (Figure 3.1.3 and Figure 3.1.4). Figure 3.1.5 nicely illustrates that the porosity of thermally-treated films could be varied with the chitosan, gelatin and sucrose concentrations. Overall, the measured porosity turned out to be comparable amongst zero-sucrose and 5% sucrose materials.

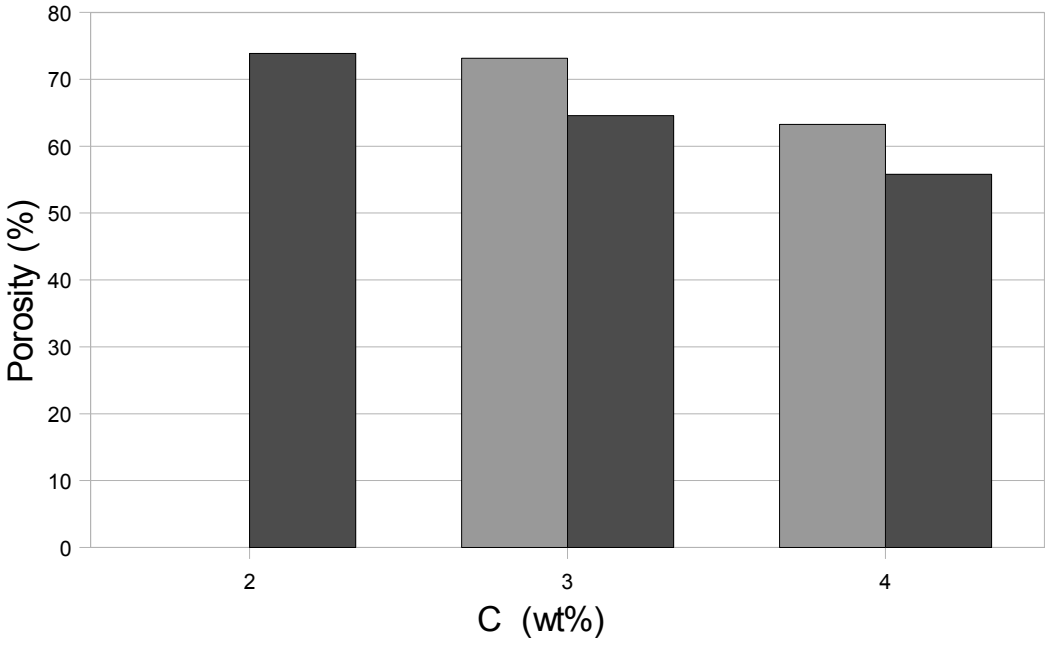

Figure 3.1.5: Porosity of crosslinked chitosan-gelatin films. C – pre-lyophilized chitosan concentration, chitosan:gelatin initial concentration ratio. Light grey Chitosan:gelatin ratio 1:1; dark grey: chitosan:gelatin ratio 1:2.

3.1.2 Mechanical properties

Attempts to accurately analyze the mechanical properties of the films proved unsatisfactory in view of the technical limitations of the available equipment and the stringent measurement constraints imposed by these small, difficult-to-handle samples. In particular, the universal tester available in-house was too large to provide the needed sensitivity. A smaller unit produced by Bose-Electroforce was also discredited by an applications expert, claiming that the sample grips of the instrument were improperly designed and would tear soft samples along the gripping regions through a pinching action, thereby preventing any true reading of the yield strength.

Another potentially suitable instrument, namely, the Netzsch 242 dynamic mechanical analyzer (DMA), was also limited by software, which in practice did not support measurements within the scope of soft hydrated biopolymers. In contrast, relatively adequate data was obtained using dry and 96% ethanol-perfused films, but the results lacked accuracy and reproducibility. In particular, non-crosslinked dry controls proved too fragile to analyze, whereas crosslinked dry samples proved brittle (~0.5% elongation; max. dynamic load = 3N), with an elastic modulus largely exceeding the set maximum of 30MPa. Not surprisingly, 96% ethanol-perfused crosslinked films and non-crosslinked controls strained to a much greater extent under dynamic tension (8-15% elongation), but lacked the toughness of the crosslinked dry samples. For instance, one chitosan-gelatin sample (3wt%: 6wt%) that had failed under strain (max. dynamic load = 1N) displayed 10% elongation and an elastic modulus of 30MPa. The general behavior of 96% ethanol-perfused and dry crosslinked samples is presented in figure 3.1.6. PBS-perfused crosslinked samples clearly exhibited much higher flexibility and greater elongation (results not shown) compared to ethanol-perfused and dry samples, but no adequate quantification could be obtained by the means of available software. Non-crosslinked PBS-perfused films did not retain mechanical integrity and could not be measured (see section 3.3 for further discussion).

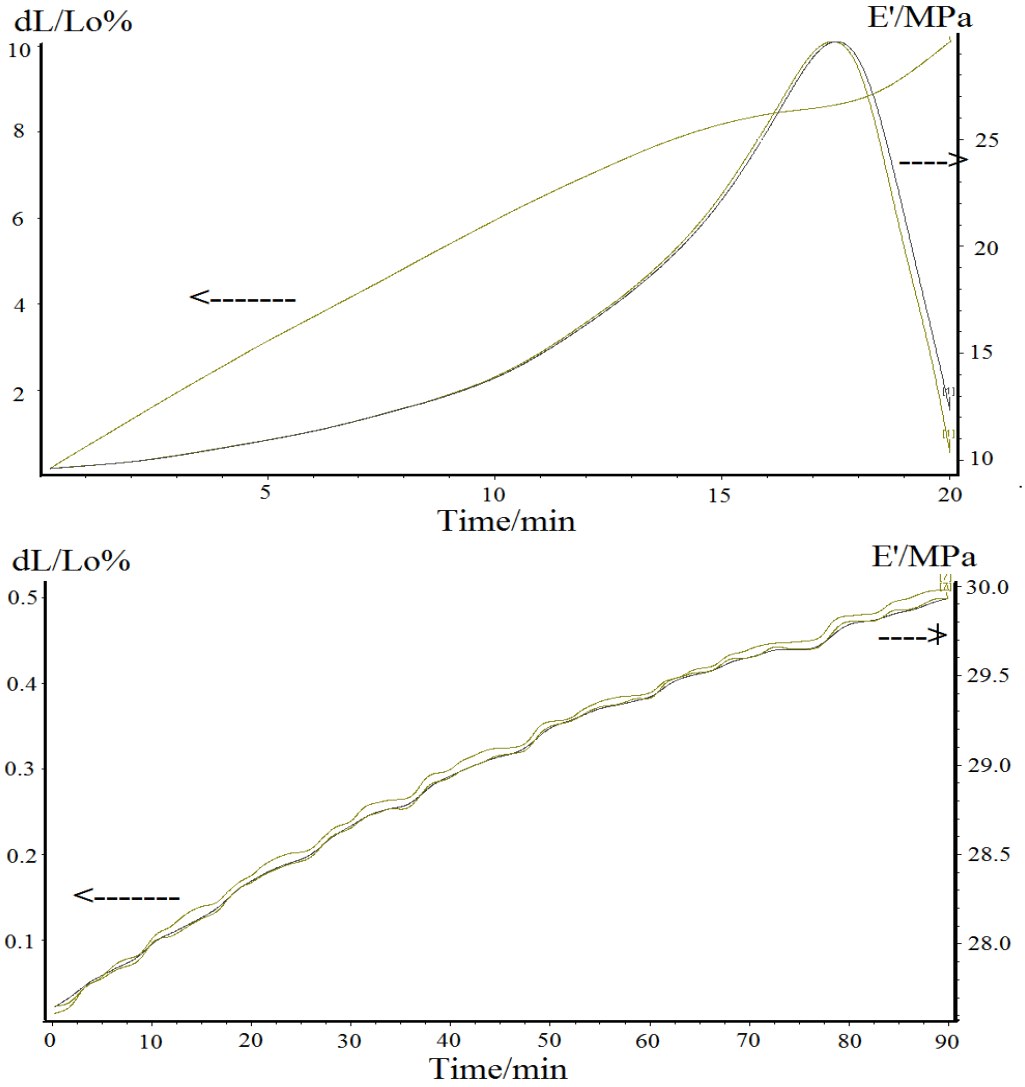

Figure 3.1.6: Mechanical behavior pattern of 96% ethanol perfused (top) and dry (bottom) crosslinked films strained against dynamic tension (35-37°C; 1Hz).

Although accurate data could not be obtained, superficial conclusions could be made. Toughness of the material was mainly dependent on the crosslinking degree of the composite and concentration of constituents prior to lyophilization.

In comparison to native dry chitosan-gelatin composites, thermal crosslinking was found to improve the mechanical toughness of such dry films. Looking to Figure 3.1.7, both a higher initial concentration of chitosan-gelatin in the preparative solution and a lower porosity led to an increased toughness of the material.

On the other hand ductile-brittle behaviour transition has been found to occur upon varying of chitosan gelatin ratio and the hydration degree of the final crosslinked material.

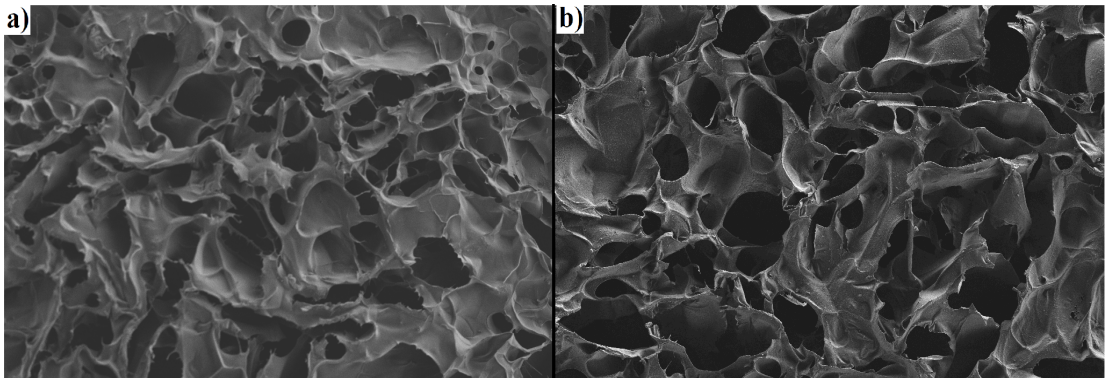

Figure 3.1.7: Morphology of crosslinked chitosan-gelatin film a)2%:4% b) 3%:6% (14h dehydrothermal treatment). Horizontal dimension = 220μm.

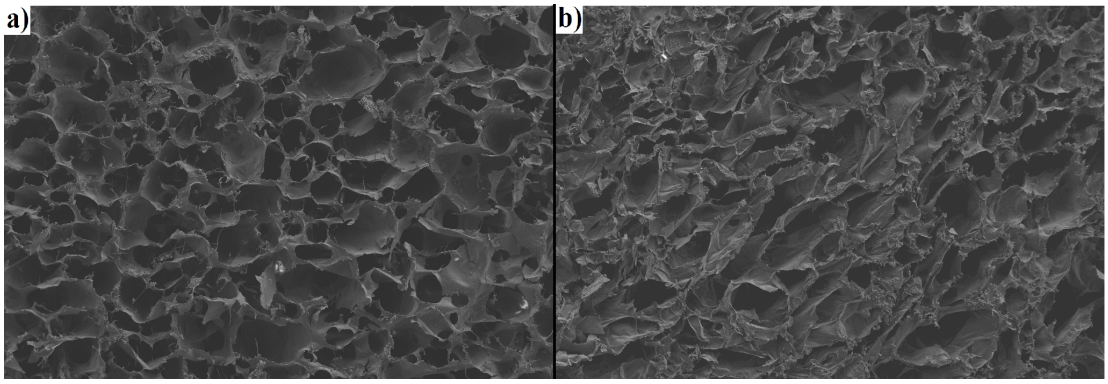

Figure 3.1.8: Surface morphology of crosslinked chitosan-gelatin film a) 4%:2% b) 2%:4% (14h dehydrothermal treatment). Horizontal dimension = 330μm.

As a rule of thumb, the chitosan-gelatin ratio appeared to play a significant role in predicting the physical properties of the material. A higher chitosan concentration led to an increase of brittleness, even upon partial re-hydration, as noted by the roughened appearance of the surface following re-hydration-dehydration tests, while the predominance of gelatin in the composite generally permitted reversibly deformable materials (Figure 3.1.8).

While dry and 100% ethanol perfused materials possessed brittle behavior upon fracture, ductile behavior was introduced with increase of hydration degree (Figure 3.1.9). As it can be observed from the images on Figure 3.1.9. a typical crosslinked dry material (a) has fractured without visible elongation, while introduction of 4% water containing solvent (b) has increased the flexibility significantly leading to elongation upon tension and eventual necking. PBS perfused material (c) has shown a dramatic increase in flexibility. It was determined that material flexibility increased in proportion to the degree of re-hydration and at the slight expense of strain performance.

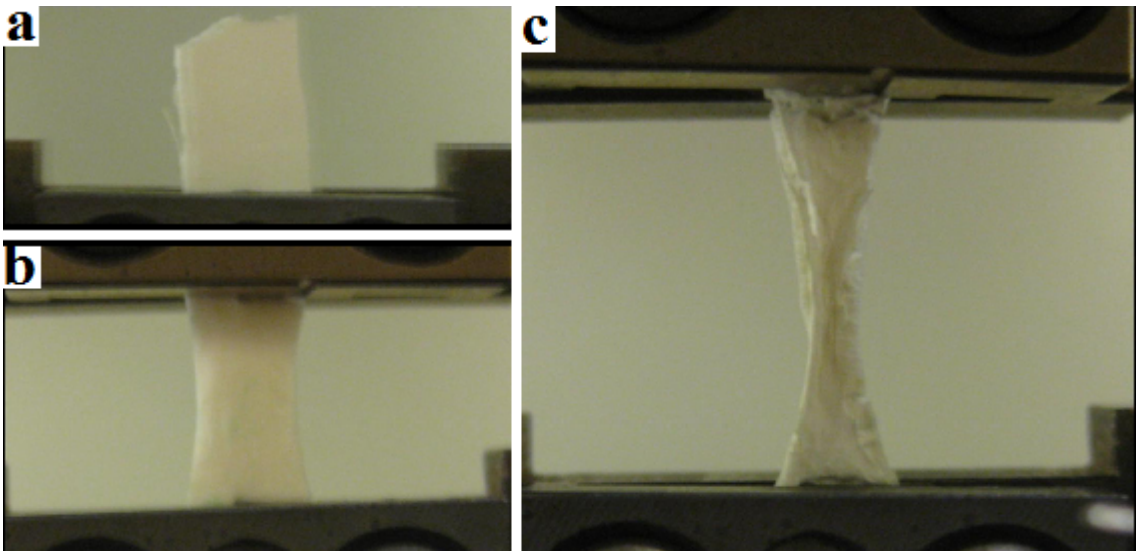

Figure 3.1.9: Photograph images of a) dry b) 96% ethanol perfused c) PBS perfused strained against dynamic tension (35-37°C; 1Hz).

3.2 Molecular structure

Prediction of structural changes on molecular level of the material has been discussed in detail in the introduction part of this thesis, based on previous studies of dehydrothermal crosslinking method. Herein FTIR spectroscopy analysis is applied as yet another evidence of structural change upon dehydrothermal treatment of the material.

The FTIR spectra of crosslinked films (14h heat treatment) with different initial chitosan-gelatin concentrations were compared against the corresponding non-crosslinked films. As well, the effect of crosslinking time was assessed spectroscopically using samples comprised of 4%wt-8%wt chitosan-gelatin. Unwashed crosslinked films were used in the analysis as positive controls, in order to avoid possible errors occurring from loss of the material during the washing procedure. The moisture content in each film was normalized by simultaneous incubation of the materials in a desiccator containing a small amount of water under partial vacuum.

Within the range of the chitosan-gelatin compositions tested, a consistent change was observed in the IR spectrum upon thermal treatment, indicative of a change in the structure, be it predominantly physical, chemical or both. Looking to Figure 3.2.1, the FTIR spectra of crosslinked and non-crosslinked films with an initial (i.e., pre-lyophilization) chitosan-gelatin concentration of 3wt% - 6wt% have been presented.

Herein, amide I (1636cm-1) and II (1541-1547cm-1) bands appeared to display a minor relative difference of intensity. In attempting to claim a structural change on the basis of this spectroscopic difference, a related time course experiment was conducted, which showed a definite reversal of amide I and II band intensities as the crosslinking time increased (Figure 3.2.2).

These changes of the FTIR analyses of amide I (i.e., C=O stretching) and amide II (i.e., N-H bending) bands appeared to reflect incremental secondary structural changes as a function of crosslinking time. Presented graphically (Figure 3.2.3), the relative increase of the amide I versus amide II band intensity over time delineated a hyperbolic profile. Although inadequate spectral resolution prevented an in-depth analysis, small but certain relative changes in bond polarization pointed to hydrogen bond redistribution. Within the limits of resolution, amide II bands also appeared to have shifted to a minor degree from their initial position at 1541cm^{-1}. Another consistent trend was a peak shift from 1400 cm^{-1} to 1450 cm^{-1}.

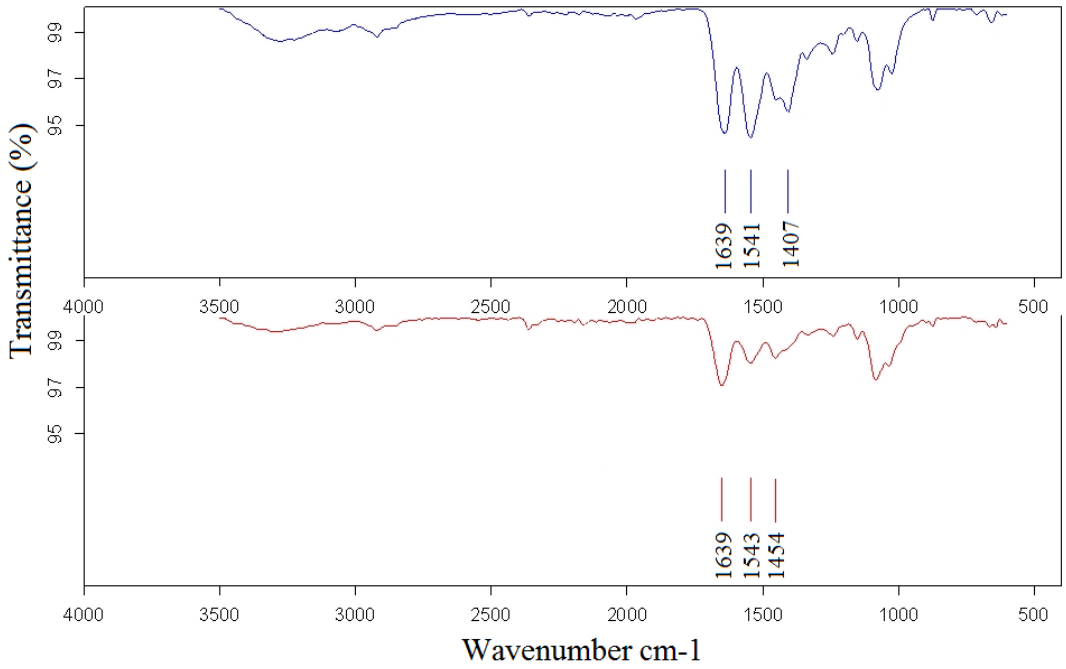

Figure 3.2.1: FTIR spectra of non-crosslinked (top), and crosslinked (bottom) chitosan-gelatin film (3wt%:6wt%).

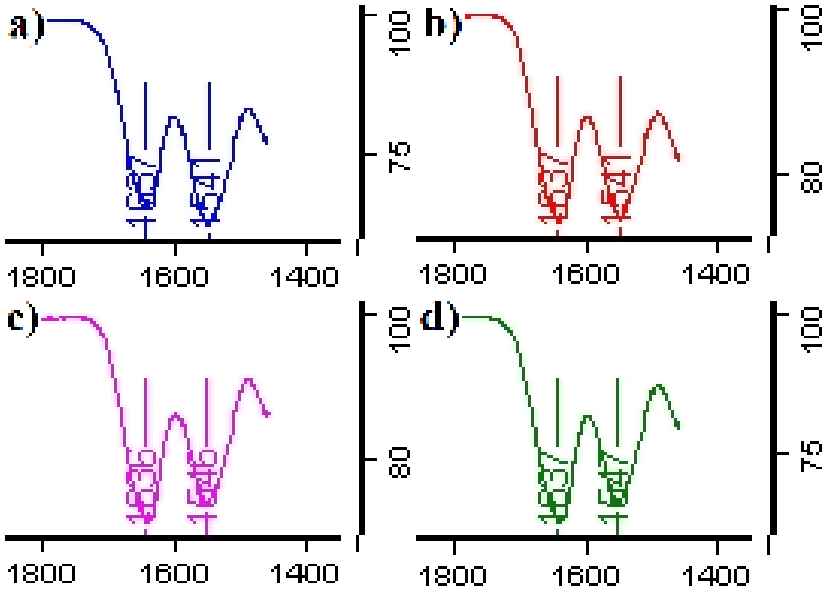

Figure 3.2.2: Expanded amide region of FT-IR spectra upon in-vacuo heat treatment for a) 0h b) 2h c) 4h d) 8h of co-lyophilized chitosan-gelatin (4%wt:8%wt).

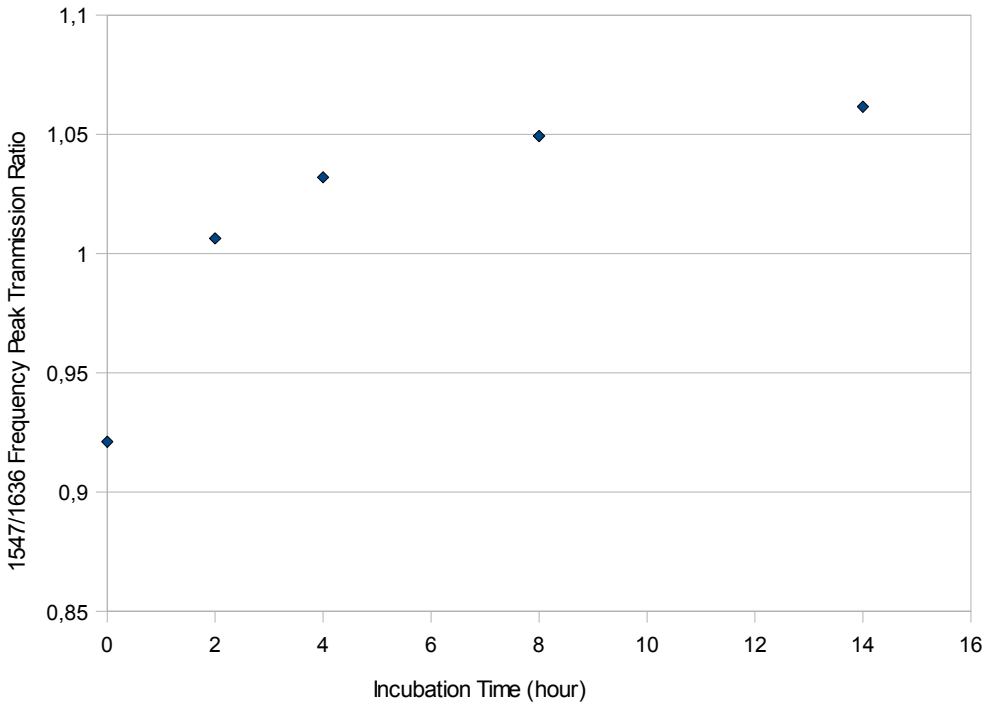

Figure 3.2.3: Ratio of IR band intensities vs. crosslinking time of chitosan:gelatin film (4%wt:8%wt).

Since the samples were permeated with water vapor before analysis, the spectral differences noted were not likely related to a differential dehydration effect expected of films heated for different times. Observed instead were spectral differences most likely related to minor covalent changes, i.e., changes which likely influenced structural compliancy, the course of re-hydration, and protein-protein hydrogen bonding. A gradual "tightening" of the inter-protein structure was envisaged, as such a scenario would reflect not only the incremental formation of crosslinks but also the spectroscopic changes. Altered non-covalent inter-protein bonding would also almost certainly be expected to contribute to the observed spectral profile of this "tightened" protein model.

3.3 Stability tests

3.3.1 Stability in aqueous media

Crosslinked samples and non-heated controls of fixed weight were incubated in PBS for different time periods, all the while agitated at 450 rpm and RT. The extent to which composite materials redissolved was followed colorimetrically by quantifying the amino groups found in solution using the ninhydrin test. As observed from the graph (Figure 3.3.1), a non-crosslinked control (square) afforded maximum color yields within a 1.5-hour PBS-incubation period, signifying the maximum extent of PBS-solubilized material. This material was presumed to be essentially gelatin, as chitosan was shown to be insoluble under the PBS incubation conditions and hence it was not expected to be retained in the supernatant prior to the addition of ninhydrin. In approximately the same time, residual uncrosslinked material within a heat-treated film (diamond, Figure 3.1.1) appeared to have dissolved, thereby establishing the porous structure of sucrose-free crosslinked preparations. Such heat-treated samples retained mechanical integrity in PBS at RT for months at a time.

Apart from the attribute of remarkable mechanical stability, no bacterial or fungal growth could be observed by inspection on these samples or in the aqueous media. This resilience towards colonization was attributed to the antimicrobial properties of chitosan, which clearly retained efficacy upon heat treatment. In both control and crosslinked samples, dissolution had reached completion in the same time frame, strongly suggesting that diffusion of solute into the film and exit of solubilized material was not rate-determining.

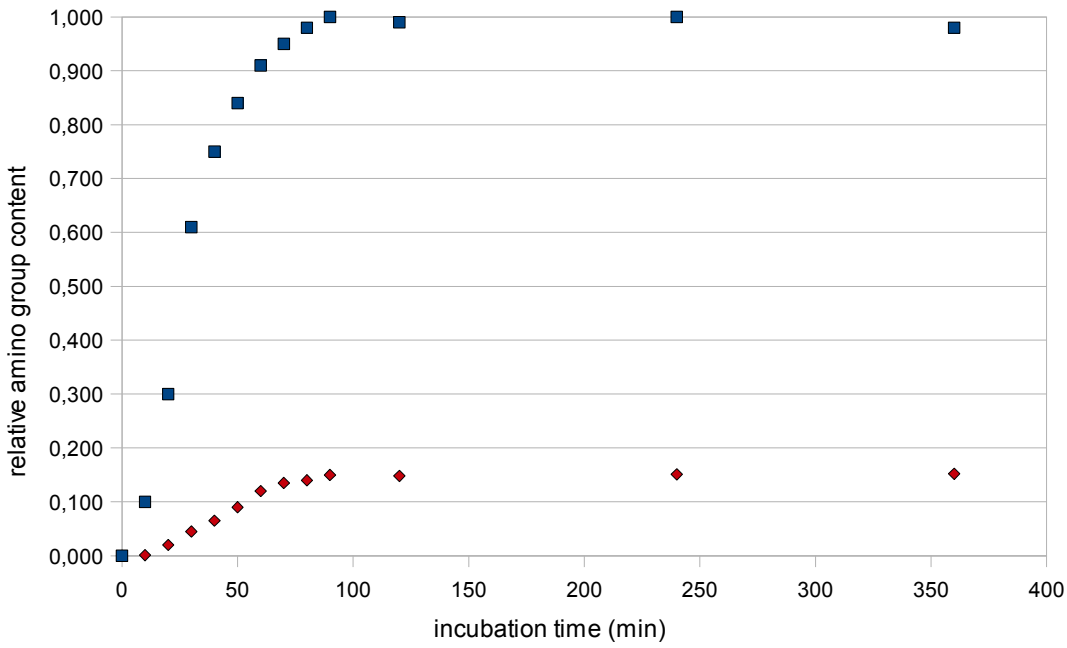

Figure 3.3.1: Relative dissolution of co-lyophilized chitosan-gelatin (2wt%: 2wt% originally) when suspended in PBS medium before (■) and after (♦) in vacuo thermal treatment.

3.3.2 Enzymatic degradation

To obtain insight into the *in vivo* biodegradation possibilities of this material, *in vitro* proteolysis tests were conducted using bovine trypsin. Among the many available enzyme formulations, purified trypsin was chosen in view that it would yield relatively few gelatin fragments and hence potentially form the basis of a convenient ninhydrin test. In any typical time-course assay, the heat-treated film was incubated with trypsin and proteolysis was terminated by removing the film from the digest solution. Since the dissolution of larger film components had been determined as being non-diffusion limited (section 3.3.1), a possibility remained that fragments entering solution might be incompletely proteolyzed. The risk being thus revealed, a skewed ninhydrin result was avoided accordingly by employing a relatively abundant trypsin concentration and long incubation times. The digest solution could then be worked up using the ninhydrin reagent. To prevent reading errors arising from insoluble dispersed materials, the solution was spun down following color development and the resultant supernatant was quantified spectrophotometrically.

As noted in the time-course tryptic digest profile (Figure 3.3.2), crosslinking had enabled films to resist but not to preclude proteolysis. The large burst of amino groups within the first hour appeared to correspond to that fraction of material, which had not crosslinked and hence could be proteolyzed more rapidly as a homogenous phase reaction. The strong time correlation between figures 3.3.1 and 3.3.2 also attested to this likelihood. From a physical standpoint, tryptic activity appeared to have caused minor "erosion" of the crosslinked films. In particular, a "surface polishing" effect was noted when pitting the appearance of a trypsin-treated film against its zero time control (Figure 3.3.3). As anticipated, non-crosslinked control films were rapidly degraded by trypsin (results not shown).

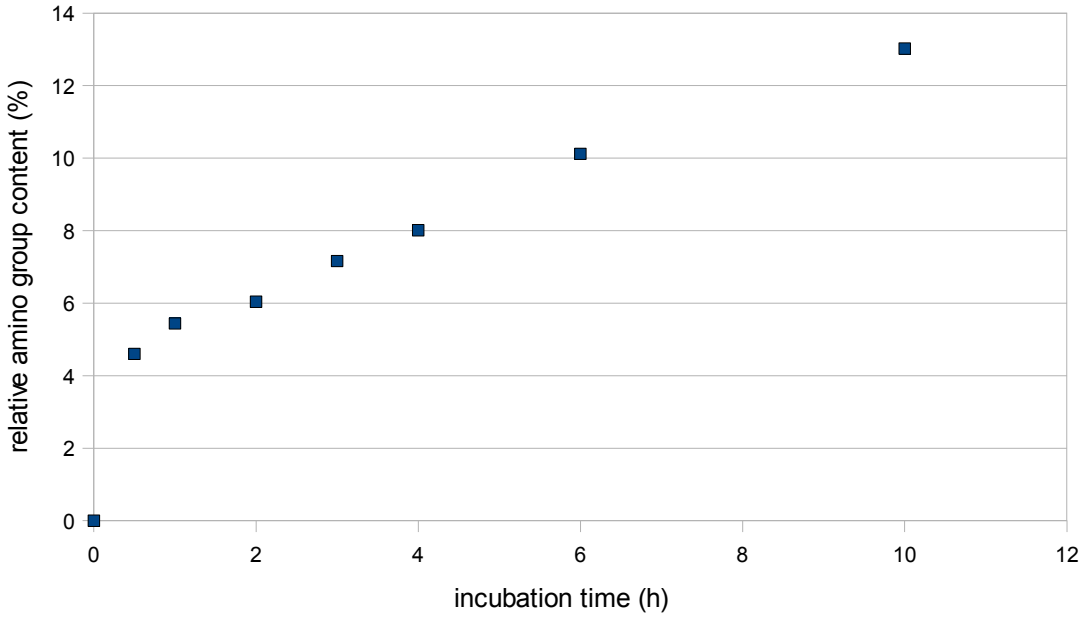

Figure 3.3.2: Liberation of ninhydrin-positive material from *in vacuo* heated chitosan-gelatin films (3wt%: 6wt%) during tryptic digestion (37°C, 450 rpm).

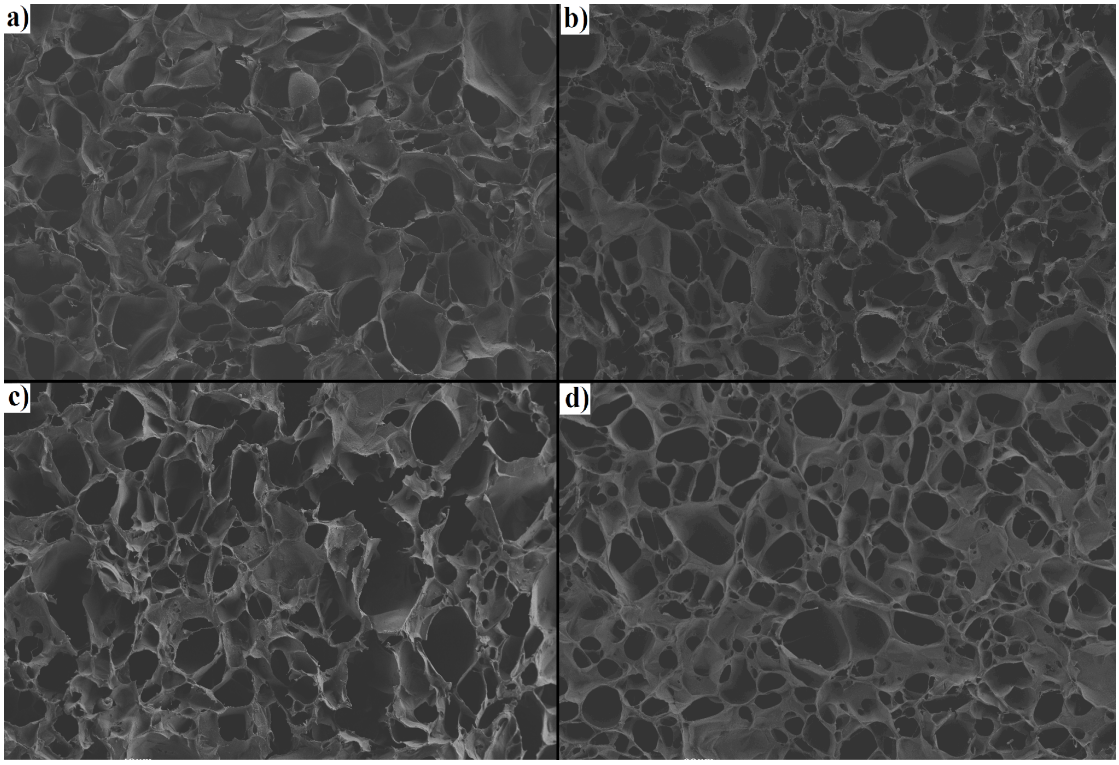

Figure 3.3.3: Surface morphology of *in vacuo* heat-crosslinked chitosan-gelatin film (3wt%: 6wt%) following tryptic digestion (37°C, 450 rpm): a) 0h; b) 2h; c) 4h; and d) 14h. Horizontal dimension = 330μm.

3.4 Cell culture studies

In order to investigate compatibility of the fabricated material with tissue in terms of cell adhesion to the surface, pore availability and stability of the films, in vitro cell culture studies were performed. Figure schematically illustrates the procedures performed

Figure 3.4.1 illustrates cell growth on/within crosslinked films placed at the base of culture plate wells (37ºC, 5% CO_2 atmosphere, 4 and 8 days). To better appreciate the extent of cell proliferation, blank wells were also inoculated and used as controls. Since green fluorescent protein (GFP) was expressed in the cell culture used, cell growth could be very conveniently monitored using the technique of inverse fluorescence microscopy. That being said, obtaining well-resolved images was problematic, as cell proliferation had clearly occurred along the film and within the film in multiple layers,

easily exceeding the focal depth of the microscope. In comparison, the film-free control well experienced cell growth in a conveniently much thinner layer.

It also appeared that colonization was somewhat influenced by the concomitant slow degradation of the film. By the 4th day of incubation, for instance, the general proliferation of cells into sub-layers of the film could not be unequivocally confirmed via microscopy, albeit some 3-D growth was clearly observed (Figure 3.4.2 b). On the 8th day, however, pore enlargement and extensive colonization of cells throughout the film pores (Figure 3.4.2 d and e) could be observed. Degradation of the film upon cell proliferation experiment was confirmed via SEM analyses (results not shown).

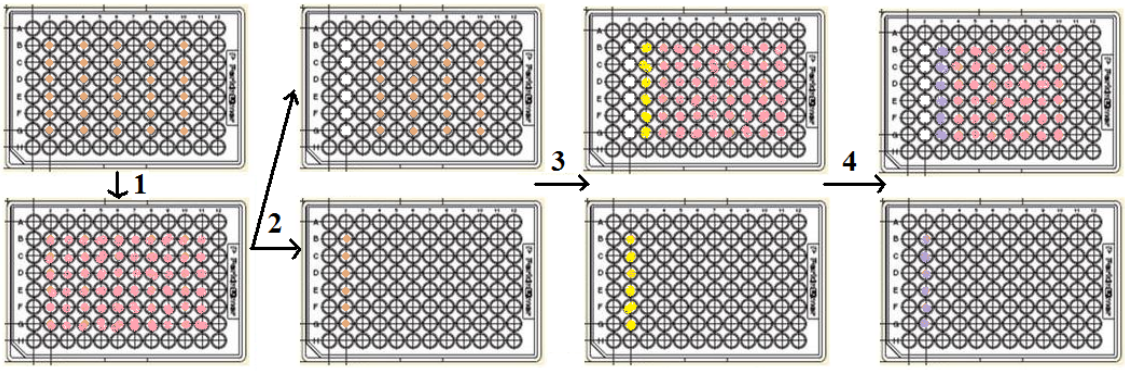

Figure 3.4.1: Cell culture experiment diagram: Sterilized cut films are placed into the wells of the plate and cells are cultured over both film-containing and control (blank) wells; next the cell culture is incubated for 2 days and fluorescence microscope images are acquired (1); the medium is removed from each of the wells, and films from the first column are transferred into a corresponding column in a separate plate (2); the MTT reagent is added to the first sample and control columns, and the fresh medium is then poured over the rest of the wells (3); after 4 hours of incubation, the solubilizing reagent is added to the first sample and control columns, and a spectrophotometric analysis is performed upon overnight incubation (4). The same procedure is also carried out for each of the other columns over different incubation periods, medium is changed every 2 days.

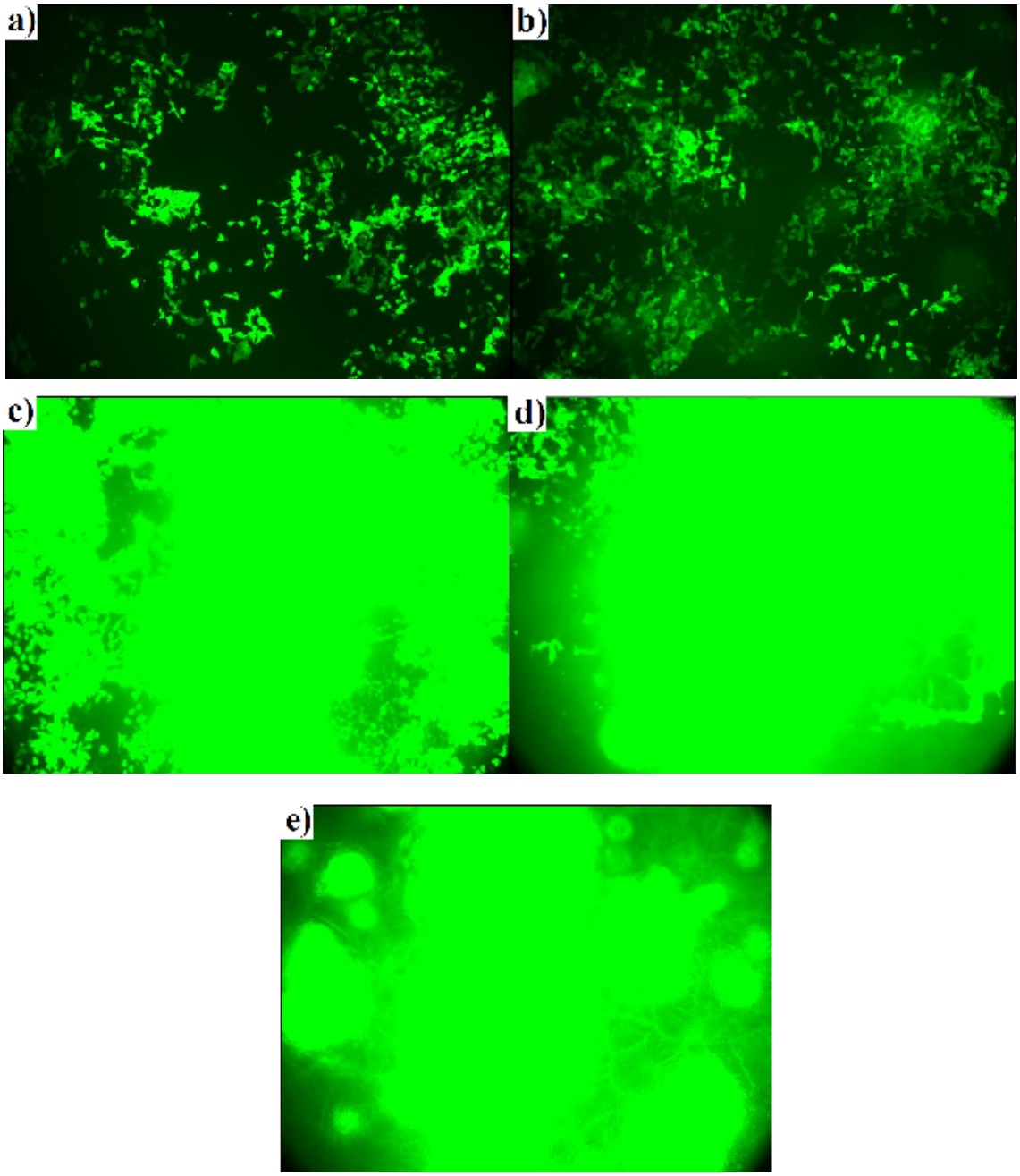

Figure 3.4.2: HEK293-GFP cells cultured on a) blank plate well, 4 day incubation; b) crosslinked film material, 4 day incubation; c) blank plate well, 4 day incubation; d) crosslinked film material, 8 day incubation; e) crosslinked film material, 8 day incubation. Images are obtained by focusing on the bottom of the well (a-d) or within layers of the film (e). Inverse phase contrast fluorescence microscope images are presented at 10X magnification.

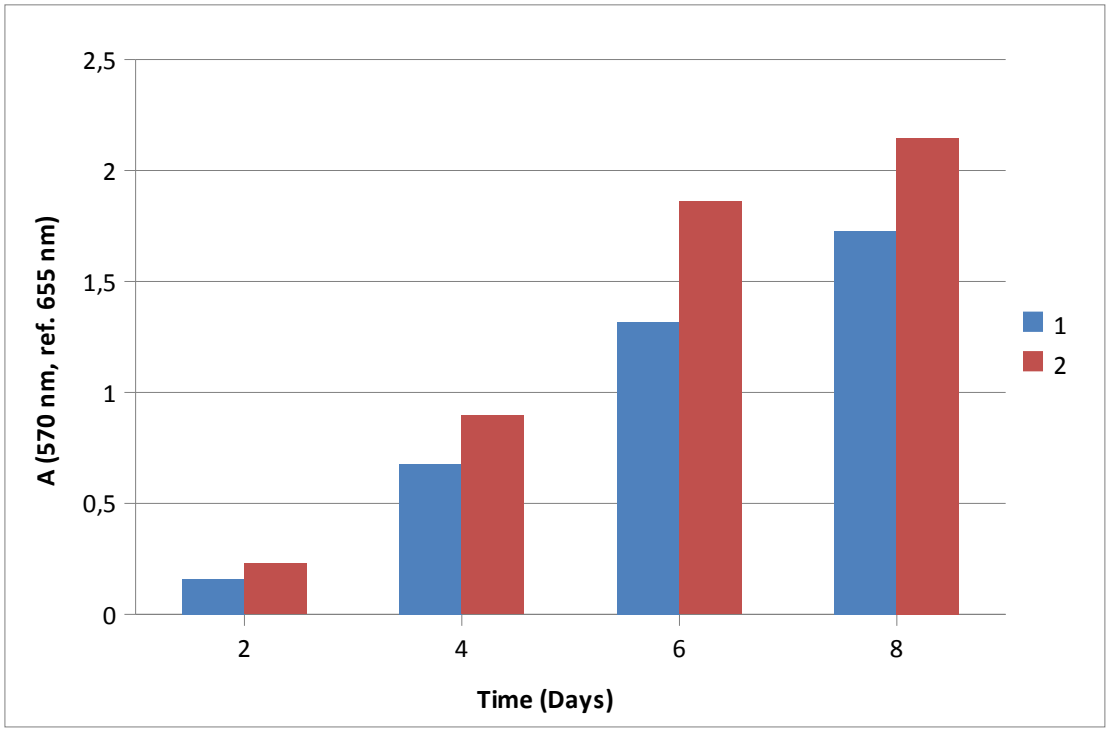

Figure 3.4.3: MTT test results versus incubation time HEK293-GFP cells adhered to blank plate wells (1) or within the crosslinked film (2). (10^4 cells/ml initial density)

Subsequent to colonization, cell growth along the film or blank well was quantified using the cell proliferation MTT assay. The assay is based on the reduction of the tetrazolium salt ((3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, MTT) by viable cells followed colorimetric detection of solubilized product (formazan) at 570 nm, with a background subtraction performed at a reference wavelength of 665 nm. It can be seen from figure 3.4.3 that cell growth was more pronounced when supported by the film. In view of established work in this area, the distinguishing factor presumably was the porous structure of the film, which provided a larger surface area for cell attachment and enhanced exchange of chemicals. Nearing the end of the assay in particular, the difference in growth of viable cells was observed to have decreased. As the culture medium had been changed infrequently (every 2 days), an insufficient nutrient loading may have prompted a drop of the growth rate, particularly in the cell-abundant film samples. Nonetheless, the ability of cells to proliferate and migrate into the film was clearly noted. This observation implied appropriate cell adhesion and demonstrated the potential merit of the crosslinked film material in promoting good tissue response.

3.5 Bovine serum hemoglobin (Hb) immobilization

The primary objective in choosing to further incorporate a third biopolymer, namely, hemoglobin, into the crosslinked film material was to better assess any potential risks of dehydrothermal crosslinking on biological function within the film. Previous work has already shown that powdered proteins in the dry state are remarkably thermally stable but no such evaluation had been previously conducted using biomaterial-based films. Since the previously prepared chitosan-gelatin films did not posses catalytic activity, there was no convenient means to directly investigate adverse (and potentially very subtle) structural changes encountered by the material upon heat treatment. It followed to reason that incorporating a non-thermophlilic catalytic protein into the structure of the film, such as bovine hemoglobin (Hb), could serve to better probe the effects of *in vacuo* thermal treatment by way of the hydrogen peroxide/o-phenylenediamine (OPDA) colorimetric assay.

The o-phenylenediamine-hydrogen peroxide assay conditions were optimized using native Hb/PBS solutions, as noted in Figure 3.5.1. The optimum reaction time was initially established as being 1 hour. For a fixed OPDA/peroxide ration, the rate of reaction was observed to be linear up to a concentration of 1mg Hb/ml PBS buffer. The observed linearity was later used to assess the "apparent" Hb concentration in the film, albeit the kinetics of immobilized Hb was expected to be complex and unpredictable, demanding the use of "apparent" in describing this strategy. A second and practical reason in specifically choosing bovine hemoglobin was the fact that it is readily abundant and sufficiently blood compatible to be potentially used as an oxygen carrier in next-generation biomaterials. The film prepared herein outlines a simple example, namely, an antimicrobial, porous and interconnected wound dressing to potentially accelerate cell proliferation and healing, where high oxygen levels presumably exert an antimicrobial and synergistic effect when combined with chitosan.

In commencing this line of work, the hope was to establish that hemoglobin could be covalently incorporated into a previously crosslinked and washed chitosan-gelatin film without losing structural integrity to a degree that would suppress activity and

potentially put into question the merit of *in vacuo* heat treatment. As dehydrothermal crosslinking will presumably transform ammonium carboxylate bridges into covalent amide bonds (see introduction), the functional groups expected to mediate intermolecular bonding were the α amino group of the N-terminal amino acid residue, the ε amino groups of lysyl residues, the α carboxyl group of the C-terminal amino acid residue, and the β and γ carboxyl groups of aspartyl and glutamyl residues. Since chitosan was also present in the film, amino groups located at position 3 of each glucosamine unit were also plausible contributors. While lying outside the scope of the current work, the potential formation of intermolecular ester bonds was not directly ruled out. If such a scenario applied herein, the additional residues potentially involved in crosslinking would correspond to tyrosyl, serinyl and threonyl amino acids.

To begin, bovine hemoglobin was thermally immobilized on the pre-crosslinked and pre-washed chitosan-gelatin films. The resultant composite film was incubated thrice and at length in PBS to remove any last traces of non-immobilized hemoglobin, which could have produced an erroneous assessment of the film bioassay. This strategy gained the necessary credibility when it was observed that the first wash buffer possessed active hemoglobin, whereas the second and third wash buffers showed no activity whatsoever. Moreover, this method combined with the colorimetric assay permitted a reasonable but indirect estimation of the immobilized hemoglobin. For instance, the *in vacuo* thermal treatment of a 20mg film was carried out after pre-incubating the film in 1ml of 1mg Hb/ml PBS. Free hemoglobin was detected (0.00862mg) in an aliquot of the first wash solution. Since a correction factor was available to account for the entire wash buffer volume and since the residual hemoglobin content of the incubation medium had been previously assayed after loading the film, the amount of film-immobilized hemoglobin was subtractively estimated as being 0,6258mg. It should be pointed out that the colorimetic assay only permitted the estimation of active hemoglobin. As such, amounts were estimated from a typical standard curve as that expressed in Figure 3.5.3, which depicts the color yield as a function of the hemoglobin concentration following a 1-hour incubation period.

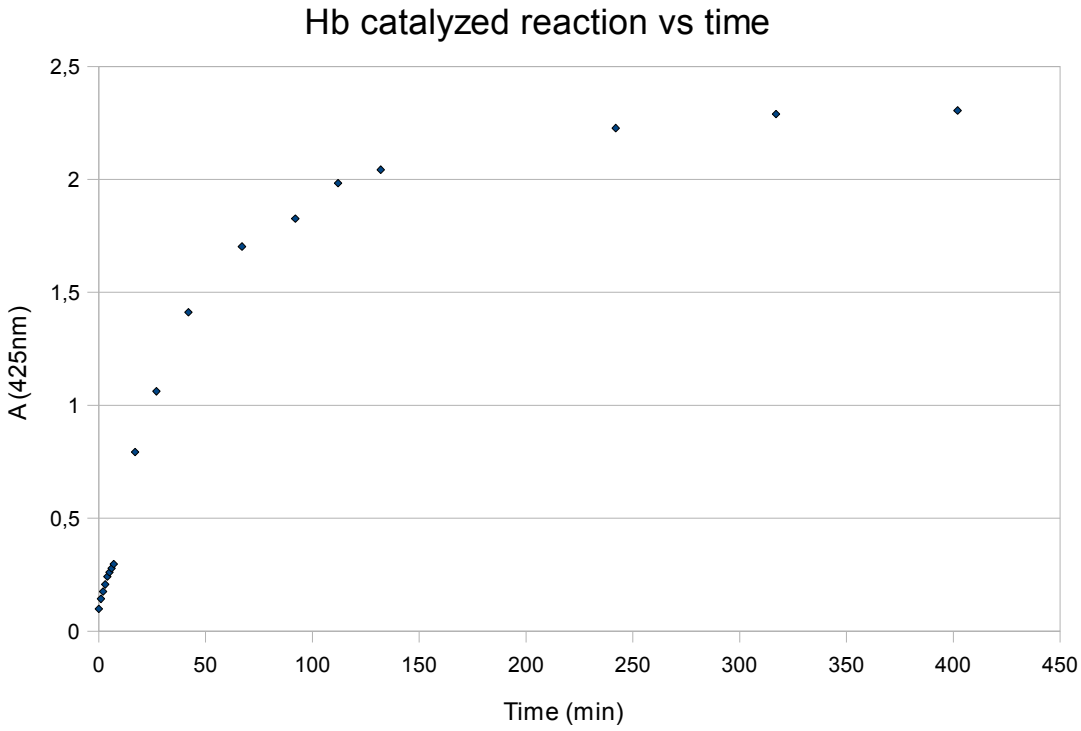

Figure 3.5.1: o-phenylenediamine (OPDA) (2x10-3 mol/L) - hydrogen peroxide (4x10-3 mol/L) reaction catalyzed by Hb/PBS solution (0,05mg/ml) detected directly in the cuvette without mixing

Figure 3.5.2: Cropped photograph images of Hb immobilized crosslinked films before (left) and after (right) the OPDA - hydrogen peroxide colorimetric assay.

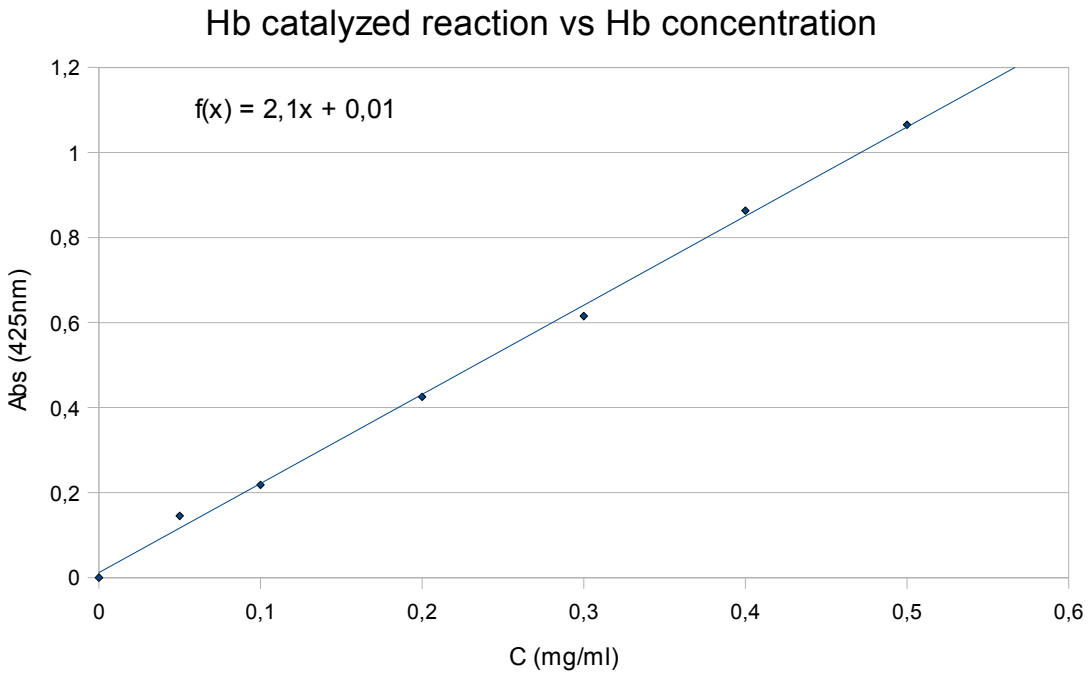

Figure 3.5.3: o-phenylenediamine (OPDA) (2x10-3 mol/L) - hydrogen peroxide (4x10-3 mol/L) reaction catalyzed by Hb/PBS solution versus Hb solution concentration, reaction period-1h, stirred.

Attempts to directly assess immobilized hemoglobin activity were rife with unavoidable errors related to any potential matrix effect on native activity, the uncharacterized role of diffusion, and the partitioning of phenylenediamine and the chromophore product into the film. Indeed, the change of color between pre- and post-assayed films clearly show a partitioning related to the buildup of choromophoric products (Figure 3.5.2). Clearly, the current method proved sub-optimal to directly characterize bioactivity within the film. Nevertheless, the method did indirectly permit an estimation of the hemoglobin, which had been loaded and crosslinked to the film. The results further proved that catalytic activity was retained by the immobilized Hb, albeit the fraction of catalytically competent units could not be appreciated. However, in view of related work with dry protein powders, it can be assumed that the essential elements of the hemoglobin structure were preserved considerably upon dehydrothermal treatment. The assessment of heat-induced structural changes will form a subject of ongoing work.

4. General Conclusions

A method to crosslink biopolymers, forming films, was developed. The reagent-free approach forming the subject of this study eliminates any safety concerns related to introduction of chemical reagents into a biomaterial in terms of direct toxicity as well as the potential or established threat posed during *in vivo* degradation by their breakdown products. Reagent-free inter-molecular covalent crosslinking was applied to intrinsically antimicrobial and biodegradable chitosan-gelatin composite material, yielding flexible, mechanically stable, interconnected-porous films.

Chitosan-gelatin films were prepared in lyophilisate form, with starting solutions flash-frozen in liquid nitrogen in a petri dish, and heat treated under dehydrative vacuum conditions, yielding crosslinked composite precursor films. Obtained films were washed liberating the non-crosslinked material and establishing the final porous morphology.

Several technical challenges have been faced during fabrication of the material. Unlike other typical biological materials, a dry powder consisting of chitosan and gelatin could not be directly manipulated at pH values nearing neutrality. The stocks were necessarily prepared in a stepwise fashion at lower pH values and later brought to pH 6. As such, the protocol was comparatively tedious with respect to other protein preparations such as albumin. The limited solubility of chitosan was a factor, as was the high viscosity imparted by gelatin, which made stirring, pH adjustments, and flash freezing of pre-lyophilisates very difficult. Unavoidable small air bubbles within the solution poured on the petri dish, lead to crack formation upon flash freezing and lyophilization, which prohibits fabrication of larger materials suitable for various mechanical testing procedures.

The antimicrobial properties of chitosan, seemed to be retained in the synthesized material, as no visible growth within the film and surrounding media could be observed under non-sterile conditions, without any supplemental antimicrobial reagent. The mechanical properties, morphology, porosity and pore interconnectivity of the material, being co-dependent, were optimized by varying the starting composition and experimental conditions.

Changes in molecular structure of the material upon dehydrothermal treatment, were detected via FTIR spectroscopy, results providing yet another confirmation of inter-molecular crosslink formation. Analyses of amide I and amide II bands reflected incremental secondary structural changes as a function of crosslinking time. Although inadequate spectral resolution prevented an in-depth analysis, small but certain relative changes in bond polarization pointed to hydrogen bond redistribution. Since the samples were permeated with water vapor before analysis, the spectral differences would only reflect spectral differences most likely related to minor covalent changes, i.e., changes which likely influenced structural compliancy, the course of re-hydration, and protein-protein hydrogen bonding. Suspected gradual "tightening" of the inter-protein structure would point to incremental crosslink formation.

Minor inter-molecular crosslinking could also be concluded from the observable swelling of the material in PBS buffer and its inability to re-dissolve under the most stringent of non-destructive reducing conditions. The long term stability in aqueous media was largely improved, even under proteolytic conditions. While non-crosslinked films did not preserve their integrity in the aqueous media, crosslinked films have remained stable for months and shown gradual degradation in a time frame of hours in a tryptic digestion solution. Mechanical tests implied that the dehydrothermally-crosslinked films, once re-hydrated, possessed the necessary flexibility and tensile strength. Toughness of the fabricated films was directly proportional to dehydrothermal incubation period and concentration of constituents prior to lyophilization. The brittle-ductile behavior has been confirmed to be dependent on the ratio of the constituents, as was expected relying on the reference publications, with plasto-elastic properties predominating with increase in gelatin content. Furthermore, mechanical behavior of the films was strongly dependent on the hydration states; dry and 100% ethanol perfused materials resulted in brittle fracture upon tension, while ductile fracture behavior increased with increasing water content in the hydration solvent, higher flexibility being achieved at the expense of some toughness loss.

Morphology studies showed that dehydrothermally treated films bore considerable porosity and interconnectivity in the presence or absence of sucrose as porogen. Porosity of the final material, was shown to be strongly dependent on concentration of constituents prior to lyophilization, and to a lesser extent on constituent ratio and sucrose incorporation. Increase in porosity is related to decay in the mechanical strength, thus optimization of these properties should be optimized according to necessities of a particular application. Incorporation of sucrose resulted in smaller, more evenly distributed pore structure, as opposed to larger irregular pore structure of the material formed without porogen incorporation, at corresponding initial solution concentrations. Satisfying interconnectivity has been observed in all formulations of the material obtained by the procedures described.

In vitro cell culture studies showed cell proliferation and migration within the pores of the film, implying good cell adhesion and demonstrating the potential merit of the crosslinked film material in promoting good tissue response. Proliferation has been observed to proceed in parallel with gradual degradation of the film structure.

Hemoglobin was immobilized to fabricated crosslinked chitosan-gelatin films, using the same crosslinking strategy. The activity assay has shown retained oxygen-binding catalytic activity of immobilized hemoglobin, attesting to the benign nature of this crosslinking technique, and yielding a material with potentially enhanced wound healing properties.

According to the evidence obtained in this study and relying on the data of the reference materials, the films fabricated are suitable to be used in many biomaterial applications. Given the advantageous properties of the films, arising from the material constituents and synthesis method employed, unfavorable consequences of conventionally applied wound healing treatment methods, such as scarring, allergic foreign body reaction due to seepage of chemicals, metabolic stresses due to chemical treatment, and infection, can be prevented. The initial results show that the film can be suitable as a specialized wound dressing, however internal applications are also possible and will be the subject of future work.

Publications arising from this thesis work

1. Zakhariouta A, Taralp, A: Development of reagent-free covalent crosslinking of chitosan-gelatin scaffolds. 10th Essen Symposium on Biomaterials and Biomechanics: Fundamentals & Clinical Applications, pp.50-51, March 5–7 2008, Universität Duisburg-Essen, Campus Essen.

2. Zakhariouta A, Taralp, A: Reagent-free covalent crosslinking of chitosan-gelatin films for medical applications. 11th Essen Symposium on Biomaterials and Biomechanics: Fundamentals & Clinical Applications, pp.168-170, March 5–7 2009, Universität Duisburg-Essen, Campus Essen.

3. Zakhariouta A, Taralp, A: Reagent-free covalent crosslinking of chitosan-gelatin films for medical applications. Part 2. 12th Essen Symposium on Biomaterials and Biomechanics: Fundamentals & Clinical Applications, March 17–19 2010, Universität Duisburg-Essen, Campus Essen. Accepted.

4. Zakhariouta A, Taralp, A: Reagent-free covalent crosslinking of chitosan-gelatin films for medical applications. Paper submitted.

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