Figure captions

Figure 1. SEM images of a honeycomb-patterned film. (a) top view, (b) tilted view, (c) side view, and (d) schematic of the double-layered structure of the film.

Figure 2. (a) Topographic AFM image , (b) cross-sectional profile along the dotted line in (a), and (c) CLSM image of Fn adsorbed on a flat film observed after 48 h incubation in 1 ml of 240 μg/ml Fn/PBS solution at 25 ℃

Figure 3. AFM and CLSM images showing the morphology of adsorbed Fn as a function of incubation time in a PBS solution of Fn. (a) AFM image at 0 hr incubation time. (b) AFM and (c) CLSM images at 20 h incubation time. Dashed line indicates the boundary separating two domains where morphology and fluorescence intensity differ. (d) AFM image at 48 h incubation time. (e) AFM image of surface after several scans on the surface of (d), where ring-like structures were scraped off by the scanning (compare areas indicated by white arrows in (d)). (f) Close-up image of area enclosed by dashed line in (d). (g) Cross-sectional profile along dotted line in (f). (h) CLSM image at incubation time of 48 h.

Figure 4. CLSM images of (a) endothelial cells (ECs) (shown in red) and cardiac myocytes (CMYs) (shown in green) immunofluorescence stained for vinculin (focal contacts) on honeycomb-patterned film and flat film after 72 h incubation. (a) and (c) on a honeycomb film, (b) and (d) on a flat film. Locations of focal contact points around the edges of pores are clearly evident.

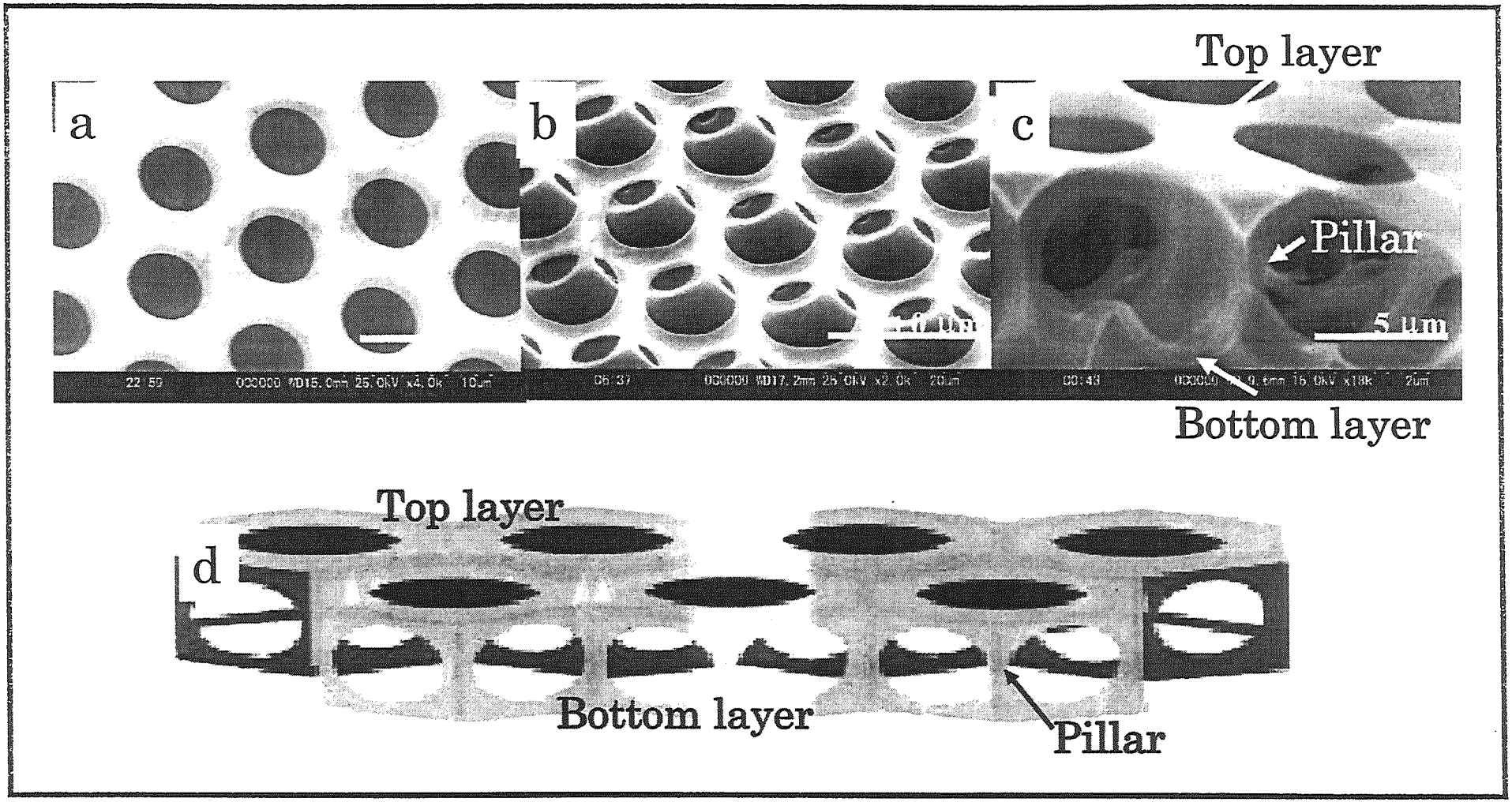
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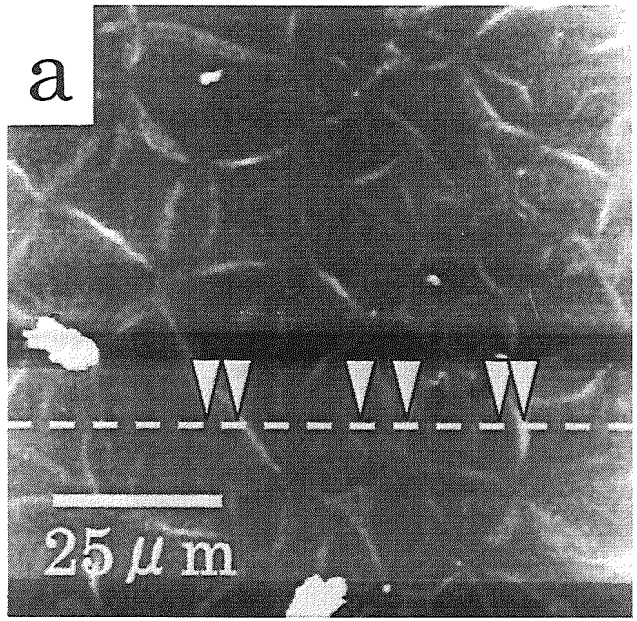
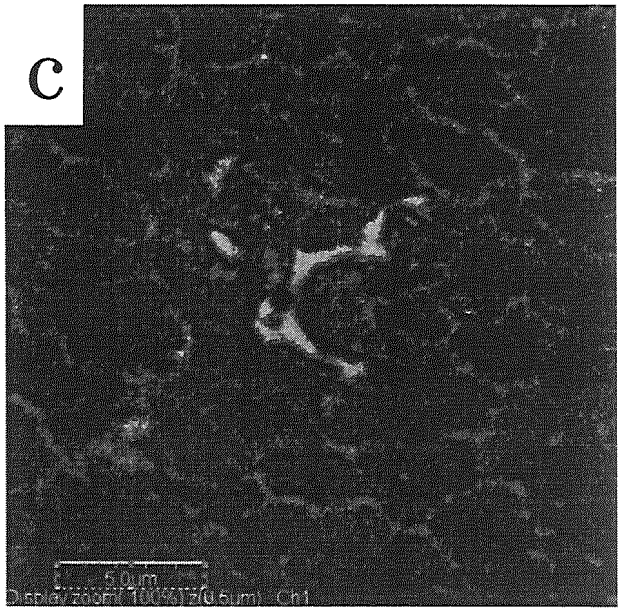
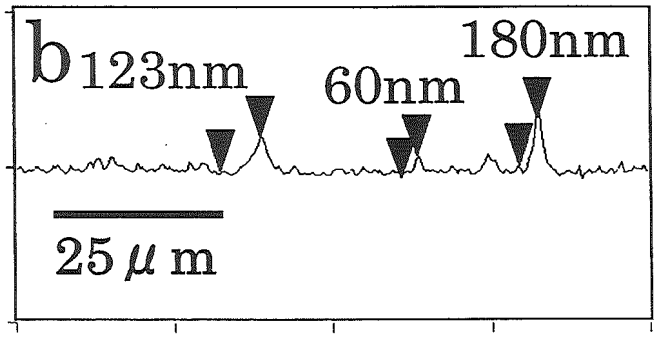
Figure 1







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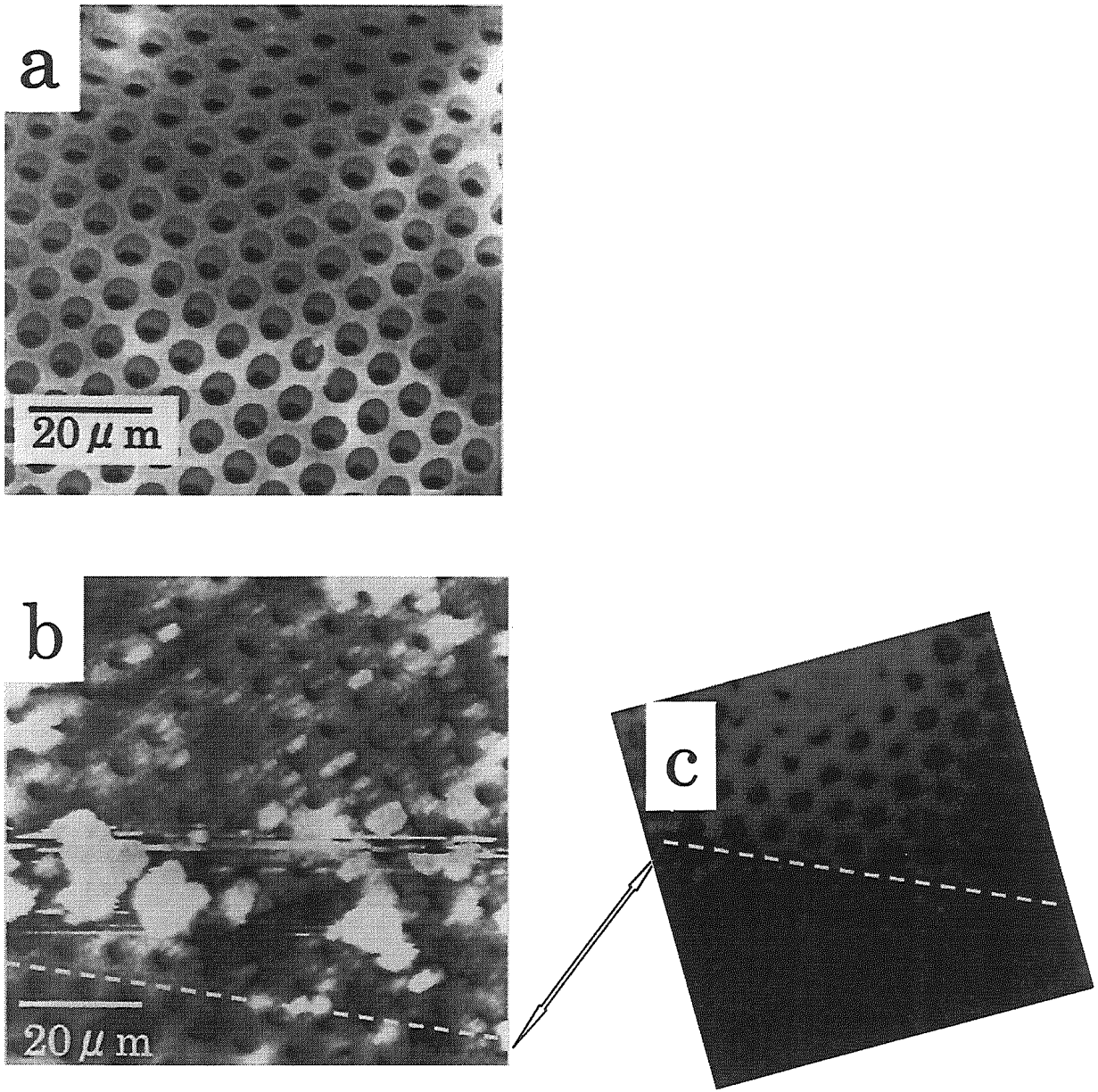

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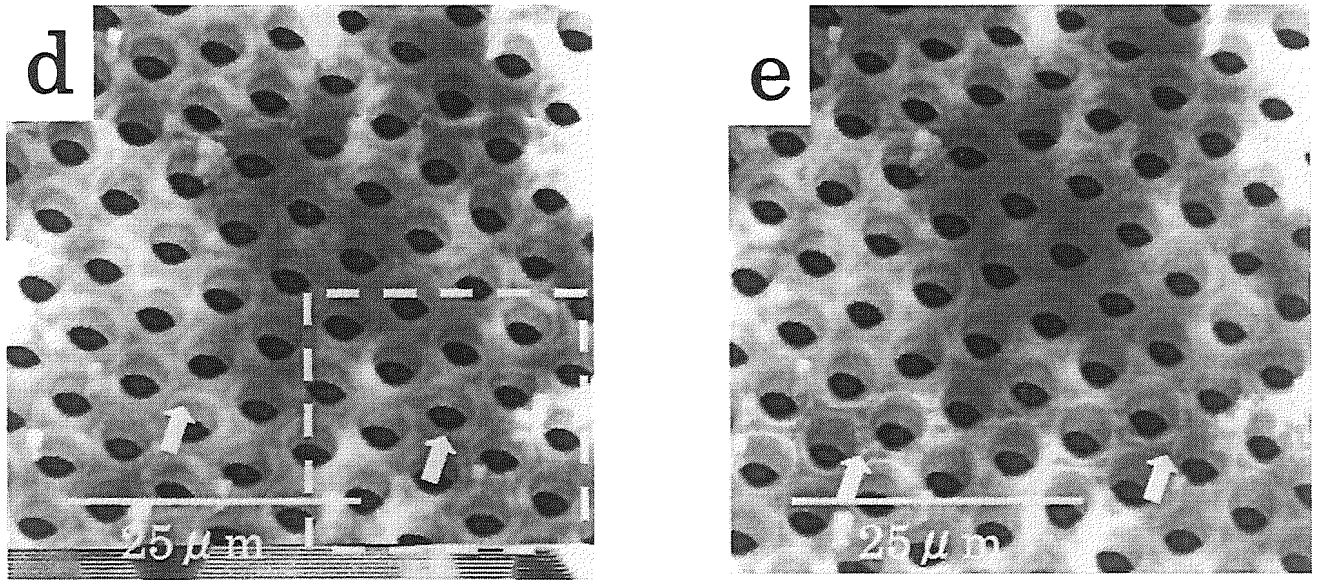

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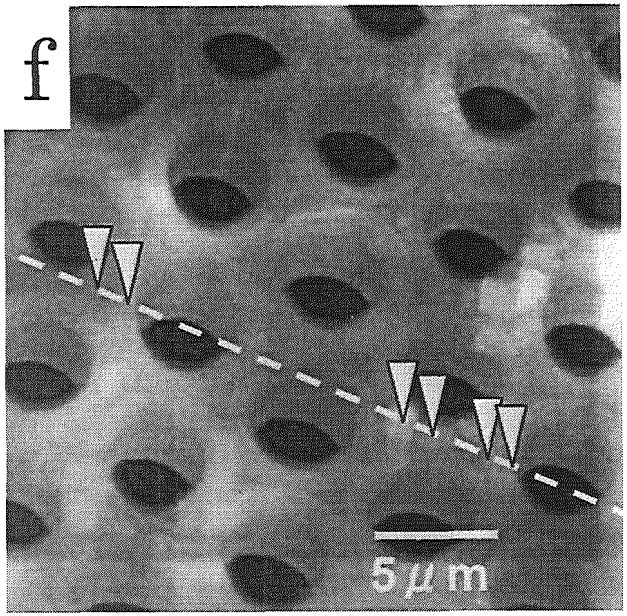

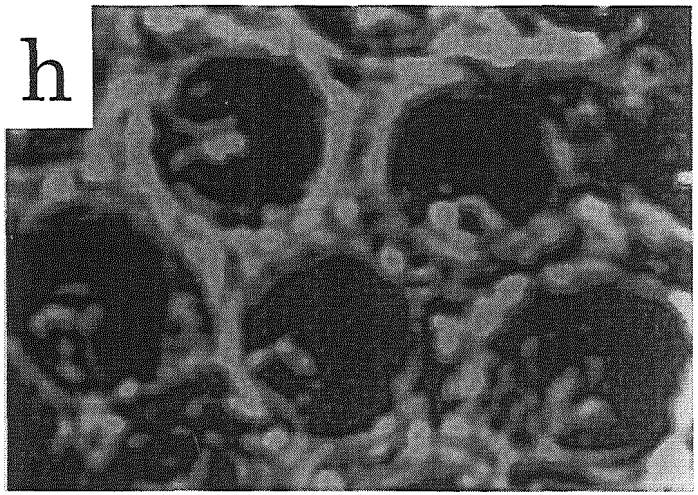

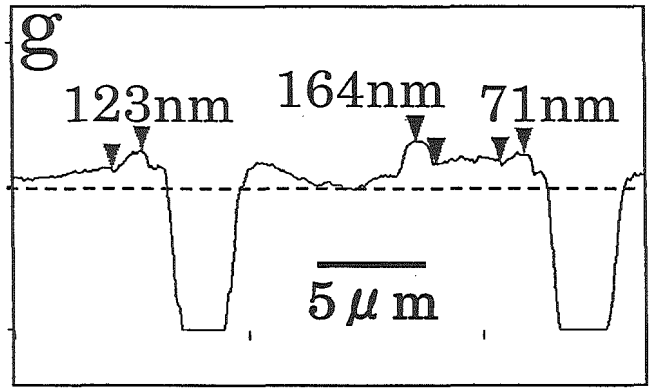

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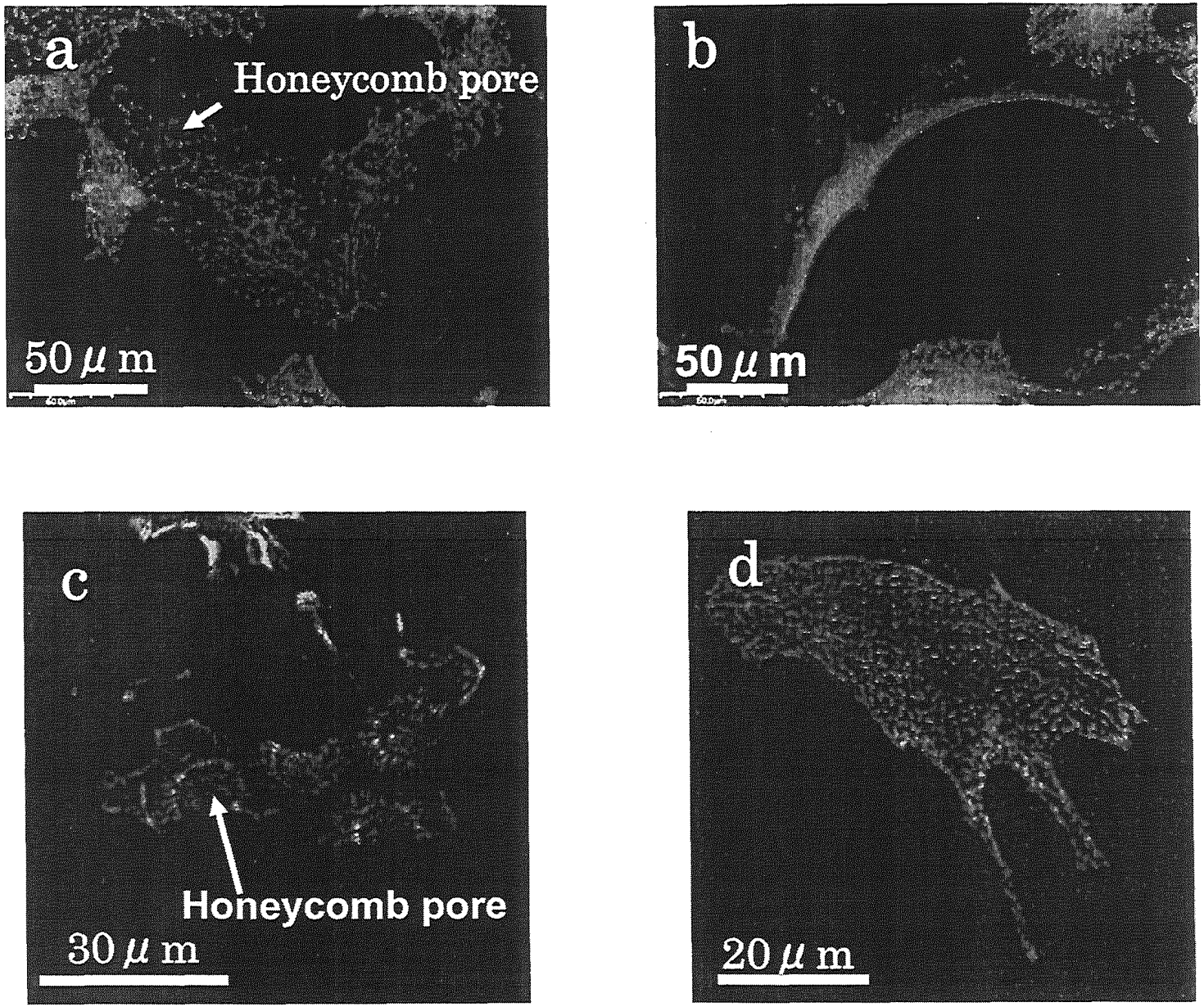

Figure 4

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Review

Gelatin as a delivery vehicle for the controlled release of bioactive molecules

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Abstract

Gelatin is a commonly used natural polymer which is derived from collagen. The isoelectric point of gelatin can be modified during the fabrication process to yield either a negatively charged acidic gelatin, or a positively charged basic gelatin at physiological pH. This theoretically allows electrostatic interactions to take place between a charged biomolecule and gelatin of the opposite charge, forming polyion complexes. Various forms of gelatin carrier matrices can be fabricated for controlled-release studies, and characterization studies have been performed which show that gelatin carriers are able to sorb charged biomolecules such as proteins and plasmid DNA through polyion complexation. The crosslinking density of gelatin hydrogels has been shown to affect their degradation rate in vivo, and the rate of biomolecule release from gelatin carriers has been shown to have a similar profile, suggesting that complexed gelatin/biomolecule fragments are released by enzymatic degradation of the carrier in vivo. This review will emphasize how biomolecules released from gelatin controlled-release systems are able to retain their biological activity, allowing for their use in tissue engineering, therapeutic angiogenesis, gene therapy, and drug delivery applications.

Keywords: Gelatin; Biomolecule carrier; Polyion complexation; Enzymatic degradation; Drug delivery

Abbreviations: aFGF, acidic fibroblast growth factor; AM, adrenomedullin; bFGF, basic fibroblast growth factor; BMP-2, bone morphogenetic protein-2; DMSO, dimethylsulfoxide; EDC, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide; EPR, enhanced permeability and retention; GA, glutaraldehyde; GEA, gastroepiploic artery; HGF, hepatocyte growth factor; HPLAC, high performance liquid affinity chromatography; IEP, isoelectric point; IGF-1, insulin-like growth factor-1; MPS, mononuclear phagocyte system; OPF, oligo(poly(ethylene glycol) fumarate; PBS, phosphate-buffered saline; PEI, polyethyleneimine; PEG, poly(ethylene glycol); pSV, SV40 promoter; RITC, rhodamine isothiocyanate; TGF-β, transforming growth factor-β; VEGF, vascular endothelial growth factor; WSC, water-soluble carbodiimide.

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Contents

1. Introduction

For the last few decades, advances in biomaterials science have continued to expand the scope of the field from the original paradigm of designing nonviable materials used in medical devices [1], to a more general approach of studying the physical and biological interactions between any material and the biological environment which surrounds it. By encompassing aspects of biology, chemistry, medicine, and materials science, biomaterials research has been used to address a wide diversity of issues ranging from artificial organ design in tissue engineering, to the fabrication of DNA microarrays in genomics.

More specifically, innovations in biotechnology have helped increase the cost efficiency of producing numerous peptides, proteins, and oligo- and polynucleotides, making them attractive candidates for sustained release applications from both a clinical and financial point of view. Thus, research in biomaterials has also been applied to the classic pharmaceutical challenge of designing systems for the sustained release of bioactive substances. In an effort to develop these controlled-release systems with reproducible and predictable release kinetics, a variety of methods have arisen to address this requirement such as: diffusion-controlled, water penetration-controlled, chemically-controlled, responsive, and particulate systems [2].

Although an extraordinary amount of synthetic (i.e. poly(glycolic acid), poly(L-lactic acid), etc.) and natural materials (i.e. collagen, alginate, fibrin, etc.) have been used as biomaterials in controlled-release applications [3], the purpose of this review is to focus on the contributions of gelatin in this area of research. As will be seen, depending on the fabrication method, variations in the electrical and physical properties of gelatin-based controlled-release systems can be achieved. It is this flexibility in processing that has allowed gelatin-based controlled-release systems to find diverse applications in fields ranging from tissue engineering, to drug delivery and gene therapy.

The significance of this ability to tailor the electrical nature of gelatin relates to the concept of sustained release of proteins from polymer matrices.

During the fabrication of a protein-polymer release system, harsh processing conditions may irreversibly denature proteins through exposure to heating, organic solutions, or sonication [4–6]. This post-fabrication loss of bioactivity has been a significant challenge for protein release technology in the past.

In order to address this issue, mild formulation conditions have been used to fabricate a multitude of polymer hydrogels as matrices for protein release, with the intent of maintaining the bioactivity of a protein drug during production. By incorporating the protein within an inert polymer delivery vehicle, the therapeutic agent is protected against enzymatic degradation and immunologic neutralization in vivo, thus allowing for prolonged release of the protein.

However, the release rate from such hydrogels typically relies on diffusion of the protein through aqueous channels. In general, this diffusion-controlled mechanism reduces the potential for sustained release, and biodegradation of the polymer scaffold decreases the construct's extended therapeutic effect even further [7].

By taking into account these aforementioned characteristics of typical diffusion-controlled designs, a shift away from the paradigm of utilizing a scaffold which is inert towards the protein should be considered. In other words, to achieve a degree of immobilization, molecular interactions between the carrier material and a therapeutic agent should be encouraged so that effective protein release kinetics can be realized [8].

One strategy which has been used to produce such interactions is polyion complexation. Polyion complexes are formed by electrostatic interactions between positively or negatively charged, high molecular weight electrolytes and their oppositely charged partners. Such interactions are quite stable since it would be statistically unlikely for all the ionic interactions between charged residues on the molecules to dissociate simultaneously. As a result, the secondary bonds forming polyion complexes are not dissociated as easily as the bonds between low molecular weight electrolytes [9].

In theory, polyion complexation can be used for the sustained release of numerous charged therapeutic agents such as proteins, polysaccharides, and oligo- and polynucleotides. Two mechanisms of polyion release from a biodegradable, charged polymeric carrier have been described [7]. Initially, a positively charged therapeutic agent is bound to the negatively charged polymeric chains of the carrier through electrostatic interactions. In the first instance, release of the agent from the carrier complex occurs because of an environmental change, such as in high ionic strength conditions. The second mechanism of release is degradation of the polymer carrier itself. However, because biodegradation of the carrier matrix would be the more likely mechanism of release in vivo, designers can control drug release kinetics by adjusting the rate of polymeric carrier degradation.

Considering the factors discussed involving polyion complexation, an ideal polymeric carrier designed for the controlled release of polyion therapeutic agents should allow control over its biodegradability and overall electrical charge, have a proven record of clinical safety, as well as allow for fabrication conditions that preserve the bioactivity of the therapeutic agent to be delivered in vivo.

2. Properties of gelatin

Gelatin is a natural polymer that is derived from collagen, and is commonly used for pharmaceutical and medical applications because of its biodegradability [7,10–12] and biocompatibility in physiological environments [13,14]. These characteristics have contributed to gelatin's proven record of safety as a plasma expander, as an ingredient in drug formulations, and as a sealant for vascular prostheses [15].

Two different types of gelatin can be produced depending on the method in which collagen is pretreated, prior to the extraction process [7]. The alkaline process, also known as "liming", targets the amide groups of asparagine and glutamine, and hydrolyses them into carboxyl groups, thus converting many of these residues to aspartate and glutamate. In contrast, acidic pre-treatment does little to affect the amide groups present.

The result is that gelatin processed with an alkaline pre-treatment is electrically different in nature from acidic-processed gelatin. This is because the alkaline processed gelatin possesses a greater proportion of carboxyl groups, rendering it negatively charged and lowering its isoelectric point (IEP) compared to acidic-processed gelatin which possesses an IEP

similar to collagen. By utilizing this technique, manufacturers now offer gelatin in a variety of IEP values.

3. Gelatin as a carrier matrix for polyion complexation

The isoelectric point of gelatin can be modified during its extraction from collagen to yield either a negatively charged acidic gelatin, or a positively charged basic gelatin. This allows for flexibility in terms of enabling polyion complexation of a gelatin carrier with either positively or negatively charged therapeutic agents. For instance, acidic gelatin with an IEP of 5.0 should be used as a carrier for basic proteins in vivo, while basic gelatin with an IEP of 9.0 should be used for the sustained release of acidic proteins under physiological conditions [7].

3.1. Fabrication methods of gelatin carriers

The fabrication methods for three fundamental gelatin carrier designs have been previously described: gelatin block hydrogels [9,12,16–25], porous gelatin block hydrogels [26], and gelatin microspheres [8,10,27–31].

3.1.1. Selection of a crosslinking agent

These three methods all utilize gelatin crosslinking in order to insolubilize the carrier in water, improve the thermal and mechanical stability of the carrier under physiological conditions, as well as control the rate of degradation in vivo [18].

Two fundamental methods of crosslinking have been described for gelatin: physical and chemical. Physical methods include UV-irradiation and dehydrothermal treatment, although these are inefficient and make it difficult to control the crosslinking density of the gelatin matrix [15].

Chemical crosslinking agents have been categorized into two types: non-zero length and zero-length. Non-zero length crosslinkers are bi-functional or polyfunctional and operate by bridging free carboxylic acid residues or amine groups between adjacent protein molecules. Examples include aldehydes (i.e. formaldehyde, glutaraldehyde, glyceraldehyde), polyepoxides, and isocyanates [15].

Zero-length crosslinking agents activate carboxylic acid residues to react directly with amine groups on adjacent protein chains. No intervening molecules are introduced between the crosslinked residues, so this process is able to achieve gelatin matrix crosslinking without integrating foreign molecules into the network. Crosslinking agents in this category include acyl azides [15] water-soluble carbodiimides [9].

Crosslinking density can be determined by performing a tensile test on gelatin sheets [29]. Another measure of crosslinking extent for hydrogels is water content, which is defined by the weight percentage of water in a swollen hydrogel. Hydrogel weight before and after swelling in 37 °C in PBS can be measured in order to calculate water content [18].

Control over the crosslinking density of gelatin hydrogel carriers is possible with the use of glutaraldehyde as the crosslinking agent for example [29]. By either prolonging the crosslinking reaction period or increasing the glutaraldehyde concentrations, the crosslinking density of the gelatin can be increased, thus allowing for the fabrication of gelatin hydrogel carriers with specific extents of crosslinking by optimization of the crosslinking reaction conditions.

Once the crosslinking agent is chosen, the electrical nature of the gelatin carrier must be considered with respect to the concept of polyion complexation. Keeping this in mind, a basic gelatin should be chosen as a carrier for acidic proteins, while acidic gelatin can be used for sustained release applications of basic proteins.

3.1.2. Gelatin block hydrogel fabrication

The fabrication of block matrices begins with the preparation of the gelatin aqueous solution, where the gelatin is dissolved in deionized water (5 g gelatin per 50 ml H_2O) at 60 °C. A variety of crosslinkers are available for this application such as glutaraldehyde (GA) [22] or a water-soluble carbodiimide (WSC) [9].

Once the desired crosslinking time has been reached, the crosslinking reaction is quenched within the newly formed hydrogels. If GA was used, then the hydrogels are immersed in an aqueous solution of glycine at 37 °C for 1 h to block residual aldehyde groups of GA, and then rinsed with water [22]. If WSC was used for crosslinking, then the hydrogels are immersed in an aqueous solution of hydrochloric

acid (pH 3.0) for 1 h and then washed with water to deactivate and remove any unreacted crosslinker.

A variety of shapes can be fabricated from these block hydrogels, ranging from disks, to cubes, or strips by punching out or cutting them using a knife. More complex shapes such as tubes can be formed by running the crosslinking reaction within a mold of the correct configuration. After thorough rinsing, the fabricated hydrogels are freeze-dried and sterilized in ethylene oxide gas.

3.1.3. Porous gelatin block hydrogel fabrication

High porosity controlled-release scaffolds are recommended for tissue engineering applications because of their increased surface area, which allows for enhanced cell seeding densities prior to implantation if desired. In addition, increased porosity can accelerate the biodegradation of a scaffold if required, while encouraging tissue in-growth and vascularization of the developing tissue.

A method has been developed to introduce porosity into gelatin block hydrogels using the fundamental techniques of crosslinking, swelling, and lyophilization [26]. Briefly, glutaraldehyde is added to an aqueous solution of gelatin and the mixture is poured into a polypropylene mold for 12 h at room temperature to allow for crosslinking. The hydrogel is then treated with an aqueous glycine solution to quench the crosslinking reaction and washed with double-distilled water. The swollen hydrogel is then frozen, allowing ice formation to act as a porogen. Scaffolds are then lyophilized with the help of a freeze dryer for 4 days to completely dry them.

Scanning electron micrographs of porous gelatin hydrogels fabricated using this technique reveal uniform sized pores ranging in diameter from 45 to 250 μm depending on the method of freezing utilized [26]. It has been shown that hydrogels frozen in liquid nitrogen have a two-dimensionally ordered structure, while those placed in −20 °C freezers have larger pores and a three-dimensional, interconnected structure, suggesting that the porosity of these constructs can be controlled by the size of ice crystals formed during the freezing process [26].

3.1.4. Gelatin microsphere fabrication

Gelatin microspheres can be produced via glutaraldehyde crosslinking of a gelatin aqueous solution in a water-in-oil emulsion technique [29]. In order to create the water-in-oil emulsion, an aqueous solution of gelatin (10 wt.%) preheated to 40 °C is added dropwise into olive oil at 40 °C under stirring at 420 rpm for 10 min.

Spontaneous gelation of the gelatin droplets is then driven by a 15 °C decrease in emulsion temperature followed by 30 min of continued stirring. 100 ml of acetone is then added to the emulsion which is stirred for an additional 1 h. The resulting microspheres are then washed three times in acetone, recovered by centrifugation at 5000 rpm at 4 °C for 5 min, and fractionated according to size through the use of sieves with different apertures.

Following air-drying, the microspheres are crosslinked by placing them into an aqueous solution of glutaraldehyde and stirred at 4 °C for 15 h. Collection of the crosslinked microspheres is performed by centrifugation at 5000 rpm, for 5 min at 4 °C, and the crosslinking reaction is quenched by agitating the microspheres in 10 mM aqueous glycine solution at 37 °C for 1 h. Lastly, the microspheres are washed three times with double-distilled water and freeze dried in preparation for sterilization by ethylene oxide.

3.1.5. Biomolecule loading of gelatin carriers

The advantage to using gelatin as a carrier for controlled release is that polyion complexation can be used to load the therapeutic agent into the matrix under mild conditions. If the protein to be released is present in the aqueous solution of gelatin during crosslinking, its therapeutic effect will most likely be lost because of chemical deactivation. A method has been developed which preserves protein bioactivity by simply preparing an aqueous solution of the protein and dropping it onto the freeze-dried gelatin carrier, allowing for sorption of the protein to the matrix and its subsequent sustained release in vivo through degradation of the carrier [12].

In addition, this method provides a highly reproducible way of quantitatively loading charged biomolecules such as basic fibroblast growth factor into gelatin hydrogels regardless of their crosslinking extent, as long as the hydrogel mesh size is large enough to allow for inward diffusion of the biomolecule [7]. Freeze-dried gelatin hydrogels are rehydrated with a solution of the protein of interest, however the volume used is much less than theoret-

ically required to fully swell the crosslinked hydrogel. This has allowed investigators to effectively study the release profiles of charged biomolecules from gelatin controlled-release systems.

3.2. Characterization studies of gelatin controlled-release systems

3.2.1. Assessing biomolecule loading

Polyion complexation has been proposed as the mechanism which allows gelatin carriers to bind charged biomolecules. This hypothesis has been tested by measuring the turbidity change of a solution composed of gelatin and protein at various temperatures [32]. Turbidimetric titrations at various concentrations of basic fibroblast growth factor (bFGF) were performed in 1/15 M phosphate-buffered solution containing various concentrations of acidic (IEP of 5.0) or basic (IEP of 9.0) gelatin at 25 °C. The time allowed for complexation between the bFGF and gelatin was varied from 6 to 72 h following initial mixing. When bFGF was mixed with the basic gelatin, no turbidity in the solution was observed. In contrast, bFGF mixed with acidic gelatin demonstrated a maximum turbidity around a bFGF/gelatin molar ratio of 1.0 and the solution's turbidity increased with complexation time.

Similar results have been observed between acidic proteins and basic gelatin mixtures which show increasing turbidity with time, while basic proteins and basic gelatin solutions lack turbidity altogether [7]. These findings suggest that polyion complexation is able to take place between proteins and gelatin of opposite charges, while similarly charged proteins and gelatin cannot form such complexes in aqueous solution.

Heparin high performance liquid affinity chromatography (HPLAC) has also been used to investigate polyion complexation by examining changes in bFGF's affinity for heparin due to gelatin complexation [32]. When bFGF is mixed with acidic gelatin in solution, HPLAC analysis shows a decrease in the peak area of intact bFGF with increasing complexation time and the appearance of a new peak at a shorter retention time. Conversely, the HPLC peak of intact bFGF is unaffected by mixing bFGF with basic gelatin. These results can be explained by polyion complexation, in which the affinity of bFGF is decreased for heparin because it forms complexes with acidic gelatin, which appear as a new peak at shorter retention times. Complexation of bFGF with acidic gelatin also decreases the amount of free, intact bFGF in solution, which manifests as a decrease in the peak area of intact bFGF. Since basic gelatin does not complex bFGF, changes in the bFGF peak for mixtures of bFGF and basic gelatin are not seen with HPLAC.

An environmental change such as increasing the ionic strength of the solution will counteract polyion complexation. This has been illustrated as an increase in the HPLC peak area of the bFGF-acidic gelatin complex with decreasing ionic strength, as well as a smaller rate of turbidity increase with complexation time of a bFGF-acidic gelatin solution with increasing ionic strength [32]. Thus, electrostatic interaction between bFGF and acidic gelatin is the driving mechanism behind polyion complexation in aqueous solution mixtures.

Protein sorption into gelatin hydrogels in vitro has also been investigated using basic fibroblast growth factor as a model [23]. Three types of gelatin were used to make hydrogels: acidic gelatin (IEP 5.0), basic gelatin (IEP 9.0) and anionized gelatin. The anionized gelatin was produced by converting amino groups present in the acidic gelatin into carboxyl groups in order to increase the density of negative charges present. This was accomplished by adding dehydrated dimethylsulfoxide (DMSO) containing various amounts of succinic anhydride to a solution of acidic gelatin. Following agitation for 1 h at 37 °C, the anionized gelatin was collected via precipitation through the addition of acetone, followed by washing in acetone 3 times, and vacuum drying. Anionized gelatin hydrogels were then fabricated from the final product as previously described.

In this study, bFGF sorption to gelatin hydrogels was assessed both qualitatively and quantitatively. Visualization bFGF sorption depth into gelatin hydrogels was performed using bFGF fluorescently labeled with rhodamine isothiocyanate (RITC). RITC-labeled bFGF solution was dropped onto gelatin hydrogel discs and left for either 1 or 6 h at 4 °C to allow for protein sorption. Examination of serial longitudinal cryo-sections using fluorescent microscopy revealed that bFGF was almost entirely sorbed into the interior of the acidic gelatin hydrogel by 6 h in a homoge-

neous manner, suggesting the bFGF molecules were able to diffuse through the swollen hydrogels.

Quantitative analysis of bFGF sorption into hydrogels was performed by placing swollen gelatin hydrogels in 100 μg/ml bFGF aqueous solution. The time profile of bFGF sorption was determined by using HPLAC to measure the concentration of bFGF remaining in the solution surrounding the hydrogels at various time intervals.

Basic FGF sorption into the acidic gelatin hydrogels was shown to increase with time, while no sorption occurred to basic gelatin. Furthermore, increasing the amount of anionization of acidic gelatin enhanced the ability to sorb bFGF, while increasing the ionic strength of the solution reduced bFGF sorption, reflecting the contribution of polyion complexation to the sorption process.

However, it should be noted that there are variations in the nature of gelatin such as molecular weight, amino acid sequence, and isoelectric point, because gelatin production involves the chemical desaturation of collagen [12]. Thus, additional factors which may contribute to the intermolecular interactions between a loaded growth factor and the gelatin carrier include Coulombic, hydrophobic, and hydrogen-bonding interactions. It has been shown that 20% of ^{125}I-label basic fibroblast growth factor and transforming growth factor-β1 are sorbed into acidic gelatin hydrogels even in solutions of high ionic strength [12]. However, increasing the solution ionic strength reduces the sorption of growth factors to gelatin carriers. Thus, although electrostatic interaction is not the only source of intermolecular interaction between a charged biomolecule and a gelatin hydrogel carrier, it indicates a considerable contribution to sorption behaviour.

3.2.2. Effect of crosslinking density on gelatin carrier degradation rate

Although polyion complexation has been discussed as the main contributor to biomolecule loading and retention within gelatin carriers, the key determinant of controlled release from these constructs is the rate of gelatin degradation. Given that crosslinked gelatin does not undergo any appreciable degradation in aqueous solution, in vivo models have been used to study the effect of crosslinking density on the rate of gelatin degradation [18]. While the rate of gelatin degradation could be studied in vitro using enzymes in aqueous solution, an accurate simulation of the degradation profile in vivo would be difficult since the numerous types and concentrations of hydrolytic enzymes involved in the process is unclear.

To address this concern, in vivo hydrogel degradation has been studied by examining the fate of gelatin hydrogels implanted subcutaneously on the backs of mice [18]. The hydrogel discs used for this study were crosslinked with glutaraldehyde for various times from 6 to 48 h at 4 °C prior to implantation in order to obtain groups with various crosslinking densities. In addition, the hydrogels loaded were radiolabeled with ^{125}I so that both weight and radioactivity loss of ^{125}I from the gelatin hydrogels could be observed in order to assess their in vivo degradation profile.

Wet hydrogel weight was shown to decrease with implantation time, regardless of the crosslinking extent. However, the hydrogels which were crosslinked for less time with a resulting higher water content (98.8%) were degraded more quickly when compared to the hydrogels which exhibited a water content of 96.9% (i.e. higher crosslinking density). After 10 days of implantation, the 98.8% water content hydrogels had disappeared from the implantation site, while the 96.9% water content hydrogels remained until day 35.

A similar trend was observed with the time course of remaining radioactivity after in vivo implantation. The radioactivity remaining in the gelatin hydrogels decreased over time, irrespective of crosslinking extent, but hydrogels with the lower water content (96.9%) were able to retain their radioactivity for a longer duration than the 98.8% water content hydrogels.

Comparison of the changes in gelatin hydrogel radioactivity and wet weight over time revealed similar diminishing profiles, indicating that the main contributor to in vivo degradation of gelatin hydrogels is their water content, and hence, their crosslinking extent. Essentially, the faster degradation rate of hydrogels with a higher water content is due to a lesser extent of crosslinking.

3.2.3. Biomolecule release from gelatin carriers

From our discussion of the mechanisms underlying the binding of charged biomolecules to gelatin hydrogel carriers, it is apparent that polyion complex-

ation can allow for a relatively simple and non-destructive method for effectively loading the carrier with a therapeutic agent. In addition, it has been shown that by varying the extent of crosslinking during gelatin hydrogel fabrication, the degradation rate of the carrier can be controlled after implantation in vivo.

Bearing these results in mind, numerous studies have been conducted in order to determine the relationship between gelatin hydrogel crosslinking density and the release profile of their bound biomolecules both in vitro and in vivo [8,9,12,29]. Yamamoto et al. [12] studied such in vitro release profiles by preparing acidic (IEP=5.0) and basic (IEP=9.0) gelatin hydrogels through glutaraldehyde crosslinking and loading them with either ^{125}I-labeled basic fibroblast growth factor (bFGF), transforming growth factor-β1 (TGF-β1), bone morphogenetic protein-2 (BMP-2), or vascular endothelial growth factor (VEGF). The growth factor-loaded gelatin hydrogels were placed in phosphate-buffered saline (PBS) solutions of ionic strength ranging from 0 to 1.5 and shaken at 37 °C. At various time points up to 3 h, the supernatant PBS was removed to measure its radioactivity. Approximately 20% of the bFGF and TGF-β1 incorporated into acidic gelatin hydrogels was released into PBS within 1 h, with no substantial release noted thereafter. In contrast, greater than 80% of the incorporated BMP-2 was rapidly released from acidic hydrogels within 30 min, while approximately 60% of VEGF was released within 50 min, with both growth factors showing a plateau in release rate after these time periods.

Similar in vitro release profiles were seen for gelatin microspheres loaded with rhodamine isothiocyanate-labeled insulin (RITC-Ins) [29]. The RITC-Ins-loaded microspheres were placed in PBS and the suspension was incubated at 37 °C for up to 14 days. At various time points, the suspension was centrifuged at 12,000 rpm and a sample of the supernatant collected so that its fluorescence intensity could be determined by fluorospectrophotometry.

The release of RITC-Ins from gelatin microspheres was shown to have a biphasic profile in vitro, irrespective of microsphere crosslinking density. An initial "burst release" of RITC-Ins was observed on the 1st day, followed by a relative plateau in release rate for the remaining 13 days. In addition, the total amount of RITC-Ins released over the 14-day period decreased for microspheres of increased crosslinking density, with a larger percentage of RITC-Ins released during the "burst phase" for microspheres of lesser crosslinking density.

The data from these two studies suggest that in vitro, little control over the release rate of peptide from gelatin carriers is achievable since crosslinking density does not affect the biphasic release profile, and only the total amount of peptide released over time. In addition, it seems that for some proteins, the amount released during the burst phase depends on the isoelectric point of the protein, such that the initial burst in release from acidic gelatin carriers is suppressed for proteins with an IEP>7.0, in comparison with that of proteins with an IEP<7.0, with the opposite relationship seen for proteins released from basic gelatin carriers [8]. Thus, for proteins such as bFGF, the observed in vitro burst release from acidic gelatin hydrogels is most likely due to loss of uncomplexed bFGF from the carrier during the initial period, followed by little or no release of bFGF thereafter.

Nonetheless, it should be noted that this relationship cannot be applied to all proteins. BMP-2 for example has an IEP value higher than 7.0, but may exhibit weakened electrostatic interactions with acidic gelatin due to sugar residues covering the BMP-2 molecule [12], illustrating the fact that electrostatic interactions are not the sole intermolecular interaction occurring during peptide binding to gelatin carriers.

As expected, the in vivo release profiles of growth factors from gelatin carriers differ from their in vitro counterparts [12,29]. Yamamoto et al. [12] placed subcutaneous implants of acidic gelatin hydrogels loaded with ^{125}I-labeled bFGF, TGF-β1, BMP-2, or VEGF into the backs of mice, with 100 μl of aqueous solution of the ^{125}I-labeled growth factors at the same dose injected subcutaneously on the back as a control. At various time points up to 15 days, the hydrogels and surrounding tissues were explanted and their radioactivity measured to evaluate the time profile of growth factor release. It was found that more than 80% of the growth factor injected as an aqueous solution was eliminated from the site of injection within 1 day, regardless of growth factor type. The hydrogels loaded with the radiolabeled bFGF and TGF-β1 exhibited a much slower decrease in radio-

activity compared to controls, while those loaded with radiolabeled BMP-2 and VEGF showed only slightly prolonged retention compared to injected controls. In addition, comparison of these in vivo growth factor release profiles to degradation profiles of ^{125}I-labeled gelatin hydrogels loaded with the same growth factors revealed a good correlation between the two, when the influence of the initial burst phase of growth factor release was excluded.

The in vivo release profiles of ^{125}I-labeled insulin from gelatin microspheres by Iwanaga et al. [29] were observed following injection of the loaded microspheres into the femoral muscles of mice, with the injection site tissues harvested at various times up to 14 days post-injection. The amount of ^{125}I-insulin decreased rapidly during the 1st day, followed by a slow disappearance from the dosing site over the following 13 days. Furthermore, it was found that a decreased amount of insulin was released during the burst phase in gelatin microspheres that had been crosslinked more extensively.

By interpreting the in vivo release profiles from these two studies, and correlating them with the rate of gelatin carrier degradation and crosslinking density, it is apparent that controlled release of certain growth factors from gelatin carriers can be explained by the mechanisms of polyion complexation and gelatin degradation, which in turn, can be controlled by the choice of the electrical nature of gelatin used and the extent to which the gelatin hydrogel carrier is crosslinked.

Polyion complexation is responsible for the binding of charged proteins such as bFGF to the oppositely charged gelatin hydrogel carrier. Once implanted in vivo, an initial burst phase of protein release is observed as any un-complexed protein is released from the carrier. Following this phase, a slower rate of protein release is observed, as the gelatin carrier undergoes enzymatic hydrolysis. The growth factor release rate is controlled by the extent of crosslinking because enzymatic degradation of the gelatin hydrogel is less effective against hydrogels with a higher crosslinking density. Thus, in varying the extent by which the gelatin carrier is crosslinked, one can control hydrogel degradation, which in turn affects protein release since the bound growth factor is only released into the surrounding environment still complexed with gelatin. Such a scenario is plausible given that the decrease in radioactivity profiles is well correlated for gelatin hydrogels loaded with radiolabeled proteins and radiolabeled hydrogels loaded with non-radioactive proteins. Proteins such as bFGF which have a high "complexation susceptibility" conform to this paradigm of controlled growth factor release more so than factors such as BMP-2, whose intermolecular interactions with gelatin carriers may not be dominated by polyion complexation to as great an extent.

Aside from protein release from gelatin matrices, the concept of polyion complexation has also been applied to the controlled delivery of plasmid DNA for gene therapy applications [33–35]. Fukunaka et al. [33] investigated the in vivo release of negatively charged, ^{125}I-labeled plasmid DNA from hydrogels of cationized gelatin implanted in the femoral muscle of mice.

By adding ethylenediamine and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride salt (EDC) to basic gelatin, amine residues are introduced to the carboxyl groups of gelatin, resulting in an overall increase in positive charges, or "cationization" [33]. An expression vector consisting of the coding sequence of LacZ and an upstream SV40 promoter (pSV-LacZ) was utilized as the plasmid DNA in this study. ^{125}I-labeled pSV-LacZ in aqueous solution was loaded into freeze-dried cationized gelatin hydrogels in a process analogous to the loading of proteins. The hydrogels were then implanted into the femoral muscle of mice and the tissues explanted at various time points up to 21 days in order to measure their radioactivity and evaluate the release profile of plasmid DNA. A solution of ^{125}I-labeled pSV-LacZ in PBS was injected into the femoral muscle as a control. In an additional group of mice, the tissues at the implanted site were harvested and gene expression in the treated muscle was evaluated by measuring β-galactosidase activity.

Analysis of the in vivo release profile of pSV-LacZ following implantation revealed a decrease in residual radioactivity of the plasmid DNA in the hydrogels with time, with the rate of decline strongly dependent on the water content of the cationized gelatin hydrogels. An increase in hydrogel crosslinking extent resulted in longer radioactivity retention in hydrogels, in contrast to the rapid disappearance of the injected ^{125}I-labeled pSV-LacZ solution control by day 3. The

amount of pSV-LacZ radioactivity remaining over time was also found to correlate well with the radioactivity remaining in ^{125}I-labeled hydrogels.

Moreover, gene expression levels were significantly enhanced by implantation of hydrogels loaded with the plasmid DNA while only transient gene expression was observed in the injected pSV-LacZ solution control. Lastly, hydrogels with higher extents of crosslinking were able to maintain a longer period of gene expression than those with lower crosslinking density. The results seen in this study are similar to those observed in protein release from non-cationized gelatin hydrogels, in which in vivo degradation of biomolecule-loaded gelatin hydrogels is determined mainly by their water content, and hence crosslinking density.

Similarities in the diminishing radioactivity profiles of hydrogels loaded with radiolabeled DNA versus radiolabeled hydrogels loaded with non-labeled plasmid DNA suggests that complexed pSV-LacZ was released from the implanted hydrogels because of gelatin biodegradation. The presence of plasmid DNA/cationized gelatin complexes released by degradation most likely promoted gene expression because of a prolonged increase in local concentration at the site of implantation. It has been theorized that such cationic delivery systems enhance the efficiency of plasmid DNA transfection by neutralizing the anionic charge of the DNA plasmid, as well as reducing its molecular size [36]. Thus, this study was able to show that an increased period of gene expression could be achieved by decreasing the rate of plasmid DNA release.

Naturally, the question of "bioactivity" of released biomolecules still remains, since the suggested mechanism of controlled delivery discussed for growth factors and plasmid DNA involves the release of biomolecule-gelatin fragments from the delivery vehicle. This issue is currently being assessed in a variety of applications.

4. Applications of biomolecule controlled release from gelatin carriers

4.1. Tissue engineering

Significant tissue losses resulting from trauma, age-related degeneration, or resection for the treatment of pathology have conventionally been treated with non-biological, prosthetic implants or biological implants such as whole organ transplantation [37].

By their very nature, prosthetic implants are unable to restore the original architecture of the lost tissue, while organ transplantation or the use of tissues from one's own body is limited to a select few because of donor organ shortages and the possibility of donor site complications [38].

The field of tissue engineering has striven to address the shortcomings associated with such conventional treatment modalities by the development of biological substitutes which restore, maintain, or improve tissue function. Although researchers have investigated the potential of tissue engineering a wide variety of tissue types, the application of certain broad principles remains the same, in that the design of a tissue engineered construct should consider the incorporation of cells, biologically active factors, and a suitable scaffold material as components for successful tissue regeneration. The following sections will illustrate how biomolecule release from gelatin hydrogels and microspheres are used within this tissue engineering paradigm.

4.1.1. Hard tissue regeneration

Although much research has been conducted in the field of bone biology and regeneration, current bone grafting materials available for clinical use are still far from ideal. Bone defects encountered in craniofacial, orthopedic, and dental surgery are currently reconstructed with a variety of biomaterials including autologous bone, allogeneic bone obtained from cadavers, xenogeneic bone obtained from animals, or synthetic alloplastic materials such as titanium, methyl methacrylate resins, and bioactive ceramics [22].

Autologous bone is still considered by many clinicians to be the "gold standard" of bone grafting materials, although patients must undergo the pain and possible complications associated with a harvesting procedure. In addition, allogeneic and xenogeneic bone grafts carry with them a theoretical risk of disease transmission, as well as increased cost. Thus, synthetic alloplastic materials show the most promise as bone grafting materials because their physical and biological properties can be precisely controlled, they are not constrained by the limitations of additional

surgical procedures for their fabrication, and they pose no risk of disease transmission.

Biodegradable gelatin hydrogels have been used as controlled-release devices for a variety of growth factors known to enhance bone formation such as TGF-β1, bFGF, and BMP-2 [20,22,24]. Yamada et al. [22] examined the potential efficacy of bFGF release from gelatin hydrogels implanted in skull bone defects. Although bFGF facilitates proliferation of osteoblasts and periosteal cells in vitro, a solution of bFGF injected in vivo rapidly disappears from the injection site [12], precluding its ability to effectively generate a biological effect at the site of interest. Thus, bFGF was incorporated into acidic gelatin hydrogels to allow for its localized release into a rabbit skull defect over an extended time period.

Complete closure of the defect was seen 12 weeks after implantation of bFGF-impregnated hydrogels, while bFGF solution applied to the defect as a control was unable to enhance bone regeneration, resulting in soft tissue in-growth. Measurements of bone mineral density at the skull defect also revealed that although bFGF-loaded gelatin hydrogels enhanced bone mineral density over controls, a significantly higher bone mineral density was seen in rabbits treated with the more crosslinked (85% vs. 98% water content) bFGF-loaded hydrogel. Similar studies utilizing the same 6 mm skull defect in a rabbit found that gelatin hydrogels loaded with recombinant human BMP-2 showed increased bone mineral density in the skull bone defects with time, compared to an injected BMP-2 solution control and a non-loaded gelatin hydrogel control [39].

Bone regeneration with the use of gelatin hydrogels has also been approached from an ex vivo gene therapy perspective [40]. The basic goal of sustained growth factor and cytokine delivery into a bone defect is to stimulate the migration, proliferation, and differentiation of the proper cell lineages into phenotypes that promote de novo bone generation. However, because the cultivation of osteoblasts in vitro from bone marrow stromal cells and osteoprogenitor cells is a relatively straightforward endeavor, ex vivo gene transfer can be performed on osteoblasts so that they may be re-implanted at a later date to deliver the required signaling molecules.

Kim et al. [40] examined the efficacy and cytotoxicity of in vitro cationic-agent-mediated non-viral gene transfer into osteoblasts. Plasmid DNA encoding red fluorescent protein was loaded into various cationic agents such as lipid, gelatin, and polyethyleneimine (PEI) and these complexes were transfected into a human fetal osteoblastic cell line, rat bone-marrow-derived primary osteoblasts, and NIH 3T3 fibroblasts. The human fetal osteoblastic cell line had a higher transfection efficacy with cationic lipid and gelatin than with PEI. In addition, cationic gelatin was associated with cell survival rates over 60% in any cell type, irrespective of the dose used for transfection, which illustrates the potential of cationized gelatin carriers for use in bone tissue engineering applications.

Cartilage tissue engineering has also been examined as a potential application for biomolecule delivery from synthetic polymer/gelatin constructs [41–43]. Severe cartilage degeneration is a widespread problem due, in part because of the poor intrinsic ability of cartilage to repair itself, coupled with a tendency toward repeated injury caused by sports activities or improper joint loading [42]. The low cellularity and relative avascular nature of cartilage has driven traditional surgical therapies to provide regenerative factors and cells into cartilaginous defects by accessing the underlying subchondral bone in an attempt to aid cartilage repair.

A less invasive strategy to promote cartilage repair has been presented [42], in which bioactive molecules can be delivered to the cartilage lesions through the use of injectable composites based on the synthetic polymer (oligo(poly(ethylene glycol) fumarate) (OPF) embedded with gelatin microspheres. Transforming growth factor-β1 (TGF-β1) and insulin-like growth factor-1 (IGF-1) were chosen as bioactive molecules for sustained release. TGF-β1 has been shown to promote progenitor cell chondrogenic differentiation, increase cartilage extracellular matrix synthesis, and enhance chondrocyte proliferation, while IGF-1 has been shown to stimulate proteoglycan and type II collagen synthesis [42].

These growth factors were loaded into gelatin microspheres which in turn, were embedded into an OPF hydrogel, creating a composite, dual growth factor delivery system. By encapsulating the growth factor-loaded microspheres in a crosslinked OPF network, additional control over release kinetics was achieved, by reducing the final cumulative release of

IGF-1 to 70.2% in collagenase-containing PBS from 95.2% seen from IGF-1-loaded acidic microspheres alone. In addition, it was found that the release profiles of TGF-β1 and IGF-1 could be manipulated in this dual growth factor delivery composite. The burst release of TGF-β1 could be altered by either loading TGF-β1 into the gelatin microsphere phase (10.8% burst release) or loading it into the OPF hydrogel phase (25.2% burst release). Concurrently, slow release of IGF-1 over a 4-week period was achieved by loading it into the microsphere phase.

Another interesting application of cartilage tissue engineering was presented by Kojima et al. [30] who examined the potential of gelatin microspheres to deliver transforming growth factor-β2 (TGF-β2) in order to develop helical engineered cartilage equivalents for a functional tracheal replacement. Bone marrow stromal cells were harvested from sheep and cultured to confluence, at which point they were seeded onto non-woven polyglycolic acid meshes and cultured in vitro with TGF-β2 and IGF-1 for 1 week to promote their differentiation towards the chondrogenic phenotype. These cell-seeded meshes were then placed in a helical template, coated with gelatin microspheres loaded with TGF-β2 , and subcutaneously implanted on the backs of rats for a 6-week duration. These cell-seeded constructs formed stiff cartilage with a helical shape after 6 weeks of implantation, while control constructs lacking the TGF-β2-loaded microspheres were much stiffer with the presence of a mineralized matrix. The tissue engineered trachea was similar to a normal trachea, with histological specimens showing mature cartilage present, and levels of glycosaminoglycans and hydroxyproline content similar to native cartilage.

4.1.2. Soft tissue regeneration

In the field of soft tissue engineering, growth factor delivery from gelatin carriers has been utilized in a diverse number of settings, from adipose tissue engineering [44], to regeneration of the periodontal ligament surrounding teeth [45]. Current treatment available for soft tissue augmentation of depressed regions or scar tissue in the breast and facial areas includes the use of autologous fat grafting, although common complications include absorption or fibrosis of the graft.

In an effort to address the shortcomings of conventional surgical approaches for harvesting fat tissue, Kimura et al. [44] utilized the tissue engineering paradigm of combining the appropriate cells, growth factors, and scaffolding material to regenerate adipose tissue in vivo. By utilizing precursor cells with the potential to differentiate into adipose cells, and combing them along with basic fibroblast growth factor (bFGF), which has been shown to promote the angiogenic response necessary for adipogenesis [46], it was theorized that adipose tissue generation could be achieved by utilizing a sustained release construct which would help create an environment for transplanted adipose precursor cells that would be conducive to adipogenesis in vivo.

Preadipocytes isolated from human fat tissue were suspended together with bFGF-loaded gelatin microspheres in a collagen sponge. This cell-seeded construct was then implanted subcutaneously on the backs of nude mice for 6 weeks. At the time of harvest, the implant site was found to have adipose tissue formation, the extent of which varied with the number of preadipocytes transplanted and the dose of bFGF loaded in the gelatin microparticles as part of the implanted construct. The control group (collagen sponge embedded with preadipocytes and bFGF in solution) generated an insignificant amount of adipose tissue in comparison. Immunohistochemistry of the harvested specimens confirmed the presence of mature human adipocytes in the newly formed fat tissue. This study illustrated the potential of combining bFGF-sustained release from gelatin microspheres, with preadipocytes and a collagen sponge scaffold to achieve successful adipose tissue generation.

Interestingly, a similar model has been used to regenerate periodontal ligament tissue [45]. A collagen sponge scaffold and bFGF-loaded gelatin microspheres were combined in a "sandwich membrane" controlled-release system, which was placed in three-walled alveolar bone defects associated with the canine teeth of beagle dogs. At 4 weeks, the non-bFGF-loaded control group exhibited defects filled with connective tissue, as well as epithelial downgrowth and root resorption, indicating a failure to regenerate the original architecture of the periodontal ligament. In contrast, throughout the 4-week period of the study, vascularization and osteogenesis were observed in the bFGF treated group only, with

histological analysis revealing new cementum formation at 4 weeks on exposed root surfaces, and functional recovery of the periodontal ligament exhibiting perpendicularly oriented collagen fibers.

The success of gelatin controlled-release systems in an array of diverse biological environments illustrates gelatin's versatility. Its ability to serve as either a hydrogel or microsphere carrier for bioactive molecules, combined with its ability to be combined with synthetic and natural polymers in composite systems, gives it great potential for use as a controlled-release vehicle in tissue engineering applications, as well as a tool to assess release kinetics of growth factors on tissue regeneration by varying its crosslinking density.

4.2. Therapeutic angiogenesis

Although basic fibroblast growth factor (bFGF) has been used for bone tissue engineering applications, it was originally characterized in vitro as a growth factor for fibroblasts and capillary endothelial cells, making it a natural candidate for in vivo controlled-release studies involving neovascularization [9]. FGF molecules are known to have a strong affinity for acidic glycosaminoglycans such as heparin and heparin sulfate. This complexation has been theorized as a mechanism to protect FGF from denaturation and enzymatic degradation in vivo [9], resembling a "natural" system for controlled release. Gelatin sustained release hydrogels have attempted to mimic this system by utilizing the concept of polyion complexation to promote angiogenesis, which is defined as the formation of blood vessels in situ and involves the orderly migration, proliferation, and differentiation of vascular cells [47].

Early studies by Thompson et al. [47] implanted Gelfoam commercial gelatin sponges loaded with acidic fibroblast growth factor (aFGF) in the neck and peritoneal cavities of rats to induce angiogenesis in situ. Within 1 week after implantation of the aFGF-treated Gelfoam, a significant angiogenic response was observed. Blood vessels were seen macroscopically within the gelatin sponge, which was confirmed histologically by H&E staining at both 1 and 2 weeks. In contrast, control sponges without aFGF were unable to induce neovascularization throughout the 2-week implantation period.

Similar results were seen by Tabata et al. [9] who tested bFGF-loaded acidic gelatin hydrogels as subcutaneous implants in mice. The amount of tissue hemoglobin was used as a quantitative marker for vascularization, while histology provided a qualitative assessment of angiogenesis. Predictably, in vivo degradation of hydrogels with time was dependent on their water content, and hence crosslinking density. Significant neovascularization and tissue granulation within the implant site were seen, while bFGF injected as a solution did not increase tissue hemoglobin values. An increased duration of the neovascularization effect was also seen in bFGF-loaded hydrogels of lower water content, suggesting a prolonged release profile for this group.

With the results of these two studies demonstrating the effectiveness by which bFGF delivery from gelatin hydrogels was able to stimulate angiogenesis, numerous therapeutic applications using this technique have been studied [28,48–51]. Sakakibara et al. [50] investigated the use of gelatin microspheres loaded with bFGF to induce coronary collateral growth, a novel technique that shows promise for patients who are not candidates for standard revascularization procedures such as coronary angioplasty, or coronary artery bypass graft surgery.

Myocardial infarction-induced heart failure was performed on rats and pigs. In the rat model, 4 weeks after infarction, radiolabeled ^{125}I-bFGF-loaded microspheres were injected into the myocardium, with ^{125}I-bFGF solutions injected as controls. The pig model examined bFGF-loaded microparticles injected into the left ventricular wall of the heart, versus injection of non-loaded microparticles in control groups. Results of the rat study showed that more bFGF remained in the rat heart 72 h after injection of bFGF-loaded microspheres vs. controls, while an improvement in left ventricular function was observed following injection of bFGF-loaded microparticles in the pig study.

Hosaka et al. [28] utilized a similar system to perform site-specific intra-arterial delivery of bFGF to augment functional collateral vessels in a rabbit model of hind limb ischemia. The goal of such therapy is to efficiently restore the blood supply to ischemic tissue by inducing the formation of functional collateral vessels from a donor artery located adjacent to the ischemic tissue. By lodging bFGF-loaded acidic