Mini Review

Fabrication of three-dimensional cell scaffolds with spatial gradients of biomolecules

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Multicellular processes of development and tissue regeneration are often sophisticatedly regulated by the spatial arrangement of extracellular matrix and signaling molecules in a time- and concentration-dependent manner. Concentration gradients of biomolecules such as growth factors and transcriptional factors play a pivotal role in the *in vivo* induction and formation of tissues and organs with complex structural architecture. It is therefore conceivable that a three-dimensional bioengineered scaffold with a spatial gradient of biomolecules could allow cells to induce regeneration of tissue with the natural morphological structure. This mini review article overviews fabrication techniques for functional gradient materials, and several concrete examples are introduced to emphasize importance of gradient materials in tissue engineering.

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Introduction

Tissue engineering typically aims to induce tissue regeneration at defective tissues or organs with either transplanted cells or host cells. To successfully implement cell-induced tissue regeneration, however, it is necessary to create a local environment that promotes cellular proliferation and differentiation[1]. In healthy tissues, it has been recognized that such environments are formed by a complex orchestration of biological signal molecules, extracellular matrix (ECM) molecules, mechanical stress, and cell-cell interactions[2]. Thus, an appropriate combination of similar biological cues could also be used to manipulate cell activities for therapeutic tissue regeneration.

Several three-dimensional materials with different pore structures have been explored as potential scaffolds for cell proliferation and differentiation in tissue engineering[3]. Requirements for cell scaffold materials include good biocompatibility, the ability to support cell proliferation and differentiation, suitable bioresorbability, appropriate mechanical strength, and easy processability[4]. In addition to serving as cell attachment substrates, scaffolds can also function as delivery vehicles for growth

factors that can enhance cellular proliferation and differentiation for tissue regeneration. Many studies have demonstrated that scaffolds and growth factors can be effectively used to enhance regeneration of various tissues[2]. However, new technologies and methodologies are required to realize the regeneration of more complex tissue architectures such as interface and gradient structures. Most tissue structural architectures form native cellular environments by regulating the three-dimensional assignment of biomolecules. For successful tissue engineering, it is therefore necessary to develop a technique that can create biomolecule gradients in cell scaffolds.

In this paper, several fabrication techniques for materials with functional gradients of biomolecules are overviewed, and several concrete examples are introduced to emphasize the importance of gradient materials in tissue engineering.

Fabrication techniques of Protein Gradients

Although simple gradients of diffusible biomolecules in the vicinity of cells in solution can be experimentally achieved using a micropipette, fabrication of protein gradients immobilized on two-dimensional surfaces has been a technical challenge[5]. Several methods have been investigated to create two-dimensional protein gradients. The most well known are soft lithographic techniques, including microcontact printing[6,7], microfluidic patterning[8-10], and photomasking[11-13] (Fig.1). Microcontact printing is a technique that uses a three-dimensional stamp to create complex two-dimensional patterns on substrates in the micrometer range (Fig.1A). Based on the microcontact printing technique, ephrinA5 gradients were created on glass coverslips to study the effect of the slope and local concentration of gradients on growth cone navigation[7]. The growth cones of chick temporal retinal axons were able to integrate these ephrin gradients and stop at a distinct zone in the gradient, while still undergoing filopodial activity. The position of this stop zone could be regulated by both the steepness of the gradient and the amount of substrate-bound ephrin per unit surface area.

Whitesides et al. have developed a micofluidic patterning technique which can generate gradients of proteins immobilized on surfaces by adsorption from gradients in solution (Fig.1B). The gradients can have dimensions from micrometers to centimeters and be generated by simple physical adsorption of proteins onto the surfaces without any chemical reactions[8-10]. Linear laminin gradients created by micofluidic patterning showed that the slope of laminin gradients affects the orientation of axonal specification of neurons[8]. Another application of this technique is to create interleukin-8 (IL-8) gradients for neutrophil migration[9]. Jeon et al. have demonstrated that neutrophils exhibit strong directional migration toward increasing concentrations of IL-8 in a linear gradient.

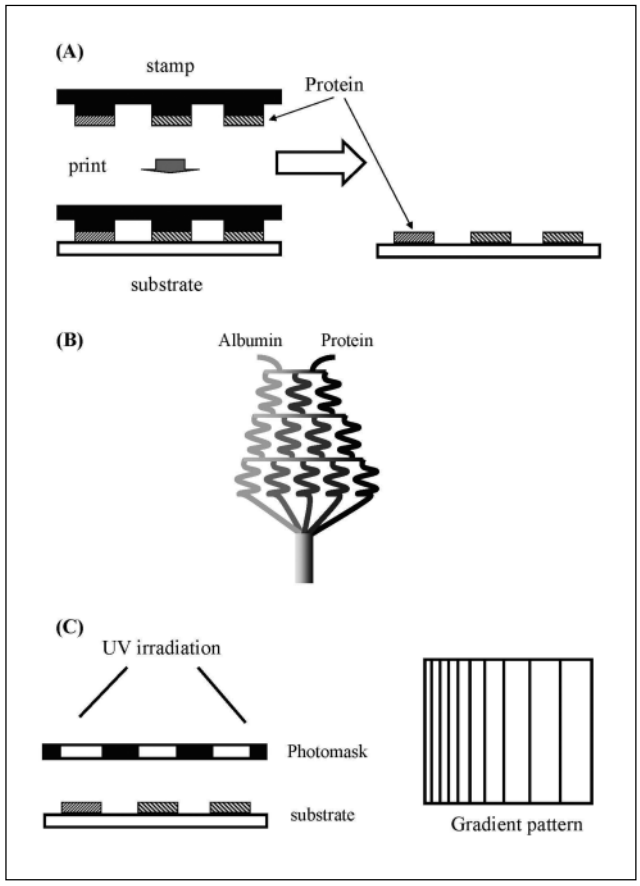

Fig.1 Fabrication techniques of immobilized protein gradients on two-dimensional surfaces.
(A) Microcontact printing, (B) Microfluidic patterning, (C) Photomasking.

Chemical immobilization of biomolecules with a concentration gradient can be achieved by using gradient-micropattern photomasks, which limit the area for photo-induced polymerization[11] or photo-induced peroxide formation[12,13] on polymeric surfaces (Fig.1C). Size-dependent transmission properties of apertures in the photomask and exposure energy are used to control photo-induced patterning reaction. Based on this approach, it has been demonstrated that the proliferation of Chinese hamster ovary cells overexpressing epidermal growth factor (EGF) receptors was enhanced only in densely immobilized regions on the EGF gradient surface[11].

In addition to the concentration gradient, gradients in physicochemical properties such as surface energy[14], polymer

crystallinity[15], and grafted-polymer chain structure[16] have been shown to affect osteoblast behavior on substrates. For example, gradients of polymer crystallinity were fabricated on films of poly(L-lactic acid) using a gradient in annealing temperature[15]. The surface roughness of the films increases with an increase in the polymer crystallinity and greatly influences the rate of osteoblast proliferation; the smoother the surface, the greater the rate of proliferation.

These studies establish that cell behavior can be modified by varying the physicochemical properties of surfaces. However, it is important to note that cell shapes are different within two-dimensional and three-dimensional environments, which can affect cellular activities. For example, fibroblasts grown in three-dimensional and two-dimensional environments exhibit different morphologies in terms of the formation of the large actin bundles and arrangement of extracellular receptors[17]. It is therefore clear that a technology to form protein gradients in three-dimensional cell scaffolds is greatly required for tissue engineering applications.

Fabrication of Three-Dimensional Cell Scaffolds with Protein Gradients

Fabrication of three-dimensional protein gradients has emerged in the research field of chemotaxis as an *in vitro* technique to investigate directed cell migration by soluble biomolecules. Over the past 40 years, several methods have been developed to study chemotaxis, including the Boyden chamber[18], collagen or fibrin gels[19], agarose[20], and Dunn chamber[21] assays. However, the gradients generated by these methods were unstable and hence could not be used for extended periods of time.

Tissue engineering applications require stabilized multiple gradients of immobilized factors in three-dimensional scaffolds. Immobilized protein gradients can mimic the spatial regulation indispensable for the process of tissue regeneration. It has already been demonstrated that scaffolds with gradients of immobilized nerve growth factor are useful in guiding nerve repair[22]. Another potential application can be found at the bone-cartilage interfaces, where spatial gradients of immobilized growth factors could be used to selectively stimulate cellular secretion of extracellular matrices at specific regions along the concentration gradient.

Peptide or protein gradients can be generated in hydrogels by adapting a gradient maker normally used to make polyacrylamide gels for electrophoresis[22-24] (Fig.2A). When covalently immobilized gradients of an adhesion peptide, RGDS, were formed on the hydrogel surface using a gradient maker, fibroblasts responded to the RGDS gradients by dramatically changing their morphology to align with the gradient axis and by migrating differentially in the direction of increasing RGDS concentrations[23]. Another application of this technique is to create concentration gradients of basic fibroblast growth factor (bFGF) covalently immobilized on hydrogel surfaces[24]. When cultured on the bFGF-gradient hydrogels, smooth muscle cells migrated more strongly with the concentration gradient when compared with control hydrogels with or without a constant bFGF concentration. Our lab has also investigated a diffusion-controlled fabrication technique to generate functional group gradients in hydrogels (Fig.2B). Briefly, a polyacrylamide (PAAm) hydrogel was exposed to a sodium hydroxide (NaOH) solution to generate a gradient of carboxyl groups in the hydrogel. The generated carboxyl groups were chemically coupled to the amino

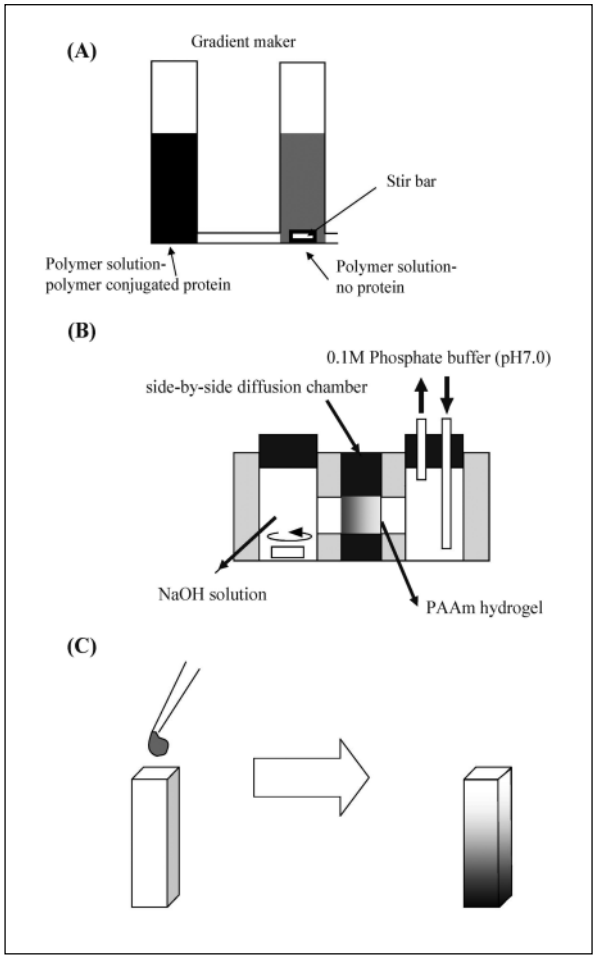

Fig.2 Fabrication techniques of three-dimensional protein gradients.
(A) Gradient maker, (B) Diffusion-controlled fabrication, (C) Diffusion-controlled fabrication for porous sponge scaffold.

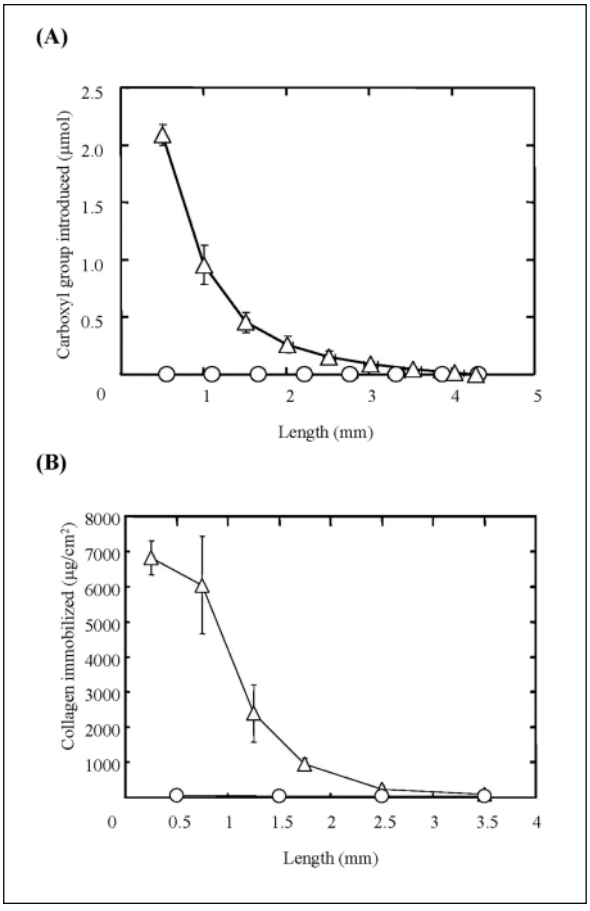

Fig.3 The generated carboxyl groups and immobilized type I collagen were stained with Toluidine Blue and Sircol Collagen staining dye to measure the gradient of (A) carboxyl groups and (B) immobilized type I collagen in PAAm hydrogels, respectively.

PAAm hydrogel was placed in the middle of a side-by-side diffusion chamber to generate carboxyl group gradients in the hydrogel, while the hydrogel contacted NaOH solutions at a concentration of 0 M (○) and 1.0 M (△) on one side and phosphate-buffered solution (pH 7.0) on the other side. The x-axis indicates the distance from the hydrogel surface contacting the NaOH solutions.

groups of type I collagen to give the hydrogel a spatial gradient of collagen immobilization. The attachment of L929 fibroblasts was then evaluated for the collagen-immobilized hydrogel. The amount of carboxyl groups in the hydrogel increased with an increase in the NaOH concentration (Fig.3A), and this carboxyl group gradient enabled us to successfully prepare a hydrogel with a gradient of immobilized collagen (Fig.3B). The number of adherent fibroblasts depended on the amount of collagen immobilized. These findings indicate that cell behavior can be influenced by a spatial gradient of biomolecules in the cell scaffold. However, hydrogels do not have the pore structures necessary for migration, proliferation, and differentiation of cells. Therefore, it is necessary to develop a technique for the fabrication of functional gradients in a sponge or non-woven fabric with porous structure.

Vepari et al. have developed a technique to generate immobilization gradients of enzymes in a porous three-dimensional silk fibroin scaffold using the principles of diffusion (Fig.2C)[25]. The activity gradient of immobilized enzymes was controlled by the volume and concentration of the enzyme solution in the carbodiimide immobilization reaction. This method could be extended to immobilize a variety of proteins and small molecules in several types of porous materials, thereby offering new options in the fields of chemotaxis and tissue engineering.

Conclusions

Several methods have been developed to create protein gradients on cell substrates for chemotaxis assays and tissue engineering. Although some sophisticated fabrication techniques can provide two-dimensional cell substrates whose controlled concentration gradients consequently regulate cell behavior, it is difficult to utilize these techniques for generating protein gradients in three-dimensional scaffolds. On the other hand, the diffusion of proteins within hydrogels or porous scaffolds can now be applied to make cell scaffolds with three dimensional concentration gradients of immobilized protein. While the bioactivity gradients of protein immobilized are still being investigated.

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