host's vascular tissue. There is no concern for limited supply and there are no problems of immunorejection because it is autotransplantation. Biotubes are expected to grow with the host's blood vessels and are an ideal vascular graft. We are planning to establish a biotube preparation method that combines surface design as described above and demonstrate the usefulness of biotubes as small-caliber artificial blood vessels by animal transplantation experiments. These results will be reported in the near future.

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Three-Dimensional Cardiac Tissue Engineering Using a Thermoresponsive Artificial Extracellular Matrix

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The purpose of this study was to try to reconstitute three-dimensional cardiac tissue using a thermoresponsive artificial extracellular matrix, poly (N-isopropylacrylamide)-grafted gelatin (PNIPAM-gelatin), as the scaffold. PNIPAM-gelatin solution gels almost immediately when heated above 34°C. We thought this property could become advantageous as scaffolding for reconstituting three-dimensional tissue. Because PNIPAM-gelatin solution gels so quickly, all seeded cells in PNIPAM-gelatin solution would become entrapped and uniformly distributed toward three dimensions. Thus it would be possible to reconstitute three-dimensional tissue by a very simple method of mixing cells and PNIPAM-gelatin solution. Fetal rat cardiac cells were mixed with PNIPAM-gelatin solution, incubated at 37°C to allow the mixture to gel, and cultured *in vitro*. To define suitable culture conditions the following parameters were tested: (1) PNIPAM-gelatin concentration, 0.04~0.125 mg/ml; (2) cell seeding density, 1~50 × 10^6 cells/ml; and (3) addition or not of hyaluronic acid. With a PNIPAM-gelatin concentration of 0.05 mg/ml, a cell seeding density of 50 × 10^6 cells/ml, and the addition of hyaluronic acid, tissue was reconstituted and it contracted synchronously. After hematoxylin and eosin staining, the cells reconstituted three-dimensional tissue, and the tissue cross-section was approximately 60 μm thick. *ASAIO Journal* 2004; 50:344–348.

Because of the shortage of donor organs for heart transplantation, cell transplantation for the end stage of chronic heart failure is expected to become reality. Recently, there have been many studies demonstrating that cells transplanted into the infarcted myocardium improved heart function.[1–3] Clinically, a myoblast transplantation has been performed,[4] but it is controversial whether these effects observed were caused by the ability of transplanted cells to create sufficient amounts of new myocardium-like tissue within the infarcted area and to participate in synchronized heart contraction.[5] In these studies, donor cells were delivered by means of direct injection into the infarcted myocardium. We believe, however, that if we expect to observe contractile activity in cells transplanted into the infarcted myocardium, we will have to transplant sufficient numbers of donor cells into the scarred and thin infarcted myocardium. We believe that it is difficult to create a functional tissue equivalent in such a myocardium without the use of scaffolding. We must, therefore, seek effective transplantation methods. A promising approach to repairing large areas of scar may be the use of tissue engineered cardiac grafts. In tissue engineering, both the cells and the scaffolding material are important for reconstituting three-dimensional tissues. In this study, we used a thermoresponsive artificial extracellular matrix, poly(N-isopropylacrylamide) (PNIPAM)-gelatin,[6–9] for scaffolding to reconstitute three-dimensional cardiac tissue. PNIPAM-gelatin solution gels quickly with simple heating above 34°C. Our hypothesis was that the cells mixed in the PNIPAM-gelatin solution would be entrapped uniformly in PNIPAM-gelatin gel by heating and that the mixture would allow reconstitution of three-dimensional cardiac tissue.

The purpose of this study was to demonstrate that three-dimensional cardiac tissue may be reconstituted by mixing fetal rat cardiac cells with PNIPAM-gelatin and to establish the optimal conditions for doing so.

Materials and Methods

Care of Animals

All animals received humane care in compliance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH Publication No. 86–23, revised 1985).

Cardiac Cells

Fetal rat cardiac cells were isolated from 18 day gestational Wistar rats. The ventricles of the fetuses were digested in three rounds of 10 minute incubations in a solution consisting of 0.05% trypsin in phosphate buffered saline. To reduce non-myocyte contamination cells, isolated cardiac cells were plated on a glass dish and cultured for 2 hours in the culture medium. The supernatant containing suspended cells was collected and centrifuged at 1,000 rpm for 5 minutes. The viability of the cells was calculated by trypan blue staining, and these cells were used for the following studies.

Scaffold

Thermoresponsive artificial extracellular matrix, poly (N-isopropylacrylamide) (PNIPAM) grafted gelatin,[6–9] here called PNIPAM-gelatin (a gift from Shoji Ohya), was used for the

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scaffold material. PNIPAM-gelatin was prepared by graft polymerization of PNIPAM on gelatin. PNIPAM-gelatin is water soluble at room temperature and immediately precipitates at temperature above approximately 34°C, so PNIPAM-gelatin solution gels as soon as it is heated above 34°C. A mixture was prepared at room temperature of various amounts of PNIPAM-gelatin and culture medium. The PNIPAM-gelatin concentrations were 0.04, 0.05, 0.075, 0.1, and 0.125 mg/ml. We studied the effects of the different PNIPAM-gelatin concentrations while using a cell seeding density of 10×10^6 cells/ml.

Three-Dimensional Cardiac Tissue

The reconstitution mixture was prepared by pouring PNIPAM-gelatin solution mixed with cardiac cells on a culture dish at room temperature. The reconstitution mixture was incubated at 37° to allow it to gel, and thereafter culture medium was added to each dish. These dishes were then placed in a humidified incubator at 37°C with 5% carbon dioxide and 95% air. The proportions of cardiac cells in the reconstituted mixture, the cell seeding densities, were studied at five different densities (1, 2, 5, 10, and 50×10^6 cells/ml). We studied the effects of various cell seeding densities using a PNIPAM-gelatin concentration of 0.05 mg/ml.

Supply of Hyaluronic Acid

Hyaluronic acid (HA) (0.003 mg/ml) was supplied to the culture medium to make HA supplied PNIPAM-gelatin solution. HA supplied reconstituted mixtures were made using the same methods as mentioned previously. We studied the effect of supplying HA for making cardiac tissue using a PNIPAM-gelatin concentration of 0.05 mg/ml and a cell seeding density of 50×10^6 cells/ml.

Macroscopic and Microscopic Observation

Macroscopic observation of the contractile activities of the reconstituted mixtures was performed daily with the unaided eye. Microscopic observation of cells' survival and the contractile activities of the reconstituted mixtures was performed daily by phase contrast microscopy. These views were recorded with a digital video camera.

Histologic Analysis

After 2 weeks of culturing, the reconstituted mixtures were fixed in 10% neutral buffered formalin, dehydrated in a graded series of ethanol, embedded in paraffin, and sectioned into 10 μm slices (perpendicular to the plane of the tissue). For basic morphology, hematoxylin and eosin (HE) staining was performed by conventional methods.

Results

PNIPAM-Gelatin Concentration

When the PNIPAM-gelatin concentration was 0.04 mg/ml, the PNIPAM-gelatin solution did not gel, even with heating above 34°C, so it could not be used as scaffold. At a PNIPAM-gelatin concentration of 0.05 mg/ml, the PNIPAM-gelatin solution did gel, and scattered asynchronously contracting isolated cells and clusters were observed in the reconstituted mixture after 2 weeks of culturing (**Figure 1**). When the PNIPAM-gelatin concentration was higher than 0.075 mg/ml, the PNIPAM-gelatin solution did gel, but the seeded cells could not survive in the reconstituted mixture (**Figure 2**).

Cell Seeding Density

When the cell seeding density was lower than 10×10^6 cells/ml, scattered asynchronously contracting isolated cells and clusters were observed in the reconstituted mixture after 2 weeks of culturing (**Figure 1**). These clusters were neither gathering nor becoming larger. When the cell seeding density was 50×10^6 cells/ml, more cells were connected, and tissue like constructs were observed in some parts of the reconstituted mixture, but they were too small for us to observe their contractions macroscopically.

Supply of Hyaluronic Acid

When HA was supplied to the reconstituted mixtures, spontaneously and synchronously contracting tissue was observed macroscopically from 1 week after culture, and the frequency of contraction was approximately between 30 and 250 beats/min as estimated by microscopic inspection. The macroscopic views were recorded by a digital video camera after 2 weeks of culturing (**Figure 3**). We also observed the same tissue using phase contrast microscopy. The tissue was made of many cells, and synchronous contractions of these cells were observed (**Figure 4**). With HE staining, cells reconstituted three-dimensional tissue with a cross-section as thick as 60 μm, and all cells in the tissue were viable and uniformly spread not only at the periphery but also in the center lesion (**Figure 5**).

Discussion

In vitro engineering of three-dimensional cardiac tissue has emerged as a technology with potential for tissue replacement therapy.[10–21] Successful engineering of cardiac tissue has been previously demonstrated by many methods of cultivation on

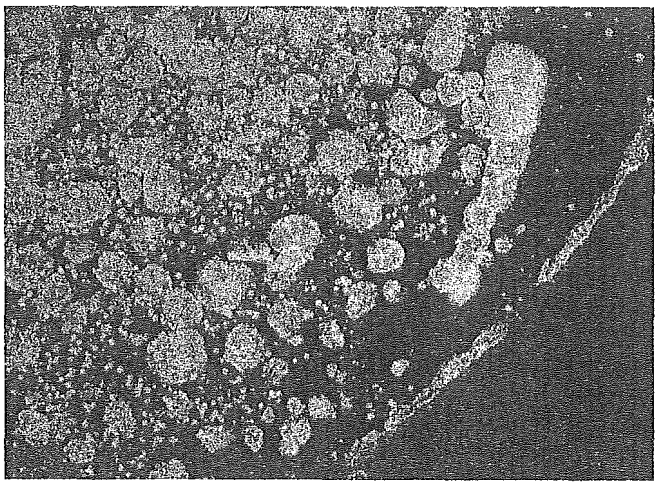

Figure 1. A phase contrast microscopic view of cardiac cells in PNIPAM-gelatin gel (PNIPAM-gelatin concentration, 0.05 mg/ml; initial cell seeding density, 10×10^6 cells/ml) after 2 weeks of culturing. Original magnification × 4. PNIPAM-gelatin, poly (N-isopropylacrylamide)-grafted gelatin.

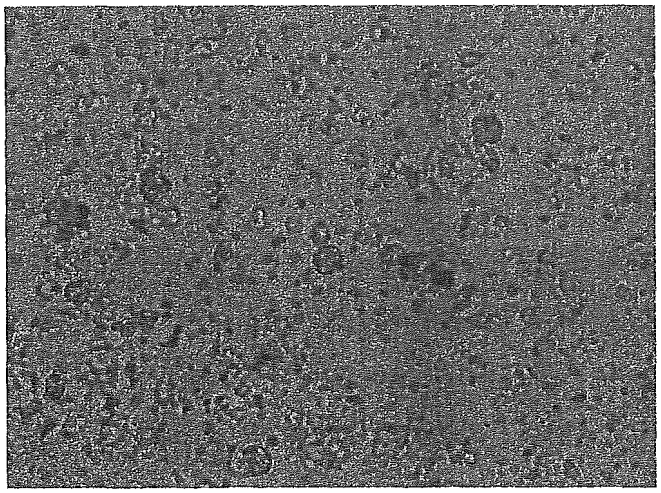

Figure 2. A phase contrast microscopic view of cardiac cells in PNIPAM-gelatin gel (PNIPAM-gelatin concentration, 0.075 mg/ml; initial cell seeding density, 10×10^6 cells/ml) after 2 weeks of culturing. Original magnification × 10. PNIPAM-gelatin, poly (N-isopropylacrylamide)-grafted gelatin.

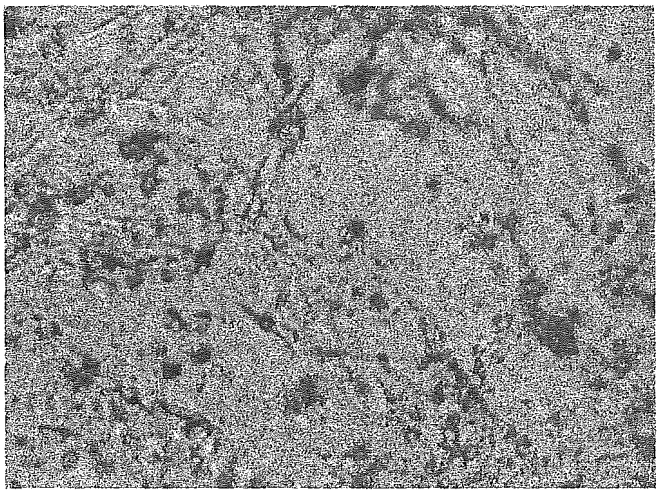

Figure 4. A phase contrast microscopic view of the tissue (PNIPAM-gelatin concentration, 0.05 mg/ml; initial cell seeding density, 50×10^6 cells/ml; hyaluronic acid, addition) after 2 weeks of culturing. Original magnification × 20. PNIPAM-gelatin, poly (N-isopropylacrylamide)-grafted gelatin.

biodegradable preformed three-dimensional mesh,[10-16] on polystyrene microcarrier beads in bioreactors,[17] in collagen gels,[18,19] on micropatterned laminin surfaces,[20] and in layered cardiomyocyte sheets.[21] To make widespread use of cardiac tissue reconstitution for tissue replacement therapy, cardiac tissues need to be made by a simple method. Cultivation on biodegradable, preformed, three-dimensional scaffolding is simple and the most popular method, but when the cells are seeded onto the scaffold, some will be unattached and lost for further tissue development.[22] Also, the problem of a spatially nonuniform distribution of cells, resulting in tissue formed only at the surface layer while the construct interior remained largely acellular,[14] remains. No new simple method of cultivation in collagen gels had been reported after Souren's report in 1992,[23] which stated that rat cardiac cells did not reconstitute cardiac tissue in collagen, but there have been recent reports of the reconstitution of cardiac tissue by the culture of cardiac cells in suspension with type I collagen[18,19] and the culture of smooth muscle cells in PNIPAM-gelatin gel.[9] PNIPAM-gelatin solution gels almost immediately by simply heating it above 34°C,[6-9] so we thought that PNIPAM-gelatin would be advantageous as a scaffold material for reconstituting three-dimensional tissue. Because PNIPAM-gelatin solution gels so quickly and easily, all of the seeded cells in PNIPAM-gelatin solution should be entrapped and uniformly distributed toward three dimensions in PNIPAM-gelatin gel. It should be possible to reconstitute three-dimensional tissue by a very simple method of mixing cells and PNIPAM-gelatin solution. We were not sure, however, whether cardiac cells would reconstitute cardiac tissue in PNIPAM-gelatin gel.

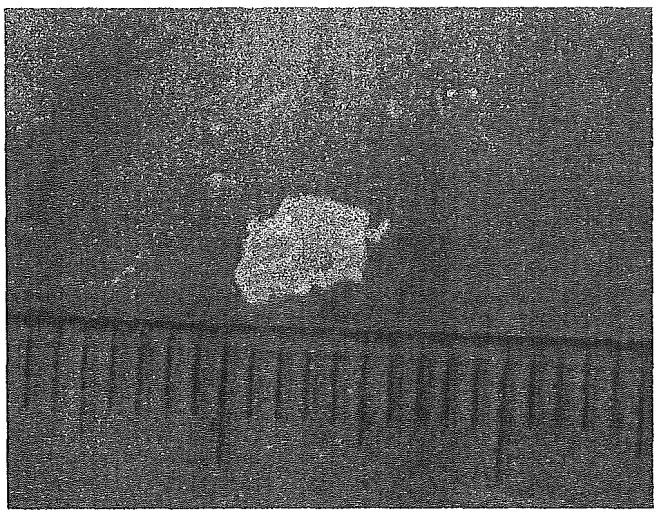

Figure 3. A macroscopic view of the tissue (PNIPAM-gelatin concentration, 0.05 mg/ml; initial cell seeding density, 50×10^6 cells/ml; hyaluronic acid, addition) after 2 weeks of culturing. PNIPAM-gelatin, poly (N-isopropylacrylamide)-grafted gelatin.

Several studies have made clear that variations in cell seeding density and culture conditions affected whether cardiac cells reconstituted cardiac tissue[23] and also affected the structure of engineered cardiac tissue.[15,16,19] In the present study, at low cell seeding density (lower than 10×10^6 cells/ml), we observed scattered asynchronously contracting isolated cells and clusters (**Figure 1**), and at high cell seeding density (50×10^6 cells/ml), synchronously contracting small tissue like constructs were observed. These results indicate that the distances between seeded cells might be too great to contact other cells at low cell seeding densities, as was noted in Souren's report.[23] High cellularity is known to enhance cell to cell contact and communication, which are factors for cardiac tissue formation *in vivo*, and this high cell seeding density might result in good distances between cells for connecting with neighbor cells. The high cell seeding density we used was closer to that in adult rat myocardium than that reported by many others, so we expected the twitch tension of this tissue to be near that of adult rat myocardium.

When the PNIPAM-gelatin concentration was higher than 0.075 mg/ml, the seeded cells could not survive in PNIPAM-gelatin gel (**Figure 2**). The cells' survival depends upon the

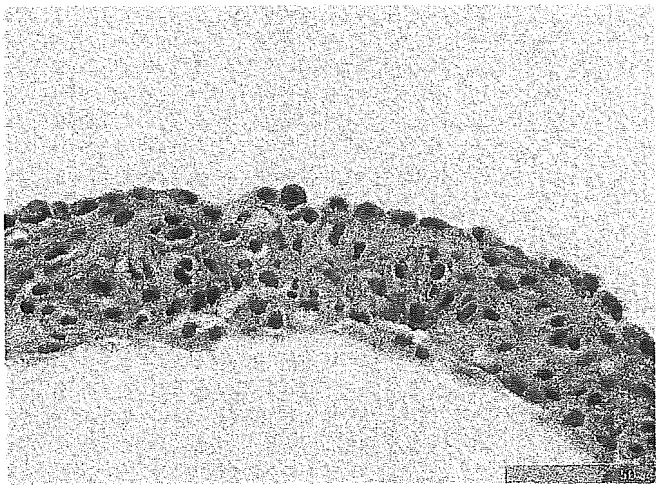

Figure 5. Cross-sectional view of the tissue (PNIPAM-gelatin concentration, 0.05 mg/ml; initial cell seeding density, 50×10^6 cells/ml; hyaluronic acid, addition) with hematoxylin and eosin staining after 2 weeks of culturing. PNIPAM-gelatin, poly (N-isopropylacrylamide)-grafted gelatin.

diffusion of oxygen and nutrition and adequate space in the three-dimensional culture. Smooth muscle cells could survive in PNIPAM-gelatin gel even at 0.2 mg/ml PNIPAM-gelatin concentration,[9] so the space in PNIPAM-gelatin gel should be sufficient for cardiac cells. We thought that cardiac cells might be more weakened by the poor diffusion of oxygen and nutrition than smooth muscle cells. At a PNIPAM-gelatin concentration of 0.05 mg/ml, PNIPAM-gelatin solution gels, and the seeded cells could survive in PNIPAM-gelatin gel (**Figure 1**). However, even with the same concentration and a cell seeding density of 50×10^6 /ml, the seeded cells make small tissue like constructs in some parts of the reconstituted mixture but not in other parts of the mixture. It seemed that that was because the microenvironment of the seeded cells must vary in different parts of the reconstituted mixture and that the variation affected the cells' growth and ability to connect with neighbor cells. Our aim was to improve the microenvironment of the seeded cells so that it would be uniform throughout the reconstituted mixture for reconstituting large cardiac tissue.

Living cells require a continuous supply of nutrients and removal of metabolites. The scaffold must have pores that are larger than 10–30 μm, preferably 200–400 μm.[24] In the present study, we did not confirm the pore size made by PNIPAM-gelatin because of technical problems, but we thought it would improve microenvironment for cardiac cells in PNIPAM-gelatin gel, and we supplied HA. HA makes the pore size larger by holding water, which has been shown to improve the control of oxygen, pH, nutrients, and metabolites. The resulting less tight network made by HA provides more free room for the formation of a new tissue and extracellular matrix synthesis.[25] By supplying HA, we were able to reconstitute large cardiac tissue, and we could observe the contractions of tissue macroscopically (**Figure 3**). HE staining revealed that cells reconstituted three-dimensional tissue with a cross-section as thick as 60 μm, and all of the cells were viable and uniformly spread at not only the periphery but also in the center lesion of the tissue (**Figure 5**). Therefore, it seemed that by supplying HA, the microenvironment of the all seeded cells was improved in all parts of the reconstituted mixture. The seeded cells survived and grew at the same place where they were distributed uniformly.

Conclusions

Spontaneously contracting three-dimensional cardiac tissue was reconstituted using a simple method of mixing cardiac cells and PNIPAM-gelatin *in vitro*. A limitation of the study was that we were not able to evaluate cell viability in the tissue by the specific property of PNIPAM-gelatin. When the tissue was incubated in trypsin solution, PNIPAM-gelatin gelled, and we could not isolate cells from the tissue to evaluate cell viability. Also in this study, we made three-dimensional tissue that was less than 100 μm thick. Some reports have noted that engineered tissues greater than 100 μm thick that are highly active metabolically and are supplied with nutrients solely by diffusion may have insufficient transport to and from the cells.[14] Further studies of cell viability in the tissue and the limitations of tissue thickness are needed.

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ORIGINAL ARTICLE

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Takehisa Matsuda, PhD

In vivo evaluation of poly(*N*-isopropylacrylamide) (PNIPAM)-grafted gelatin as an in situ-formable scaffold

Abstract We examined whether poly(*N*-isopropylacrylamide)-grafted gelatin (PNIPAM-gelatin) with a lower critical solution temperature of approximately 34°C, which was prepared by quasi-living radical graft polymerization, can serve as an in situ-formable three-dimensional extracellular matrix or cell scaffold. A mixture of fibroblasts stained with fluorescent dye and PNIPAM-gelatin in Dulbecco's modified Eagle's medium solution was injected into the subcutaneous tissue of Wistar rats, and immediately formed a white, opaque cell-incorporated gel. Fibroblasts immediately after injection were spherical in shape and were homogeneously distributed in the gel. Fibroblasts in the gel 2 weeks after injection had spread and proliferated. One day after injection, many macrophages and neutrophiles were observed around the gel. As the implantation period proceeded, the inflammation reaction subsided. One week after injection, fibroblasts in the native tissue and macrophages migrated into the gel. From 6 to 12 weeks after injection, some degree of calcification in the solid tissue was intermittently observed. The weight of the gel 6 weeks after implantation was reduced to almost one-half of the weight of the originally injected sample. The potential usefulness of PNIPAM-gelatin as an injectable scaffold is discussed.

Key words Poly(*N*-isopropylacrylamide)-grafted gelatin · Thermoresponsiveness · In situ-formable scaffold · Cell viability

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Introduction

Engineered tissues composed of cells and extracellular matrices (ECMs) have been fabricated ex vivo and in vivo to heal or replace diseased tissues or to replace lost tissues.[1–5] Biomacromolecules and synthetic polymers have been utilized for designing the extracellular space and as structural platforms or scaffolds. However, the difficulty is to precisely fabricate custom-made or complex tissue shapes. In situ-formed gel prepared from its aqueous solution can be used to fabricate hybrid tissues with desired shapes.[6,7] The materials, which are induced to form a gel by a physiologically permitted stimulus such as temperature[8–11] or pH change[12–14] and light irradiation,[15–17] have been utilized for artificial three-dimensional (3D) ECM or cell scaffolds and drug delivery vehicles.

Gelatin, denatured collagen, exhibits cell adhesiveness and biodegradability, but is soluble in water at physiological temperatures. Insolubilization of gelatin at physiological temperatures is an essential requirement for tissue-engineered platforms, cell scaffolds, and artificial ECM. However, chemically or photochemically driven cross-linking reactions leading to gelation are more or less harmful or toxic. Instead, we utilized thermoresponsive sol-to-gel characteristics with an inverse phase transition temperature. Poly(*N*-isopropylacrylamide) (PNIPAM) precipitates in water above 32°C but is water-soluble at room temperature.[18] Because of this unique thermoresponsive feature, PNIPAM has been utilized for thermoresponsive tissue culture dishes,[19–21] drug delivery vehicles,[8] hemostasis,[10] and 3D ECM materials.[9,11]

In our previous study, we prepared PNIPAM-grafted gelatin (PNIPAM-gelatin), which was obtained by quasi-living radical polymerization initiated from the dithiocarbamyl group derivatized on a lysine residue of gelatin, as a thermoresponsive 3D ECM material.[21,22] PNIPAM-gelatin gel, prepared from its buffer suspended with bovine smooth muscle cells, produced a viable cell-entrapped hybrid tissue. The cell viability and cell proliferation potential were found to be markedly influenced by the PNIPAM graft density,

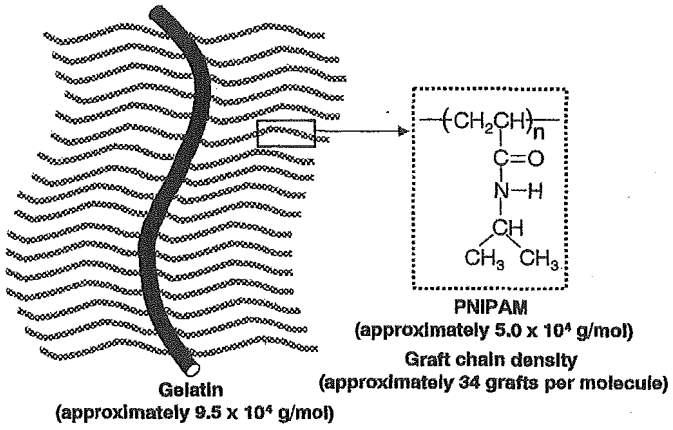

Fig. 1. Structure of poly(*N*-isopropylacrylamide)-grafted gelatin (*PNIPAM*-gelatin). The approximate number of graft chains per gelatin molecule was 34 and the average molecular weight of each PNIPAM graft was approximately 5.0×10^4 g/mol

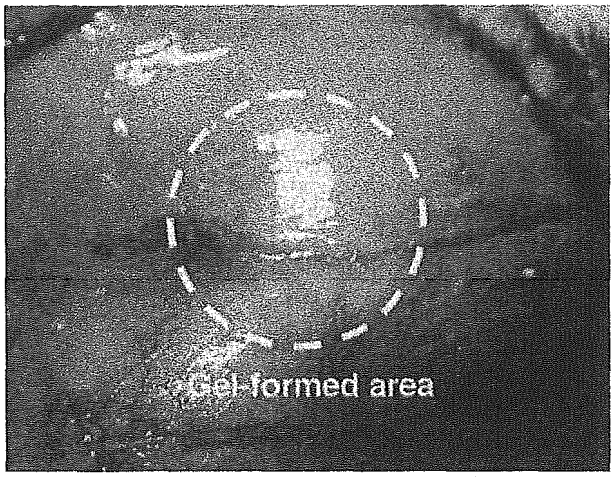

Fig. 2. PNIPAM-gelatin gel formation in rat subcutaneous tissue. A buffer solution of PNIPAM-gelatin suspended with rat fibroblasts was injected and spontaneously formed a white, opaque gel

the molecular weight of the graft chain, and the concentration of PNIPAM-gelatin. Our previous study showed that higher graft density and higher molecular weight of the graft chain in PNIPAM-gelatin enhanced cell viability and cell proliferation potential in 3D culture.[23] Among the designed PNIPAM-gelatins, the highest potential was found for PNIPAM-gelatin with each graft chain having a length of 5.0×10^4 g/mol and a graft density of approximately 34 grafts per molecule (Fig. 1).

In this study, using this PNIPAM-gelatin, the potential usefulness of PNIPAM-gelatin as an in situ-formable scaffold was examined in vivo. In situ characteristics of hybrid tissue formed in rat subcutaneous tissue, including cell viability, cell proliferation potential, natural ECM productivity, and degradability of the gel, as functions of implantation period, were studied.

Materials and methods

Materials

Gelatin (molecular weight: approximately 9.5×10^4 g/mol, from bovine bone) and sodium *N*,*N*-diethyldithiocarbamate trihydrate were obtained from Wako (Osaka, Japan). Solvents and other reagents (special reagent grade) were also purchased from Wako and used after conventional purification.

Cell morphology and matrix formation in PNIPAM-gelatin gel

We conducted the experiments in accordance with Industrial Guidance for the Care and Use of Laboratory Animals. The Wistar rats used were 10 weeks old. Rat fibroblasts harvested from rat subcutaneous tissue by the collagenase digestion method were stained with fluorescent dye of (benzoxazolium, 3-octadecyl-2-[3-(3-octadecyl-2(3H)-benzoxazolylidene)-1-propenyl], perchlorate, DiO, Molecular Probes, Eugene, OR, USA) before use. A phosphorous buffered saline (PBS) solution of PNIPAM-gelatin (concentration: 5 w/v%), prepared as previously described[21–23] and suspended with fluorescently stained fibroblasts (cell density: 1.2×10^6 cells/ml), was injected into a rat's subcutaneous tissue under anesthesia using ketamine (1 ml/Kg). Three rats were used in this study. Tissue containing gels were extirpated and fixed with 10% formalin neutral solution (pH = 7.4) at 37°C for 7 days and then rinsed with buffer. Specimens were dehydrated with a graded series of ethanol, embedded in paraffin, and cut into sections. Cell morphology in gels was observed by confocal laser scanning microscopy (Radiance 2100, Biorad, Hercules, CA, USA). The specimens for light microscopy were stained with hematoxylin and eosin (H&E) and Masson's trichrome (MT). Cell morphology and secreted collagen were observed by light microscopy (Vanox-S, Olympus, Tokyo, Japan).

Histological analysis of PNIPAM-gelatin gel

A PBS solution of PNIPAM-gelatin without cells was injected into a rat's subcutaneous tissue. Tissues containing gel were dissected 1 week after injection. Specimens were stained with H&E, MT, periodic acid schiff stain (PAS), and von Kossa's method. The interfaces between tissue and gel were observed using light microscopy.

Bioresorbability of PNIPAM-gelatin gel

The bioresorbability of PNIPAM-gelatin gels (20 w/v%) was examined by weight loss after incubation. Gels formed ex vivo were implanted into a rat's subcutaneous tissue. After a predetermined period, the weight loss of gels was measured after freeze-drying. Two rats, in which two gels were placed, were used for measuring the weight loss.

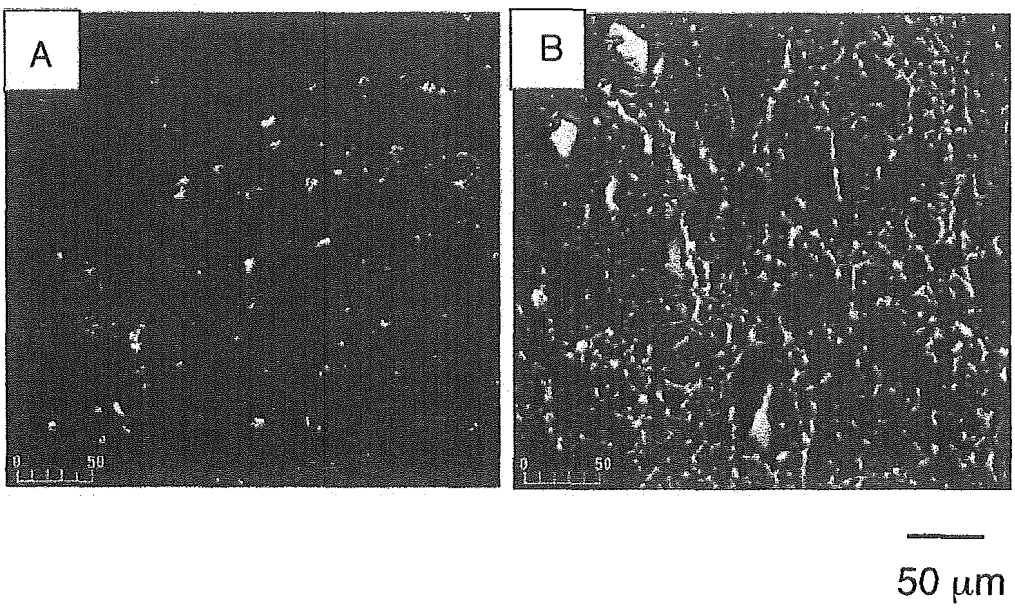

Fig. 3. Confocal laser microscopic observation of rat fibroblasts stained with fluorescent dye (DiO) in PNIPAM-gelatin gel immediately (**A**) or 2 weeks (**B**) after injection. Initially injected cell density: 1.2×10^6 cells/ml

Results

A buffer solution of PNIPAM-gelatin suspended with fluorescently stained fibroblasts was injected into a rat's subcutaneous tissue using a syringe. A white, opaque elastic gel that entrapped fibroblasts was immediately formed (Fig. 2). Confocal laser scanning microscopic observation of tissue samples showed that fluorescently labeled fibroblasts in the gel were homogeneously dispersed and remained spherical in shape when injected (Fig. 3A). Cell aggregation and elongated cell shapes were observed 2 weeks after injection (Fig. 3B). Higher cell proliferation was noticed compared with cells initially injected. Histochemical observation showed an accumulated ECM, which must have been produced by inoculated and proliferated cells and probably by cells migrating from surrounding tissues (Fig. 4).

The weight of PNIPAM-gelatinous tissues, which were easily harvested from the injected sites of native tissue and were subsequently freeze-dried, gradually decreased with incubation time, indicating that PNIPAM-gelatin was biodegraded and sorbed over the implantation period (Fig. 5). The harvested tissue weighed around only one-half of the weight of the initially injected sample on a solid basis 6 weeks after injection.

Cell-free PNIPAM-gelatin gel, prepared in rat subcutaneous tissue by injection, was histologically examined for up to 12 weeks after injection (Fig. 6). At the very early implantation period (1 day after injection), many cells responsible for inflammation such as neutrophils and macrophages were observed around the gel. One week after injection, fibroblasts and macrophages, both stained with PAS, began to penetrate into the gel. Although PAS-stained cells appeared to be rare at 1 week after injection, the cells resided on and in the biodegrading gels at 6 and 12 weeks. Masson's trichrome-stained tissue showed that collagen fibers locally accumulated around the gel and a fibrous granulation tissue was observed at 6 and 12 weeks after injection (Fig. 6). Microparticles stained by H&E and von Kossa's method, identified as calcified tissue, intermittently aggregated or scattered in the tissue, were observed 6 and 12 weeks after injection.

Discussion

In situ-formed gel, induced by a physiologically permitted stimulus, can function as cell scaffold and may be very useful in fabricating a custom-made or complex shaped hybrid tissue in tissue engineering applications. The required properties for in situ-formed 3D ECM materials are (1) rapid gelation from their aqueous solutions in living tissues, (2) high cell viability and cell proliferation potential, (3) low inflammatory reaction, and (4) suitable biodegradability, an additional property to materials required for in vitro use. Our injectable 3D scaffold is gelatin grafted with PNIPAM, thereby incorporating bioactive function and thermoresponsive gelation with the designed scaffold. In vitro study showed that cell viability and cell proliferation in the 3D gel were enhanced by a suitable graft chain density and molecular weight of graft chain.

In this study, we evaluated the potential as an in situ-formed ECM material in vivo of the PNIPAM-gelatin that exhibited the highest cell proliferation potential among PNIPAM-gelatins designed with different molecular components. A cell suspension of PNIPAM-gelatin in a medium, when injected into rat subcutaneous tissue, was immediately converted into a cell-incorporated white, opaque gel (Fig. 2). Fibroblasts in the gel extended and proliferated and secreted collagen 2 weeks after injection (Figs. 3 and 4), indicating that the PNIPAM-gelatin gel functions as a cell scaffold in vivo as expected from our previous experiments in vitro.

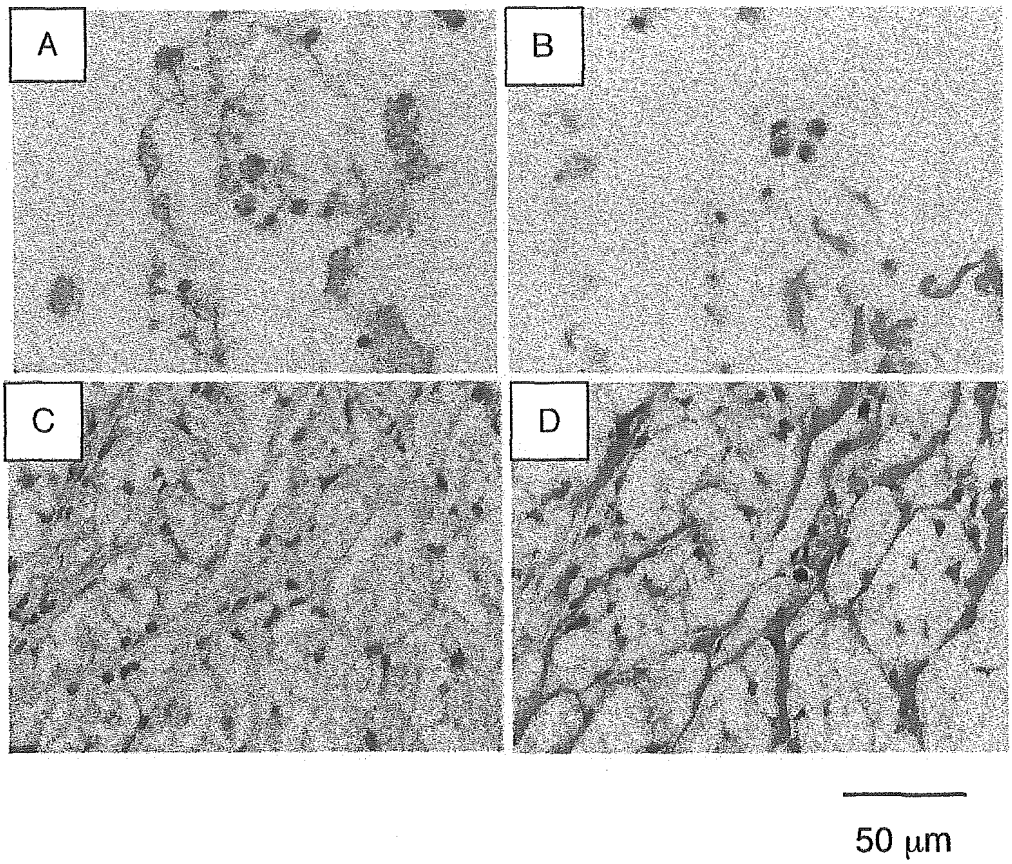

Fig. 4. Light microscopic observation of fibroblasts in PNIPAM-gelatin gel immediately (**A**, **B**) or 2 weeks (**C**, **D**) after injection. The specimens were stained with hematoxylin and eosin (**A**, **C**) and Masson's trichrome (**B**, **D**)

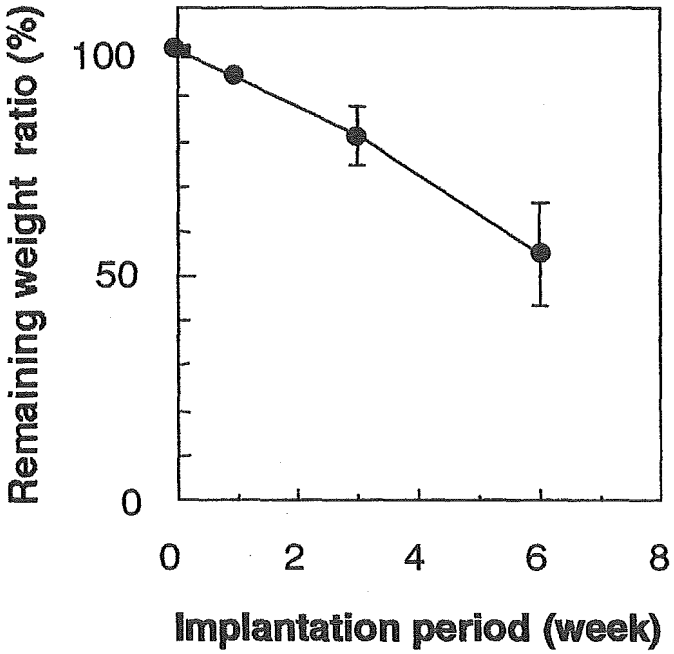

Fig. 5. Examination of dry weight loss of PNIPAM-gelatin gel in rat subcutaneous tissue. The weight gradually decreased with the implantation period

Histological evaluation of PNIPAM-gelatin gel showed that the wound healing process is accompanied by the presence of neutrophils, macrophages, and fibroblasts, and gel degradation and new tissue formation occurred throughout the implantation time. As shown in Fig. 5, the weight of the gel decreased with implantation time, resulting in approximately 50% weight reduction 6 weeks after implantation, probably the result of the balance between the weight gain of the ingrown and regenerated tissue and the weight loss caused by the degradation of the gel. Appropriate tissue formation and simultaneous gel degradation for tissue regeneration depend on the cell type and injection site. The gel degradation period may be controlled by the PNIPAM to gelatin ratio. Calcification was found 6 and 12 weeks after injection. In general, the occurrence of calcification is detrimental in a soft tissue regeneration technology. Only limited studies on gel-induced calcification have been reported. Pioneering studies by Imai[24-26] and Kopecek[27,28] et al. revealed that hydrogels such as poly(2-hydroxyethyl methacrylate) or poly(acrylamide) and its copolymer with a hydrophobic monomer (butyl methacrylate) induced hydroxyapatite formation on and in the gel upon implantation into subcutaneous tissues of young rats. In young rats, calcification was induced at very high probability regardless of the type of implanted material. However, such calcification did not occur in adult rats or other large animals such as dogs or goats, indicating that the occurrence of calcification largely depends on the age of the rat and the animal species. The rats implanted in this study were young, i.e., 10 weeks old. A separate experiment showed that no calcification occurred upon extensive implantation of injectable cartilage tissues containing chondrocytes in rabbits using PNIPAM-gelatin as an injectable scaffold over a period of 1 year.[29] To translate the evidence of the occurrence of

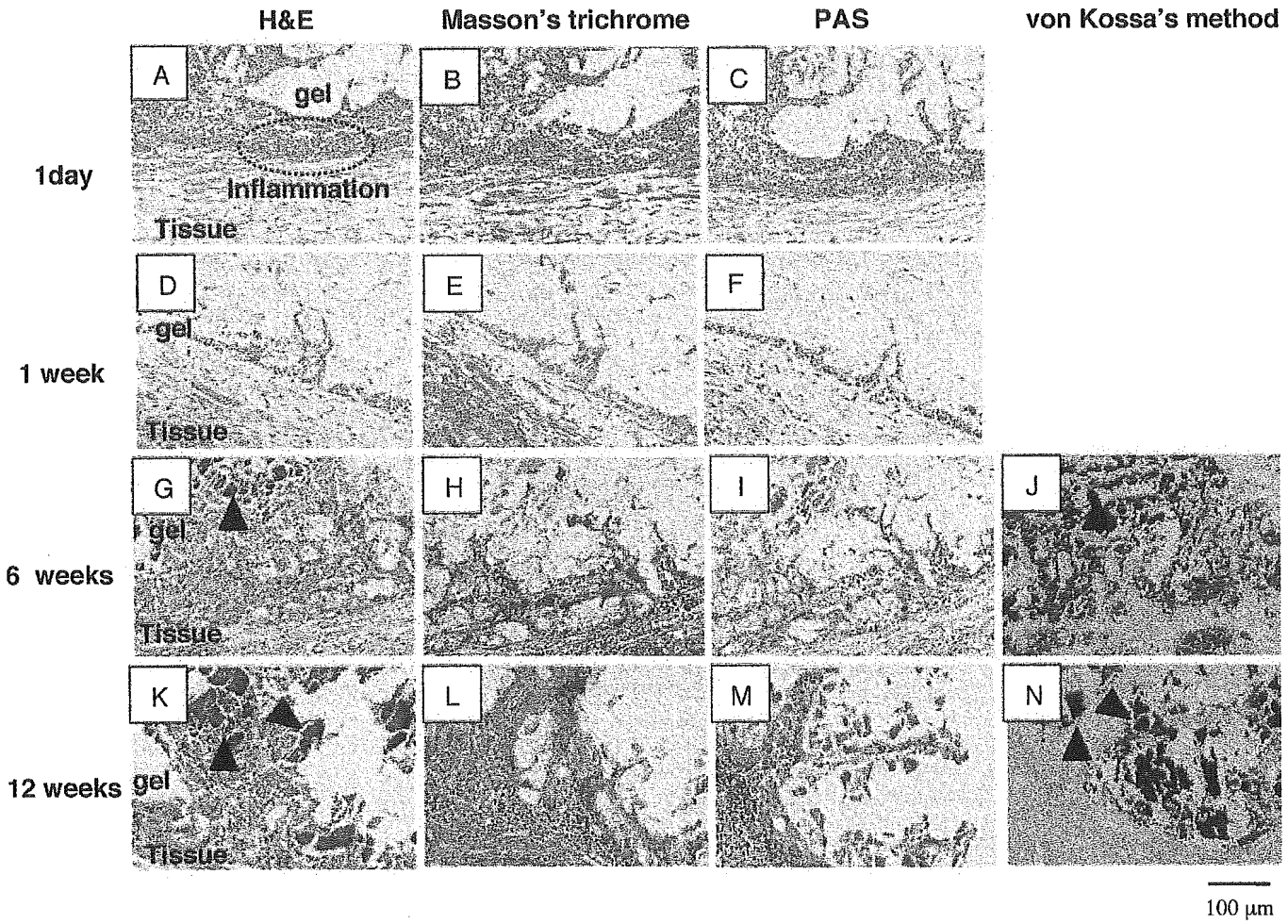

Fig. 6. Histological analysis of PNIPAM-gelatin gel in rat subcutaneous tissue 1 day (**A–C**), 1 week (**D–F**), 6 weeks (**G–J**), and 12 weeks (**K–N**) after injection. Staining was with hematoxylin and eosin (**A, D, G, K**), Masson's trichrome (**B, E, H, L**), periodic acid schiff (**C, F, I, M**), and von Kossa's method (**J, N**). *Black triangles* show the calcification points

calcification in rats in the present study to humans may not be appropriate or not valid at this time, but further study is required for elucidation. Our ongoing study using PNIPAM-gelatin is focusing on soft tissue augmentation technology, and will be reported in the near future.

Conclusions

PNIPAM-gelatin served as an injectable, in situ-formed scaffold on and in which fibroblasts spread and proliferated. PNIPAM-gelatin was biodegraded over time without excessive inflammatory reactions. It is suggested that the calcification occurring in the later period of implantation was the result of using young rats. Further study will elucidate the suitability of PNIPAM-gelatin as a soft tissue augumentation technology.

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Technical note

The potential of poly(*N*-isopropylacrylamide) (PNIPAM)-grafted hyaluronan and PNIPAM-grafted gelatin in the control of post-surgical tissue adhesions

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Abstract

Poly(*N*-isopropylacrylamide)-grafted hyaluronan (PNIPAM–HA) and PNIPAM-grafted gelatin (PNIPAM–gelatin), which exhibit sol-to-gel transformation at physiological temperature, were applied as control of tissue adhesions: tissue adhesion prevention material and hemostatic aid, respectively. The rat cecum, which was abraded using surgical gauze, was coated with PNIPAM–HA-containing PBS (concentration: 0.5 w/v%). The coated solution was immediately converted to an opaque precipitate at body temperature, which weakly adhered to and covered the injured rat cecum. One week after coating, tissue adhesion between the PNIPAM–HA-treated cecum and adjacent tissues was significantly reduced as compared with that between non-treated tissue and adjacent tissues. On the other hand, the coating of bleeding spots of a canine liver with PNIPAM–gelatin-containing PBS (concentration: 20 w/v%) resulted in spontaneous gel formation on the tissues and subsequently suppressed bleeding. Although these thermoresponsive tissue adhesion prevention and hemostatic materials are still prototypes at this time, both thermoresponsive biomacromolecules bioconjugated with PNIPAM, PNIPAM–HA and PNIPAM–gelatin, may serve as a tissue adhesion prevention material and hemostatic aid, respectively.

Keywords: Poly(*N*-isopropylacrylamide)-grafted biomacromolecules; Gel; Thermoresponsiveness; Tissue adhesion prevention; Hemostasis

1. Introduction

The prompt management of normal wound healing during and after surgical treatment may predict the post-surgical healing of tissues. Hemostatic control during surgical operation and tissue adhesion prevention after surgery are two critical issues in wound healing. To this end, various approaches and materials have been developed and tested over the years. However, "ideal" wound-healing materials have not been realized as yet.

Post-surgical tissue adhesion, which results from malignant healing response of a damaged tissue to a non-injured tissue, often causes life-threatening complications or necessitates re-operation. To reduce tissue adhesion, the use of physical barrier membranes to separate adjacent tissues during the healing process has been proposed and examined [1]. Carboxymethylcellulose [1,2], dextran [3] and oxidized regenerated cellulose [1–4] films have been clinically used as such membranes with some therapeutic effects. Hyaluronan (HA), which is an extracellular matrix component, is known to temporarily prevent tissue adhesion [5] when such a solution is coated on damaged tissue. However, HA is rapidly biodegraded by hyaluronidase and removed away from the injury sites [6].

On the other hand, tissue adhesive glue or hemostatic aids have been used when bleeding cannot be controlled during surgery. Fibrin glue has been clinically used in these cases. However, its major drawbacks are its low mechanical strength and potential infection risk inherent to blood origin. Semisynthetic and synthetic materials such as cyanoacrylate derivatives [7,8], gelatin–resorcinol–formaldehyde [8], and fluorinated hexamethylene diisocyanate-based urethane prepolymers [9] have been applied as surgical adhesives. Although they have

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appropriate tissue adhesiveness, cytotoxicity and severe inflammatory reactions with the use of the former two glues and very slow degradation with the last glue are the major drawbacks, respectively.

Regardless of tissue adhesion prevention or hemostatic aids, rapid sol-to-gel formation is necessary to cover the injured or bleeding sites of a tissue. Such a phase transition is desired to occur within a few minutes after application at physiological temperature. We previously prepared poly(*N*-isopropylacrylamide) (PNIPAM)-grafted hyaluronan (PNIPAM–HA) [10,11] and PNIPAM-grafted gelatin (PNIPAM–gelatin) [12–14]. These were soluble in water at room temperature but precipitated or gelled at physiological temperature due to thermoresponsive phase transition characteristics of PNIPAM. In this study, we explored the potential applicability of thermoresponsive PNIPAM–HA and PNIPAM–gelatin as a tissue adhesion prevention material or hemostatic aid, respectively.

2. Materials and methods

2.1. Material

Sodium hyaluronate (HANa, molecular weight: ca. 5.0×10^5 g/mol) was supplied by Seikagaku Kogyo Co. Ltd., Gelatin (molecular weight: ca. 9.5×10^4 g/mol, from bovine bone) and the solvents, which were of special reagent grade, were purchased from Wako Pure Chemical Industry Ltd., (Osaka, Japan) and used after conventional purification.

2.2. Cell adhesion on PNIPAM–HA film

An aqueous solution of PNIPAM–HA (concentration: 0.5 w/v%) was coated onto a circular cover glass (diameter: 14.5 mm, Matsunami Glass Co. Ltd., Osaka, Japan) and dried at room temperature. Rat fibroblasts at a density of 2.0×10^4 cells/ml were seeded on PNIPAM–HA films. After 3 h of incubation, cell morphology was observed by phase-contrast microscopy (Diaphoto, Nikon, Tokyo, Japan). All the procedures including cell culture were carried out at 37°C.

2.3. Tissue adhesion prevention efficacy of PNIPAM–HA

Tissue adhesion prevention efficacy was assessed using a rat cecum abrasion model [1,4]. Anesthesized Wistar rats were subjected to laparotomy. Each rat cecum was abraded with a surgical gauze. A PBS (0.5 ml) of PNIPAM–HA (concentration: 0.5 w/v%) was coated onto the cecum. One week after application, the incidence and severity of adhesions of the cecum to adjacent tissues were evaluated according to the following system: after harvesting the cecum and fixing it in formalin neutral buffer solution (pH 7.4, Wako Pure Chemical Industry Ltd., Osaka, Japan) at 37°C, the specimens stained with hematoxylin–eosin (H&E) and Masson's trichrome were observed by light microscopy (VANOX-S, Olympus, Tokyo, Japan).

0: No cecum adhesions
1: Firm adhesion with easily dissectable plane
2: Adhesion with dissectable plane causing mild tissue trauma
3: Fibrous adhesion with difficult tissue dissection
4: Fibrous adhesion with non-dissectable tissue planes.

2.4. Histological analysis of tissue adhesion prevention efficacy for PNIPAM–HA precipitate

For histological analysis, PNIPAM–HA-treated rat ceca, after seven days, were fixed with 10% formalin neutral buffer solution (pH 7.4) for more than seven days, dehydrated in a graded ethanol series, embedded in paraffin, and sectioned at 5 μm thickness. After staining with H&E or Masson's trichrome, the specimens were evaluated by light microscopy.

2.5. Hemostatic characteristics of PNIPAM–gelatin

The hemostatic characteristics of PNIPAM–gelatin were evaluated using a canine liver model (weight: 25 kg) and a Wistar rat aorta model (average weight: 250 g). The canine liver was abraded with trephine in laparotomy and the rat aorta was clamped and punctured using a 23-gage needle. A PBS of PNIPAM–gelatin (concentration: 20 w/v%) was coated on the bleeding spot. The efficacy of hemostasis was determined by gross observation.

3. Results

3.1. PNIPAM–HA

When rat fibroblasts were seeded and cultured on PNIPAM–HA film, cast from their aqueous solution, a markedly reduced adhesion and suppressed spreading (mostly round shape) were observed (Fig. 1), indicating that PNIPAM–HA is a non-cell-adhesive matrix.

The efficacy of the PNIPAM–HA film for tissue adhesion prevention was evaluated using a rat cecum abrasion model [1,4]. When a PBS solution of PNIPAM–HA was coated on a rat cecum, an opaque PNIPAM–HA precipitate was immediately formed around the cecum at body temperature. One week after coating, ceca without PNIPAM–HA coating strongly adhered to adjacent tissues (Figs. 2 and 3). When the adhesion incidence of each rat was scored according to the scoring

rate described in Section 2, the average overall score (4: severe adhesion, 0: non-adhesion) was 2.2 ± 0.7 ($n = 9$) and the experimental sample number over score 2, which shows tissue adhesion, was counted as eight out of nine rats examined (Table 1). On the other hand, the reduced adhesion of the cecum to adjacent tissues was observed, although the PNIPAM–HA-treated ceca weakly adhered to adjacent tissues (Figs. 2 and 3). The average overall score was 1.3 ± 0.5 ($n = 8$). The experimental sample number over score 2 was two out of eight rats examined (Fig. 2 and Table 1). These results indicate that the in situ formed PNIPAM–HA precipitate significantly reduced the degree of adhesion and occurrence of tissue adhesion of the rat cecum to adjacent tissues.

3.2. PNIPAM–gelatin

A PBS solution of PNIPAM–gelatin (20 w/v%) was coated on the bleeding spots generated by pricking a canine liver and a rat aorta with a needle. The solution was immediately converted to an elastic hydrogel on the

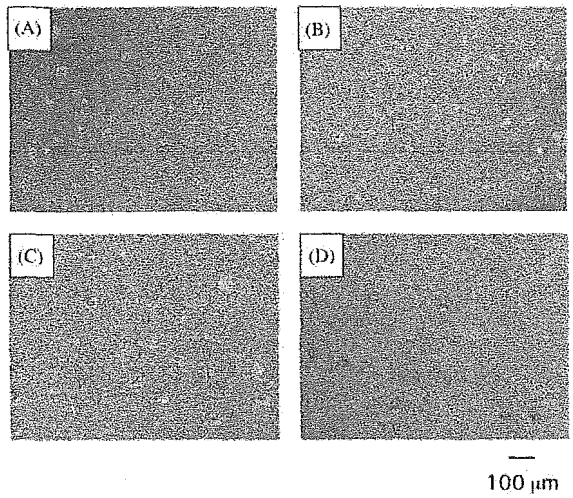

Fig. 1. Phase-contrast micrographs of rat fibroblasts (seeding density: 2.0×10^4 cells/ml) on glass (A)(C), PNIPAM–HA (B)(D) surfaces at 37°C immediately (A)(B) or after 3-h incubation (C)(D).

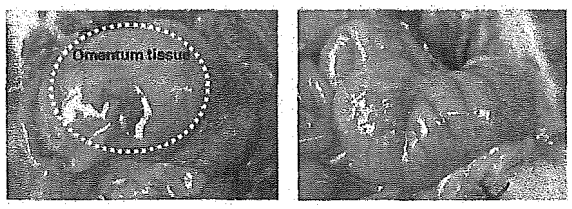

Fig. 2. Gross observation of PNIPAM–HA-treated cecum adhering to adjacent tissues one week after coating. Left: Non-PNIPAM–HA-treated cecum where omentum tissue adhered to and covered the injured cecum. Right: PNIPAM–HA-treated cecum without tissue adhesion.

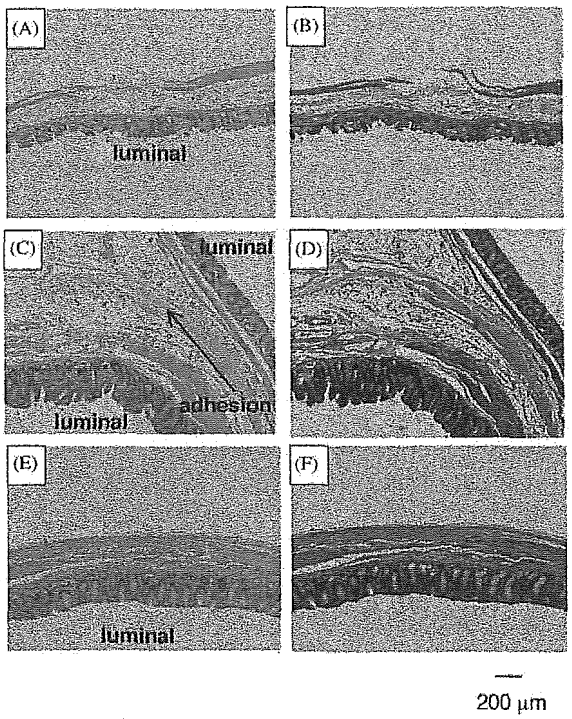

Fig. 3. Histological analysis of PNIPAM–HA-treated cecum adhering to adjacent tissues. Specimens were stained with H&E (A)(C)(E) and Masson's trichrome (B)(D)(F). PNIPAM–HA-treated cecum: (A) and (B), immediately after covering (E) and (F), 7 days after coating. Non-PNIPAM–HA-treated cecum: (C) and (D), 7 days after coating.

Table 1
Adhesion score of rat cecum

Sample	Adhesion score[a]	n	Ratio ≥score 2
Non-treated	2.2 ± 0.7	9	8/9
PNIPAM-HA-treated	1.3 ± 0.5	8	2/8

[a] Adhesion scores are as follows: 0: No cecum adhesions, 1: Firmly adhesion with easily dissectable plane, 2: Adhesion with dissectable plane causing mild tissue trauma, 3: Fibrous adhesion with difficult tissue dissection, 4: Fibrous adhesion with nondissectable tissue planes.

bleeding spots (Fig. 4). The hydrogel weakly adhered to and covered the injured sites, resulting in hemostasis, which was completed within a minute after coating (Fig. 4). Pulsation was maintained, and no bleeding was observed within the experimental time observed (1–2 h after application).

4. Discussion

Fundamental requirements for "ideal" wound-healing materials are as follows: (1) viscous liquid form to

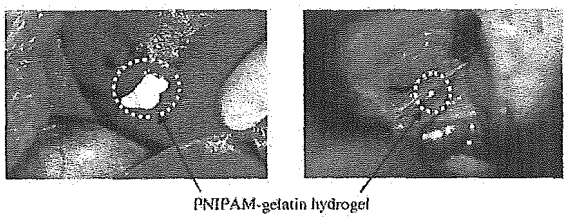

Fig. 4. Hemostases of bleeding from canine liver (left) and rat aorta (right) covered with PNIPAM–gelatin solution (20 w/v%). Spontaneous hydrogel formation and hemostases were observed. No re-bleeding was observed during 1-h after coating. Pulsatile-induced periodic dilation/contraction was observed on the dissected rat aorta.

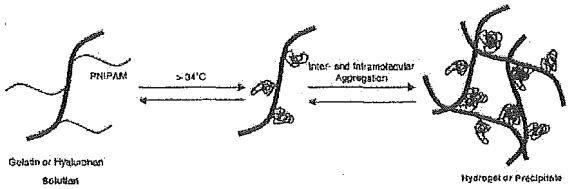

Fig. 5. Thermoresponsive gelation mechanism of PNIPAM–HA and PNIPAM–gelatin. Due to the dehydration of the hydrated amide group above lower critical solution temperature (LCST), PNIPAM graft chains were precipitated to form multimolecular aggregates. At high concentrations of PNIPAM-grafted biomacromolecules, the entire solution gelled to produce an opaque hydrogel and at low concentrations, a white precipitate was obtained.

completely cover complex-shaped tissue surfaces, (2) rapid formation of a swollen gel-like film or a precipitate when applied to injured tissues, (3) non-cell adhesiveness for tissue adhesion prevention and cell adhesiveness for hemostatic aids, and (4) appropriate biodegradability. That is, a wound-healing material should be biodegraded and sorpted with normal healing. Although various approaches and attempts have been carried out to meet the requirements listed above, in situ-applicable liquid-type materials, which include cyanoacrylate and fibrin glue, are limited (the major drawbacks associated with these hemostatic aids are described in Section 1) [7–9]. In this article, we applied thermoresponsive biomacromolecules (PNIPAM–HA and PNIPAM–gelatin) as wound-healing materials to meet the target requirements as listed above.

On a PNIPAM–HA film, cast from its aqueous solution at room temperature, the adhesion and spreading of fibroblasts were markedly inhibited (Fig. 1), which is due to the very highly swollen gel-like structure of HA (Fig. 5). When such a solution was applied to a rat cecum tissue, a slightly opaque precipitate was spontaneously formed. One week after application, markedly reduced adhesion of the PNIPAM–HA-treated cecum to adjacent tissues was observed while the non-treated cecum adhered to adjacent tissues, producing collagenous tissues (Fig. 3 and Table 1). Thus, in situ swollen precipitate of PNIPAM–HA effectively functioned in preventing tissue adhesion between the cecum and adjacent tissues.

As for thermoresponsive hemostatic aids, our previous study showed that PNIPAM–gelatin served as an artificial extracellular matrix material: the aqueous solution of PNIPAM–gelatin was immediately converted to a hydrogel, in which cells can adhere and proliferate. When PNIPAM–gelatin-containing PBS was coated on the bleeding sites of the canine liver and rat aorta, the solution was immediately converted to an opaque elastic hydrogel (Fig. 4). The hydrogel fully covered and weakly adhered to the bleeding sites, resulting in complete hemostasis on both tissues. The PNIPAM–gelatin-coated aorta pulsated well, indicating that the PNIPAM–gelatin formed quite elastic hydrogel and the hydrogel did not interfere with the periodic pulsation of a high-pressure circulatory system.

5. Conclusion

Although this article describes very limited experiments on tissue adhesion control using PNIPAM–HA and PNIPAM–gelatin, the bioconjugation of thermoresponsive synthetic materials and extracellular-matrix derived biomacromolecules, which thermally form precipitate or hydrogel, can provide a new prototype of wound-healing materials and promising procedures. Further studies to improve the properties required for both tissue adhesion and tissue adhesion prevention and to examine a longer term performances are needed. Such studies are ongoing in our laboratory.

Acknowledgements

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High Performance Gene Delivery Polymeric Vector: Nano-Structured Cationic Star Polymers (Star Vectors)

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Abstract: Nano-structured hyperbranched cationic star polymers, called star vectors, were molecularly designed for a novel gene delivery non-viral vector. The linear and 3, 4 or 6 branched water-soluble cationic polymers, which had same molecular weight of ca. 18,000, were synthesized by iniferter (initiator-transfer agent-terminator)-based photo-living-radical polymerization of 3-(*N,N*-dimethylamino)propyl acrylamide, initiated from respective multi-dithiocarbamate-derivatized benzenes as an iniferter. All polymers produced polyion complexes 'polyplexes' by mixing with pDNA (pGL3-control plasmid), in which the particle size was ca. 250 nm in diameter [the charge ratio < 2/1 (vevtor/pDNA)] and ca. 150 nm (the charge ratio > 2.5/1), and the ζ-potential was ca. +10 mV (the charge ratio > 1/1). When COS-1 cells were incubated with the polyplexes 12h after preparation under the charge ratio of 5/1, higher gene expression was obtained as an increase in branching, with a little cytotoxicity. The relative gene expression to the linear polymer was about 2, 5, and 10 times in 3-, 4-, and 6-branched polymers, respectively. The precise change in branching of polymers enabled the control of the gene transfer activity.

Keywords: Non-viral vector, star polymer, polyplex, branched polymer, gene transfection, molecular design.

INTRODUCTION

The cationic polymers, which can generate nano-particles by formation of polyion complexes 'polyplexes' with DNA irrespective of its size and kind, are highly expected as one of the major materials for non-viral vectors [1-4]. However, the primary obstacle toward implementing an effective gene therapy using the cationic polymers remains their relatively inefficient gene transfection *in vivo* than virus vectors.

To achieve an enhancement of gene transfection using cationic polymers, numerous studies have been performed by various approaches; e.g., the chemical synthetic engineering approach in which the kind and composition of the polymers are modified [5,6], biochemical approach in which targeting ligands such as galactose, mannose, transferring, or antibodies into the polymers [7-11], functional molecular engineering approach in which stimulus-response polymers with light and thermal reactivity are designed as high performance vectors [12-14], and physical engineering approach in which physical stimulation with electroporation, gene gun, ultrasound and hydrodynamic pressure are provided at the transfection [2,15,16]. However, few studies in the molecular structure of cationic polymers, which are usually synthesized by conventional radical polymerization, has been reported, except for the effects of changes in the polymer chain length and composition of polymers [17-20] and complex multi-branching polymers, of which structural analysis is impossible [21-24]. Since precise molecular design, including the molecular weight and three-dimensional structure, by conventional radical polymerization was quite difficult in general, the systematic structure-dependency of cationic polymers in gene transfection has not been established.

In this study, for examination of the effects of the molecular structure on gene expression we designed novel cationic polymers with star-shaped and symmetric structure, which is determined by 2-parameters, the degree of branching and chain length. Molecular design was performed by the iniferter (acts as *ini*tiator-trans*fer* agent-*ter*minator)-based photo-living-radical polymerization method pioneered by Otsu *et al.* [25-30]. An iniferter, benzyl *N,N*-diethyldithiocarbamate (DC) is dissociated into a benzyl radical and a dithiocarbamyl radical by ultraviolet light (UV) irradiation. The reaction involving an *N,N*-diethyldithiocarbamyl radical favors chain termination with a growing polymer chain radical end rather than a reaction with a vinyl monomer, whereas a benzyl radical reacts with a vinyl monomer to produce a polymer. These reactions proceed only during irradiation. Therefore, the chain length of the growing polymer is controlled by irradiation condition such as irradiation time or light intensity and the composition of the solution. We previously used the living radical polymerization for designing of various surface graft architectures [31-34] controlling the chain length, block graft chain, gradient chain length and regionally graft polymerized pattern surface. As the first step of the study, star polymers of the same molecular weight at a precise degree of branching of 0, 3, 4, and 6 were synthesized. The effects of the degree of branching on gene expression by measuring the luciferase activity were examined.

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MATERIALS AND METHODS

Materials

Benzyl chloride, 2,4,6-tris(bromomethyl)mesitylene, 1,2,4,5-tetrakis(bromomethyl)benzene, and hexakis(bromomethyl)benzene were obtained from Sigma-Aldrich (Milwaukee, WI). Sodium *N,N*-diethyldithiocarbamate and *N,N*-dimethyaminopropyl acrylamide were purchased from Wako Pure Chemical Ind. Ltd. (Osaka, Japan). Solvents and other reagents, all of which were of special reagent grade, were obtained from Wako and used after conventional purification. Plasmid DNA (pGL3-control), which contains the firefly luciferase gene, was obtained from Promega Inc., (Tokyo, Japan). ExGen 500 [poly(ethylene imine)] was obtained from Euromedex Inc., (Cedex, France).

Synthesis of Cationic Star Polymers

Cationic polymers including linear and three types of star polymers with 3, 4, or 6 branches per molecule were prepared by iniferter-based photo-living-radical polymerization of 3-(*N,N*-dimethylamino)propyl acrylamide as a monomer from respective inifeters such as benzyl *N,N*-diethyldithiocarbamate, 2,4,6-tris (*N,N*-diethyldithiocarbamylmethyl)mesitylene, 1,2,4,5- tetrakis(*N,N*-diethyldithiocarbamylmethyl)benzene, and hexakis (*N,N*-diethyldithiocarbamylmethyl)benzene, which were obtained by *N,N*-diethyldithiocarbamylation from respective benzyl halogenate derivatives such as benzyl chloride, 2,4,6-tris (bromomethyl)mesitylene, 1,2,4,5-tetrakis(bromomethyl) benzene, and hexakis(bromomethyl)benzene.

The general preparation method of iniferter is followed. An ethanol solution (10 ml) of chloromethyl benzene (4.8 g, 38 mmol) was added to an ethanol solution (50 ml) of sodium *N,N*-diethyldithiocarbamate (10.3 g, 46 mmol) at 0°C. After the mixture was stirred at room temperature for 24 h, the resulting sodium chloride was separated by filtration. The filtrate was concentrated under reduced pressure. The residue was added into 150 ml of water and extracted with ether (200 ml x 2) and washed successively with deionized water (100 ml x 3), followed the separation of the organic layer, drying over $MgSO_4$, condensation to give benzyl *N,N*-diethyldithiocarbamate: yield, 17.6g (93%); ^{1}H NMR (DMSO-d_6 with Me_4Si) δ 7.34 (m, 5H, C_6H_5), 4.54 (s, 2H, CH_2-S), 4.05 (q, 2H, N-CH_2), 3.73 (q, 2H, N-CH_2), 1.28 (m, 6H, CH_2CH_3).

The general procedure of iniferter-induced photo-living-radical polymerization is followed. A methanol solution (20 ml) of benzyl *N,N*-diethyldithiocarbamate (24 mg, 0.1 mmol) and 3-(*N,N*-dimethylamino)propyl acrylamide (3.9 g, 25 mmol) was placed into 50 ml quartz crystal tube. A stream of dry nitrogen was introduced through a gas inlet to sweep the tube for 5 min or more. The solution was then irradiated for 30 min with a 200 W Hg lamp (SPOT CURE, USHIO, Tokyo, Japan) in nitrogen atmosphere at 20~25 °C. Light intensity was set to 1 mW/cm^2 at the wavelength of 250 nm (UVR-1, TOPCON, Tokyo, Japan). The reaction mixture was concentrated under reduced pressure. The residue was dissolved in a small amount of methanol. The precipitate, obtained by the addition of a large amount of ether, was separated by filtration. Reprecipitation was performed in the methanol-ether system. The last precipitate was dried in a vacuum to yield poly[3-(*N, N*-dimethylamino)propyl acrylamide] as a white powder. The molecular weight, determined by GPC analysis, was 18,000 g mol^{-1}. ^{1}H NMR (DMSO-d_6 with Me_4Si) δ 7.60 (br, 1H, N-*H*), 3.22 (br, 2H, NH-CH_2), 2.30 ((br, 2H, $N(CH_3)_2$-CH_2), 2.15 (br, 6H, N-CH_3), 1.65 (br, 2H, CH_2-CH_2).

General Methods

GPC analysis was carried out on a RI-8012 (TSK_{gel} α-3000 and α-5000; Tosoh, Tokyo, Japan) after calibration with standard polyethylene glycol samples. The eluent was *N,N*-dimethylformamide. ^{1}H-NMR spectra were obtained on a Valian Gemini-300 (300 MHz) spectrometer (Tokyo, Japan). All ^{1}H-NMR spectra were recorded in DMSO-d_6 solutions using tetramethylsilane as the internal standard. Dynamic light scattering (DLS) measurements were carried out using a DLS-8000 instrument (Otsuka Electrinics, Tokyo, Japan). An Ar ion laser (λ_0 = 488 *nm*) was used as the incident beam. The sample was prepared by direct mixing of pDNA solution and the polymer in Tris-HCl buffer (pH 7.4). The DNA concentration of the mixture was then adjusted to 23 μg cm^{-3}.

Cell Culture and Transfection

COS-1 cells (ca. 3 x 10^4 cells per well) were seeded prior to treatment in 24-well plates and grown for 24 h in DMEM (Gibco, Invitrogen Corp., Carlsbad, CA) containing 10% fetal calf serum (Hyclone Laboratories Inc., Logan, UT), penicillin (200 units/ml, ICN Biomedicals Inc., Aurora, OH), and streptomycin (200 mg/ml, ICN) in an atmosphere of 5% CO_2 at 37 °C. Transfections were performed with 0.5 μg of plasmid DNA (pGL3-control) in 24-multi well dishes in 0.2 ml of OPTI-MEM I (Gibco). After 3 h of incubation, the cells were washed once with PBS, and cultured in 1 ml of DMEM containing 10% fetal calf serum for an additional 48 h. The medium was removed and the cells were washed twice with PBS. The cells were lysed with 0.2 ml of cell lysis buffer (Promega, Madison, WI) and mixed by vortexing. The lysate was centrifuged at 15,000 rpm for 1 min at 4 °C and 5 μl of the supernatant was analyzed for luciferase activity using a Luminous CT-9000D (Dia-Iatron, Tokyo, Japan) luminometer. The relative light unit/s (RLU) were converted into the amount of luciferase (pg) using a luciferase standard curve, which was obtained by diluting recombinant luciferase (Promega) in lysis buffer. The protein concentrations of cells lysates were measured by Bio-Rad protein assay (BIO-RAD, Hercules, CA) using bovine serum albumin as a standard. The expressed luciferase represented the amount (mole quantity), which is standardized for total protein content of cell lysate. The data are presented as means±S.D. (n=5).

Cytotoxicity

Cytotoxicity was assessed by cell viability assay using WST-8 method (Dojindo, Kumamoto, Japan). COS-1 cells were seeded 24 h prior to treatment in 96-well plates at 5,000 cells per well. Cells were treated with the same conditions used for luciferase assays, with a volume of 6.2 μl of the transfection mixture including 0.124 μg of pDNA added to