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Biological responses of novel high-toughness double network hydrogels in muscle and the subcutaneous tissues

Yoshie Tanabe · Kazunori Yasuda · Chinatsu Azuma · Hiroko Taniguro · Shin Onodera · Akira Suzuki · Yong Mei Chen · Jian Ping Gong · Yoshihito Osada

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Abstract The study evaluated biological reaction of four types of novel double network gels in muscle and subcutaneous tissues, using implantation tests according to the international guideline. The implantation tests demonstrated that, although poly (2-acrylamide-2-metyl-propane sulfonic acid)/poly (*N*,*N*′-dimetyl acrylamide) (PAMPS/PDMAAm) gel induced a mild inflammation at 1 week, the degree of the inflammation significantly decreased into the same degree as that of the negative control at 4 and 6 weeks. This gel has a potential to be applied as artificial cartilage. In addition, Cellulose/Gelatin gel showed the same degree of inflammation as that of the negative control at 1 week, and then, showed a gradually absorbable property at 4 and 6 weeks. This gel has a potential to be applied as an absorbable implant. The PAMPS/polyacrylamide and Cellulose/PDMAAm gels induced a significant inflammation at each week. These DN gels are difficult to be applied as clinical implants in the current situation.

Y. Tanabe · K. Yasuda (✉) · C. Azuma · H. Taniguro · S. Onodera
Department of Sports Medicine and Joint Reconstruction Surgery, Graduate School of Medicine, Hokkaido University, Kita-15 Nishi-7, Kita-ku, Sapporo 060-8638, Japan
e-mail: yasukaz@med.hokudai.ac.jp

A. Suzuki
Department of Pathology, Graduate School of Medicine, Hokkaido University, Sapporo, Japan

Y. M. Chen · J. P. Gong · Y. Osada
Division of Biological Sciences, Graduate School of Science, Hokkaido University, Sapporo, Japan

1 Introduction

Normal cartilage tissue is a kind of multi-functional hydrogel with high toughness. Commonly, hydrogel materials consist of three-dimensional hydrophilic polymer network in which a large amount of water is interposed. However, most of the artificial hydrogel materials are suffered from a lack of strength and toughness. Since 1992, we have developed novel methods to obtain double network (DN) hydrogels with excellent properties concerning strength and toughness [1–5]. The DN hydrogels were synthesized by a two-step sequential method of polymerization. The stiff polymer network was formed at first, after that, the second component was induced to the first network by diffusion. By polymerizing the second component, the DN hydrogel was formed. Therefore, the DN hydrogel is comprised of two independently cross-linked networks that are physically entangled with each other. There is no micro-phase separation between the two components. The dynamic light scattering analysis on the DN gel has shown a bimodal structure of the gel, which consists of the first densely cross-linked stiff domain as the hard phase and the second flexible domain as the soft phase [6]. This bimodal structure is considered to dramatically toughen the DN gels [7]. Therefore, the structure and the mechanical toughness of the DN hydrogel are completely different from that of the common interpenetrated polymer networks [8, 9] or a fiber-reinforced hydrogel, which is only a linear combination of two component networks.

Recently, the authors developed four types of unique DN hydrogels as potential materials for artificial cartilage: The first gel is PAMPS/PDMAAm DN gel, which consists of poly (2-acrylamide-2-metyl-propane sulfonic acid) and poly (*N*,*N*′-dimetyl acrylamide). The second gel is PAMPS/PAAm DN gel, which consists of poly

(2-acrylamide-2-metyl-propane sulfonic acid) and polyacrylamide. The third gel is Cellulose/PDMAAm DN gel, which is composed of bacterial cellulose and poly-dimetylacrylamide. The forth gel is Cellulose/Gelatin DN gel, which consists of bacterial cellulose and gelatin. Specifically, the PAMPS/PDMAAm DN gel has an amazing wear property as a hydrogel that is comparable to the UHMWPE in pin-on-flat-type wear testing [10], and this gel is hardly degraded when it is implanted into a living body [11]. In addition, the PAMPS/PAAm DN gel exhibits a high degree of ultimate strength with about 20 MPa, and shows an extremely low friction coefficient of approximately 0.01 [1, 12], which is comparable to the coefficient value of human cartilage [13, 14]. Cellulose/PDMAAm and Cellulose/Gelatin DN gels are unique gels that include natural polymers. Then, evaluation must be made as to what kind of tissue reaction is induced by implantation of these gel materials in the living body and how strong the degree of the reaction is, in order to consider application of these DN gels to clinical implants. However, no studies on biological tissue reaction of the implanted DN gels have been reported as of yet.

To answer the above-described questions, the pellet implantation test into the muscle and the subcutaneous implantation test with a massive material, which can evaluate local reaction in the muscle or the subcutaneous tissue around the implanted material, are recommended [15]. Therefore, we have conducted this study composed of macroscopic observations performed in the subcutaneous implantation test with a massive material and histological examinations carried out in the pellet implantation test into the muscle. The purpose of this study is to evaluate any biological reaction of the PAMPS/PDMAAm, PAMPS/PAAm, Cellulose/PDMAAm, and Cellulose/Gelatin DN gels implanted into the para-vertebral muscle and the subcutaneous tissue, using the biomaterial implantation test with the rabbit according to the international guideline for biological evaluation of the safety of biomaterials [15].

2 Materials and methods

2.1 Materials

2-Acrylamido-2-methylpropanesulfonic acid (AMPS) (Tokyo Kasei Co., Ltd, Tokyo, Japan), as a monomer, was used as received. *N*,*N*′-Dimetyl acrylamide (DMAAm) (Kojin Co., Ltd., Tokyo, Japan), as a monomer, was purified by distillation under reduced pressure before usage. Acrylamide (AAm) (Junsei Chemical CO., Ltd, Tokyo, Japan), as a monomer, was recrystallized from pure chloroform before use. *N*,*N*′-Methylenebisacrylamide (MBAA) (Tokyo Kasei Co., Ltd), as a cross-linking agent, was recrystallized twice from pure ethanol before use. 2-oxoglutaric acid (Wako Pure Chemical Industries, Ltd., Tokyo, Japan), as a UV initiator, was used as received. Potassium persulfate, as an initiator, was recrystallized before use. 1-Ethyl-(dimethylaminopropyl) carbodiimide (EDC) (Tokyo Kasei Co., Ltd, Tokyo, Japan), as a coupling agent, was used as received. Gelatin powder from calf-skin was purchased from Junsei Chemical Co., Ltd., and used without any further purification.

2.2 Gel preparation

2.2.1 Synthesis of first network hydrogel

The PAMPS gel, as the first network of DN gel, was obtained by radical polymerization using MBAA as a cross-linker and 2-oxoglutaric acid as an initiator. Monomer concentration was 1 mol/L, cross-linker was 4 mol% with respect to the monomer concentration, and initiator was 0.1 mol% with respect to the monomer concentration. Aqueous solution containing a monomer, cross-linker, and the initiator was bubbled with nitrogen for 30 min, and then injected into a cell consisting of a pair of glass plates separated by a silicon rubber. The cell was irradiated with a UV lamp (wave length 365 nm) about 6 h.

Bacterial cellulose (BC) is an extracellular cellulose, produced from a bacterium *Acetobacter* consisting of hydrophobic ultra-fine fiber network stacked in a stratified structure [16]. *Acetobacter xylinum* (ATCC 53582) was cultured in Hestrin-Schramm medium at pH 6.0, and incubated at 28° C for 10 days. A disc-shaped BC about 10 mm in thickness and 50 mm in diameter obtained after cultivation was washed with 1% aqueous NaOH solution to remove alkali-soluble components, followed by water washing for a prolonged period at room temperature. Detailed description is given in the references [17]. The purified BC gel was immersed in a second network polymer solution for the preparation of DN gel.

2.2.2 Synthesis of double network (DN) hydrogel

PAMPS/PDMAAm and PAMPS/PAAm DN gels: The double network (DN) hydrogel was synthesized by the sequential network formation technique (two-step method). The PAMPS gel (1st network) was immersed in an aqueous solution of 3 M DMAAm, containing 0.1 mol% MBAA, and 0.1 mol% potassium persulfate for 1 day until reaching equilibrium. The 2nd network (PDMAAm) was subsequently polymerized in the presence of the PAMPS gel at 60° C for 6 h between two glass plates. After polymerization, the PAMPS/PDMAAm DN gel was immersed in

pure water for 1 week and the water was changed two times every day to remove any un-reacted materials. PAMPS/PAAm DN gel was synthesized in the same procedure as that of PAMPS/PDMAAm DN gel.

Cellulose/PDMAAm gel: The purified BC gel was immersed in an aqueous solution of 3 M DMAAm containing 0.1 mol% MBAA, and 0.1 mol% potassium persulfate for one day until reaching equilibrium. The 2nd network (PDMAAm) was subsequently polymerized in the presence of the cellulose gel at 60° C for 6 h between two glass plates. After polymerization, the obtained PAMPS/PDMAAm DN gel was immersed in pure water for 1 week and the water was changed two times every day to remove any un-reacted materials.

Cellulose/gelatin gel: The purified BC gel was immersed in an aqueous solution of gelatin (pH 7.0) at 50° C for 1 week and then immersed in an EDC aqueous solution for 4 days at room temperature to make a chemical cross-linking reaction. The concentration of gelatin was 30 wt% and its pH was adjusted by 3-(N-morpholino) propane sulfonic acid (MOPS). The EDC concentration was 1.0 M. After gelation of gelatin with EDC, samples were washed with a large amount of deionized water for 1 week at room temperature to equilibrate and wash away any residual chemicals [18].

2.3 Animal experimentation

Animal experiments were carried out in the Institute of Animal Experimentation, Hokkaido University School of Medicine under the Rules and Regulation of the Animal Care and Use Committee, Hokkaido University School of Medicine. A total of 20 mature female Japanese white rabbits, 4 months of age and weighing 3.3 ± 0.3 kg, were used. Implantation test was composed of implantation of a small gel pellet into the para-vertebral muscle and implantation of a massive gel block into the subcutaneous tissue, according to the guideline for biological evaluation of the safety of biomaterials, which had been published by the Ministry of Health, Labour and Welfare, Japan [15]. Surgery was performed under anesthesia (intravenous injection of pentobarbital, 25 mg/kg). After surgery, each animal was permitted to move freely within their cage (310 mm in width, 550 mm in length, 320 mm in height).

2.4 Pellet implantation test into the para-vertebral muscle

A total of 90 pellets, rectangular parallelepiped specimens (1 × 1 × 10 mm), were created with each DN gel material. As for negative and positive controls for histological comparisons, high-density polyethylene (Hatano Research Institute, Food and Safety Center, Hatano, Japan) and polyurethane containing 0.75% Zinc diethyldithiocarbamate (Hatano Research Institute) were used, and the same shaped pellets were prepared. Each material immersed in water was sterilized in an autoclave of 120° C for 20 min. Our previous study showed that this sterilization method did not change any properties of the materials used in this study [11].

A total of 15 rabbits were used. In each animal, six small skin incisions of 5 mm were made at the back, avoiding injury of the fascia of the para-vertebral muscle. Three of the six incisions were located on the right side, and the remaining three incisions were located on the left side. The distance between the two skin incisions was 2.5 cm or more. Each pellet specimen was pushed into the tip of a needle having a number-15 diameter (Fig. 1A). Then, the needle was inserted into the para-vertebral muscle penetrating the fascia so that the needle was inclined 60° to the fascia (Fig. 1B). Then, the pellet specimen was pushed out from the needle tip into the muscle with a stylet inserted into the needle (Fig. 1C). In each rabbit, six kinds of pellet specimens were randomly implanted in the six portions of the muscle. After the skin incisions were closed with 3-0 nylon sutures, the wound was coated with a spray-bandage (Nobecutane Spray, Welfeid, Tokyo, Japan). Five animals were sacrificed at 1, 4, and 6 weeks after implantation, respectively, by injecting a lethal dose of pentobarbital. The right and left para-vertebral muscles, in which the pellet specimens were implanted, were carefully harvested *en block*.

Each whole muscle was dissected into three sub-block specimens where three different pellet specimens were implanted respectively. Each specimen was fixed in 10% neutral buffered formalin. After fixation, the specimen was embedded in paraffin, and sliced with a microtome along the longitudinal axis of the implanted pellet. The consecutive 10 sections were stained with hematoxylin and eosin,

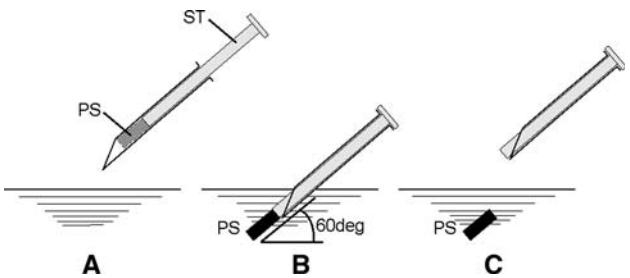

Fig. 1 How to implant a pellet specimen (PS) in to the muscle. Each pellet specimen was pushed into the tip of a needle having a number-15 diameter (**A**). Then, the needle was inserted into the para-vertebral muscle penetrating the fascia so that the needle was inclined 60° to the fascia (**B**). Then, the pellet specimen was pushed out from the needle tip into the muscle with a stylet (ST) inserted into the needle (**C**)

and observed with light microscopy (XF-15; Nikon, Tokyo, Japan).

To quantify the degree of inflammatory response of the muscle to the implanted materials, we took a photograph that showed a cross-section of the muscle. A cross-section of the gel specimen was oval-shaped or rectangular. We drew the longest and shortest axes or widths of the cross-sections (Fig. 2). On four parts on these two lines, we measured the width of the reactive inflammatory zone against the pellet specimens, according to the guideline [15]. Then, we revised the width value of the reactive inflammatory zone on the longest axis or width of the gel specimen with a calculation based on estimation that the oval-shaped or rectangular cross-sections of the gel specimens were obtained by cutting a cylindrical or square parallelepiped rod in a plane inclined by certain degrees to an completely axial section, which was a circle or a square. An average of the four revised width values was defined as the width of the reactive inflammatory zone in each section. Then, the mean of three sections randomly selected out of the 10 sections on each pellet specimen was used as a representative value of the reactive inflammatory zone width on each pellet from each animal.

2.5 Subcutaneous implantation test of massive DN gel specimens

Concerning each DN gel material, five rectangular parallelepiped specimens ($10 \times 10 \times 5$ mm) were prepared. Five mature female Japanese White rabbits were used. Four longitudinal skin incisions of 3 cm were made at the back. Two of the four incisions were located on the right side, and the subcutaneous tissue was then undermined from each incision to the right direction along with the fascia of the back muscles so that two square subcutaneous spaces (3×3 cm) were made. The remaining two incisions were located on the left side, and two subcutaneous spaces (3×3 cm) were made from each incision to the left direction using the same procedure. The distance between the four subcutaneous spaces was 8 cm or more. In each rabbit, four kinds of DN gel specimens were randomly implanted in the four subcutaneous spaces. The subcutaneous tissue and the skin were closed with 3-0 nylon sutures. At 6 weeks after surgery, each animal was sacrificed by injecting a lethal dose of pentobarbital. Macroscopic situations of the implanted massive gel specimens in subcutaneous tissues were observed in careful dissection. Then, the tissues surrounding the gel specimens were histologically examined in the same manner as used in the implantation tests into the muscle.

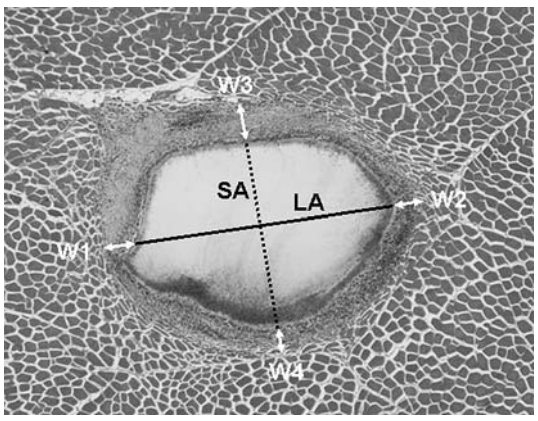

Fig. 2 Measurement of the width (W1–W4) of the reactive inflammatory zone against the pellet specimens. LA: the longest axis, SA: the shortest axis

2.6 Statistical analysis

All data were described as the mean and standard deviation values. For statistical comparisons on the biodegradation in each parameter of each DN gel material, we used the one-way analysis of variance (ANOVA). The significance limit was set at $p = 0.05$. For the statistical calculation, commercially available software (Stat View, Abacus Ontent Inc, Berkeley, CA, USA) was used. A significance level was set at $p = 0.05$ for each test.

3 Results

3.1 Pellet implantation test into the para-vertebral muscle

In each rabbit, the skin incision was well healed without any infection. At the time of sacrifice, the body weight (3.3 ± 0.5 Kg at 1 week, 3.7 ± 0.2 at 4 weeks, and 3.3 ± 0.5 Kg at 6 weeks) was unchanged as compared to that before surgery (3.2 ± 0.4 Kg). In macroscopic observation, we could not observe any signs of inflammation or abscess formation on the back.

The high-density polyethylene pellet as the negative control was surrounded by fibrous tissue including fibroblasts, macrophages, lymphocytes and a few neutrophils without any muscle necrosis at 1 week (Fig. 3A), and the pellet was surrounded by thin fibrous tissue with a small numbers of inflammatory cells at both 4 and 6 weeks (Fig. 5A, 7A). The width of the above-described inflammatory zone was 0.08 ± 0.05 mm at 1 week and 0.03 ± 0.03 mm at 6 weeks. On the other hand, around the polyurethane pellet containing zinc diethyldithiocarbamate

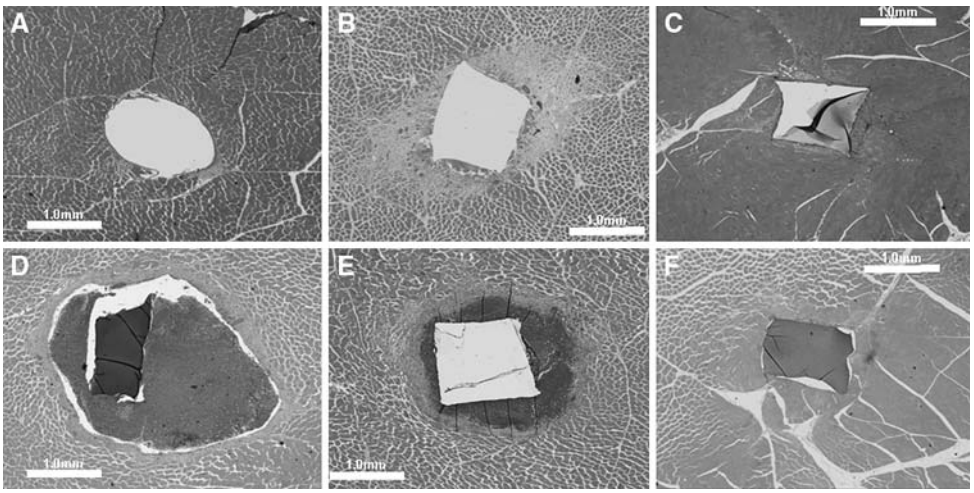

Fig. 3 Histological observations at 1 week (HE stain, ×2). (**A**) Negative control, (**B**) Positive control, (**C**) PAMPS/PDMAAm gel, (**D**) PAMPS/PAAm gel, (**E**) Cellulose/PDMAAm gel, (**F**) Cellulose/Gelatin gel

as the positive control, many granulocytes infiltrated and many of these cells were necrotized. A number of muscle cells in this area were also necrotized. This area was surrounded by thick fibrous tissue including various inflammatory cells, such as macrophages, neutrophils, lymphocytes (Fig. 3B). At 4 weeks, the number of infiltrating granulocytes was reduced, while this inflammatory zone was surrounded by the fibrous tissue including fibroblasts, macrophages, lymphocytes and eosinophils (Fig. 5B). At 6 weeks, the pellet was surrounded by a thick fibrous capsule with some lymphocytes (Fig. 7B). The width of the inflammatory zone was 0.34 ± 0.09 mm at 1 week and 0.08 ± 0.05 mm at 6 weeks.

The implanted PAMPS/PDMAAm, PAMPS/PAAm, and Cellulose/PDMAAm DN gels induced various degrees of inflammation essentially similar to that around the positive control at 1 week (Fig. 3C, D, E). Namely, the gel was surrounded by numerous neutrophils with necrotic cells, and this zone was surrounded by fibrous tissues in which a number of macrophages including foreign body giant cells and fibroblasts with some eosinophils (Fig. 4). The inflammation and the muscle necrosis around the gel was obviously milder around the PAMPS/PDMAAm gel than around the positive control, but even stronger around the PAMPS/PAAm (Fig. 3D) and Cellulose/PDMAAm gels than around the positive control (Fig. 3E). At 4 and 6 weeks, the degree of inflammation decreased around the three gels. Specifically around the PAMPS/PDMAAm gel, neutrophils disappeared and a thin fibrous capsule was formed (Fig. 5C, 7C). In contrast, around the PAMPS/PAAm (Fig. 5D, 7D) and Cellulose/PDMAAm (Fig. 5E, 7E) gels, some neutrophils still remained with necrosis and the inflammatory zone was surrounded by thick fibrous tissue with infiltration of macrophages with some lymphocytes. The ANOVA demonstrated that the width of the inflammatory zone around the PAMPS/PDMAAm gel was significantly less than that around the positive control at

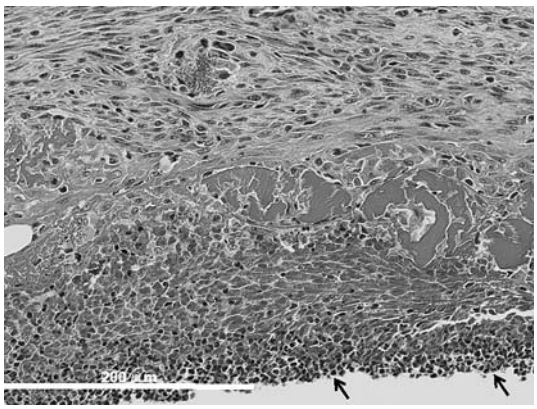

Fig. 4 Inflammation and muscle necrosis around the PAMPS/PAAm observed at 1 week (HE stain, ×20). Two arrows show an interface between the gel specimen and surrounding tissues

1 week, although it was significantly greater than that around the negative control. On the other hand, the width of the inflammatory zone around the PAMPS/PAAm and Cellulose/PDMAAm gels was significantly greater than that around the positive control at 1 week (Fig. 8). At 4 and 6 weeks, the width around the PAMPS/PDMAAm gel was the same as that of the negative control, while the width was the same around the Cellulose/PDMAAm gel as that of the negative control and significantly greater around the PAMPS/PAAm gel than that of the negative control (Fig. 8).

The reactive inflammation around the implanted Cellulose/Gelatin gel was obviously different from the area surrounding the other three gels. At 1 week, neutrophil infiltration or cell necrosis was rarely seen around the pellet, which was surrounded by a small amount of fibrous tissue with infiltration of many macrophages (Fig. 3F). At 4 and 6 weeks, conversely, the inflammatory zone around the gel became thicker than that observed at 1 week. In this area, foreignbody reaction that involved numerous

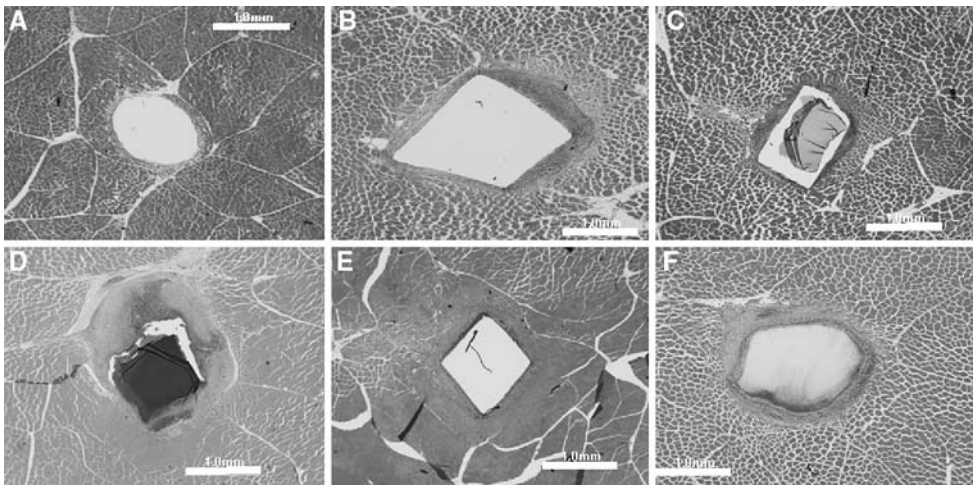

Fig. 5 Histological observations after 4 weeks (HE stain, ×2). (**A**) Negative control, (**B**) Positive control, (**C**) PAMPS/PDMAAm gel, (**D**) PAMPS/PAAm gel, (**E**) Cellulose/PDMAAm gel, (**F**) Cellulose/Gelatin gel

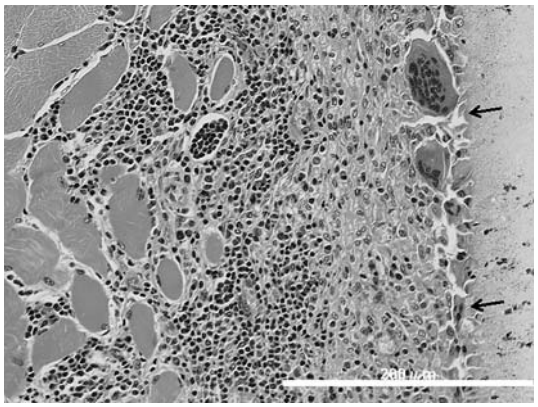

Fig. 6 Histological findings around the Cellulose/Gelatin gel observed at 4 weeks (HE stain, ×20). Numerous lymphocytes, plasma cells, and macrophages including foreignbody macrophages were observed. Two arrows show an interface between the gel specimen and surrounding tissues

lymphocytes, plasma cells, and macrophages including foreignbody macrophages, and some eosinophils were observed in this zone (Figs. 5F, 6, 7F). The ANOVA showed that the inflammatory zone width around the Cellulose/Gelatin gel was significantly less than the positive control at 1 week ($p < .0001$), while there was no significant difference from the negative control. At 4 and 6 weeks, conversely, the width became significantly greater than the negative control (4w: $p = .0158$, 6w: $p = .0443$), while there was no significant difference from the positive control (Fig. 8).

3.2 Subcutaneous implantation test with massive DN gel specimens

In each rabbit, the skin incision was well healed without any infection at 6 weeks. At the time of sacrifice, the body weight (3.5 ± 0.3 Kg) was unchanged, compared to that before surgery (3.2 ± 0.4 Kg). The skin had prominence due to each implanted gel. In macroscopic dissection, there were no inflammatory signs such as redness of the skin, effusion, or abscess formation. The PAMPS/PDMAAm DN gel specimens were surrounded by a thin fibrous capsule, while the PAMPS/PAAm and Cellulose/PDMAAm DN gel specimens were surrounded by a fibrous capsule thicker than that around the PAMPS/PDMAAm DN gel specimens. The Cellulose/gelatin DN gel specimens were surrounded by the thickest fibrous capsule. When the capsule was removed, a part of the specimens became absorbed.

In histological observations, the PAMPS/PDMAAm gel was surrounded by a thin fibrous capsule without any inflammatory cells, while the PAMPS/PAAm and Cellulose/PDMAAm gels were surrounded by fibrous tissues including many granulocytes, lymphocytes, and monocyte/macrophage-like cells. The Cellulose/gelatin DN gel specimens were surrounded by fibrous tissues with numerous lymphocytes, plasmacells, and eosinophils.

4 Discussion

Macroscopic observations in this study showed that implantation of the PAMPS/PDMAAm, PAMPS/PAAm, Cellulose/PDMAAm, and Cellulose/Gelatin DN gels into the muscular and subcutaneous tissues did not affect the health or the life of the animals, or did not induce drastic local effects including abscess formation, tissue-lysis, broad inflammation in the whole limb, and so on, even when a massive gel block was implanted. The results of histological examinations in the two implantation tests were essentially similar in spite of the differences in the surrounding tissue and the material size. Histological examinations in the pellet implantation test demonstrated that the implanted PAMPS/PDMAAm DN gel induced the

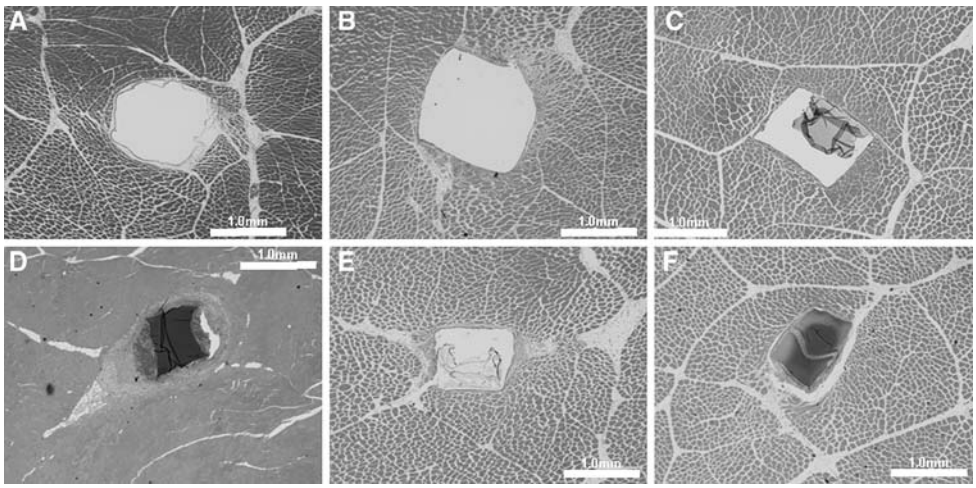

Fig. 7 Histological observations at 6 weeks (HE stain, ×2). (**A**) Negative control, (**B**) Positive control, (**C**) PAMPS/PDMAAm gel, (**D**) PAMPS/PAAm gel, (**E**) Cellulose/PDMAAm gel, (**F**) Cellulose/Gelatin gel

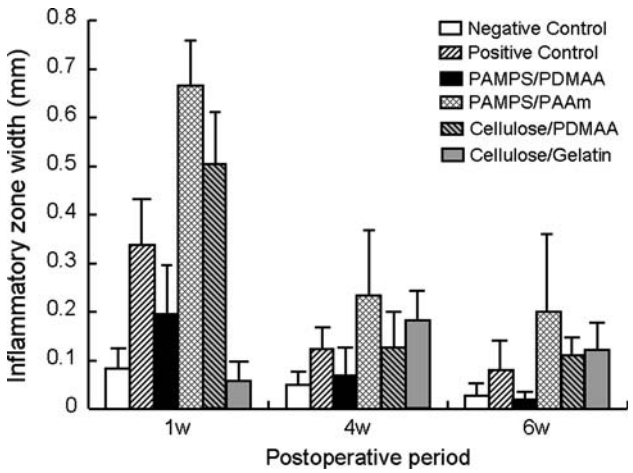

Fig. 8 Comparisons of the inflammatory zone width at 1, 4, and 6 weeks. Concerning the statistical comparisons between two width values, see Table 1

inflammation significantly less than that of the positive control at 1 week, although it was significantly greater than that of the negative control. In addition, the degree of the inflammation around the implanted PAMPS/PDMAAm DN gel significantly decreased into the same degree as that of the negative control at 4 and 6 weeks. However, the implanted PAMPS/PAAm and Cellulose/PDMAAm DN gels induced the inflammation significantly greater than the positive control at 1 week, and showed the same degree of inflammation as that of the positive control at 4 and 6 weeks. On the other hand, although the implanted Cellulose/Gelatin DN gel showed the same degree of inflammation as that of the negative control at 1 week, the inflammation increased and became the same degree as that of the positive control at 4 and 6 weeks.

It is well known that foreign body reaction in living body consists of a series of biological reaction. Namely, inflammatory cells infiltrated around a foreign body in the early phase, and, then, the foreign body is surrounded by granulation tissue and/or fibrous capsule in the late phase. The histological reactions around the four DN gels examined in the present study were essentially similar to the above-described foreign body reaction, although the degree of the reaction was different among the implanted materials. Although a few studies tried to microscopically analyze types of the cells infiltrating around an implanted material in order to quantify a degree of the tissue-damaging property of an implanted material [19], it has been considered that such cell analysis is not effective to show the degree [15]. On the other hand, Ikarashi et al. [20, 21] reported that the result of the test on cell toxicity is the most correlated to the width of the inflammatory zone, the degree of the muscle necrosis, and inflammatory cell infiltration into the space between the muscle fibers. They concluded that the width of the inflammatory zone is the most useful index to show the degree of tissue-damaging property, because it is simple and quantitative. Therefore, we used this index in the present study.

The PAMPS/PDMAAm DN gel induced a mild inflammation the degree of which was between the negative and positive controls only at 1 week, but showed the same degree of inflammation as the negative control at 4 and 6 weeks. In addition, implantation of a gel block into the subcutaneous tissue did not induce any problematic reactions. PDMAAm has been used as the material for soft contact lenses. Our previous study reported that the material properties of this DN gel are unchanged by implantation into the subcutaneous tissue for 6 weeks [11], and that the PAMPS/PDMAAm DN gel has a high degree of toughness against wear in pin-on-flat testing. Therefore, the PAMPS/PDMAAm DN gel is considered to be a potential material to be applied as a clinical implant, such as artificial cartilage, although there still remain many problems to be solved. Further evaluations from various viewpoints should be performed in the near future. On the

Table 1 This table shows *p*-values in statistical comparisons between two types of gels at each observation period. NS shows no significant difference

		Positive	PAMPS/PAAm	PAMPS/PDMAA	Cellulose/PDMAA	Cellulose/Gelatin
Negative	1w	<.0001	<.0001	.0130	<.0001	NS
	4w	NS	.0010	NS	NS	.0158
	6w	NS	.0005	NS	NS	.0443
Positive	1w	–	<.0001	.0023	.0005	<.0001
	4w	–	.0307	NS	NS	NS
	6w	–	.0076	NS	NS	NS
PAMPS/PAAm	1w	–	–	<.0001	.0037	<.0001
	4w	–	–	.0046	.0331	NS
	6w	–	–	.0005	NS	NS
PAMPS/PDMAA	1w	–	–	–	<.0001	.0059
	4w	–	–	–	NS	.0476
	6w	–	–	–	NS	.0360
Cellulose/PDMAA	1w	–	–	–	–	<.0001
	4w	–	–	–	–	NS
	6w	–	–	–	–	NS

other hand, the implanted PAMPS/PAAm and Cellulose/PDMAAm DN gels induced a strong inflammation at each period. These materials are difficult to be applied as clinical implants, if they are used in the current situation.

Around the Cellulose/Gelatin DN gel, which was composed of the two natural polymers, inflammation was very mild at 1 week, but significantly increased with numerous lymphocytes, monocyte/macrophage-like cells, plasmacells, and eosinophils at 4 and 6 weeks. In addition, the surface of the gel blocks implanted into the subcutaneous tissue was obviously absorbed. In our previous study, the ultimate stress of the Cellulose/Gelatin gel block dramatically decreased after implantation, while obvious deterioration was not observed in the Cellulose/PDMAAm DN gel [11]. These facts showed that the structure composed of gelatin was absorbed by the above described cell activity. This fact indicated that the Cellulose/Gelatin DN gel has a unique absorbable property in the living body. This material may be applicable to an absorbable implant, such as a material for drug-delivery system, although some improvement may be needed to reduce the inflammatory reaction in future studies. On the other hand, we found that strong inflammation was observed around the Cellulose/PDMAAm DN gel at 1 week, although both the Cellulose/Gelatin and PAMPS/PDMAAm DN gels induced mild inflammation. The reason for this phenomenon could not been clarified in this study, and should been studied in the near future.

This study has some limitations. First, we did not perform the long-term observation in this study. Therefore, we cannot refer to the long-term results of the implanted DN gels. Secondly, we did not analyze the molecular mechanism of the biological reaction due to each DN gels in this study. Thirdly, we did not perform the examination of inflammatory cytokines. These issues should been studied in the near future. Fourthly, the biocompatibility evaluated using cell culture tests, which is one of the most important factors for biomaterials, was not reported, although the authors simultaneously performed the cell culture tests beside the present study and confirmed that these gel materials have not cytotoxic effects. It is considered that the comprehensive results of the cell culture tests, study methods of which are extremely different from those of the present study, should be reported as an independent paper in the near future.

5 Conclusions

The implantation tests demonstrated that, although poly (2-acrylamide-2-metyl-propane sulfonic acid)/poly (*N*,*N*′-dimetyl acrylamide) gel induced a mild inflammation at 1 week, the degree of the inflammation significantly decreased into the same degree as that of the negative control at 4 and 6 weeks. Because the previous studies showed that the PAMPS/PDMAAm DN gel has an excellent wear property comparable to the UHMWPE in pin-on-flat-type wear testing [10], and that this gel is hardly degraded when it is implanted into a living body [11], we believe that this gel has a possibility to be applied as artificial cartilage in the future. However, to verify this possibility, many other factors needed for artificial cartilage repair, such as porosity, cell nutrition, changes of water content and compressive strength, capsule formation in the cartilage tissue, and so on, should be evaluated in

future studies. Secondly, Cellulose/Gelatin gel showed the same degree of inflammation as that of the negative control at 1 week, and then showed a gradually absorbable property at 4 and 6 weeks. This gel has a potential to be applied as an absorbable implant. The poly (2-acrylamide-2-metylpropane sulfonic acid)/polyacrylamide and Cellulose/poly (*N*,*N*′-dimetyl acrylamide) gels induced a significant inflammation at each week. These DN gels are difficult to be applied as clinical implants in the current situation.

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