A polyelectrolyte complex (PEC) is a compound made from an electrically neutralized molecular complex of polyanions and polycations [13]. PEC can be prepared in various forms such as a film (2D) and a hydrogel, a microcapsule or a sponge (3D), which can be used as a scaffold in tissue regeneration studies. The effects of PEC films composed of polysaccharides on cell behavior have been studied, and we have already reported that PEC can stimulate differentiation of osteoblasts and periodontal ligament fibroblasts [14–16]. These studies suggest that PEC can be used as a biomaterial for repairing or regenerating tissues. In addition, because the PEC are composed of polysaccharides, PEC is expected not to elicit immune responses against it and to have better biocompatibility with the human body, although this is yet to be proved. Therefore, it is necessary to study the interactions between PEC and cells, especially human-derived, to clarify the usefulness of PEC as a biomaterial.

In this study, normal human osteoblasts (NHOst) were cultured on various PEC prepared on a tissue culture plate from chitosan as the polycation and modified chitins or hyaluronan as the polyanion. It should be generally agreed that estimating not only functional advantages but also safety and biocompatibility of biomaterials is important to develop them for clinical use, but the latter is not always studied. Therefore, we measured changes in gap junctional intercellular communication (GJIC) as well as the cell number and differentiation. GJIC is very important function for almost all cells to maintain their homeostasis [17]. During this decade, we have studied the effects of model biomaterials on the GJIC of cells cultured on them and suggested a possibility that changes in the GJIC can be used as an index of biocompatibility of biomaterials [18–21]. Therefore, we measured changes in GJIC of NHOst on PEC in order to estimate the biocompatibility of PEC from their effects on these cell functions.

2. Materials and methods

2.1. Chemicals

Fig. 1 shows the chemical structures of the polyanions and the polycation. Chitosan as the cationic polysaccharide and carboxymethylated chitin [CM-Chitin: degree of substitution (DS) = 1.0 (1.0 anionic site/saccharide ring)] were purchased from Katokichi Co., Ltd. (Kagawa, Japan). Sulfated chitin (S-Chitin: DS = 1.5), phosphated chitin (P-Chitin: DS = 1.6), hyaluronan (HA), and sulfated hyaluronan (SHA: DS = 1.05) were prepared as previously reported [14–16,22].

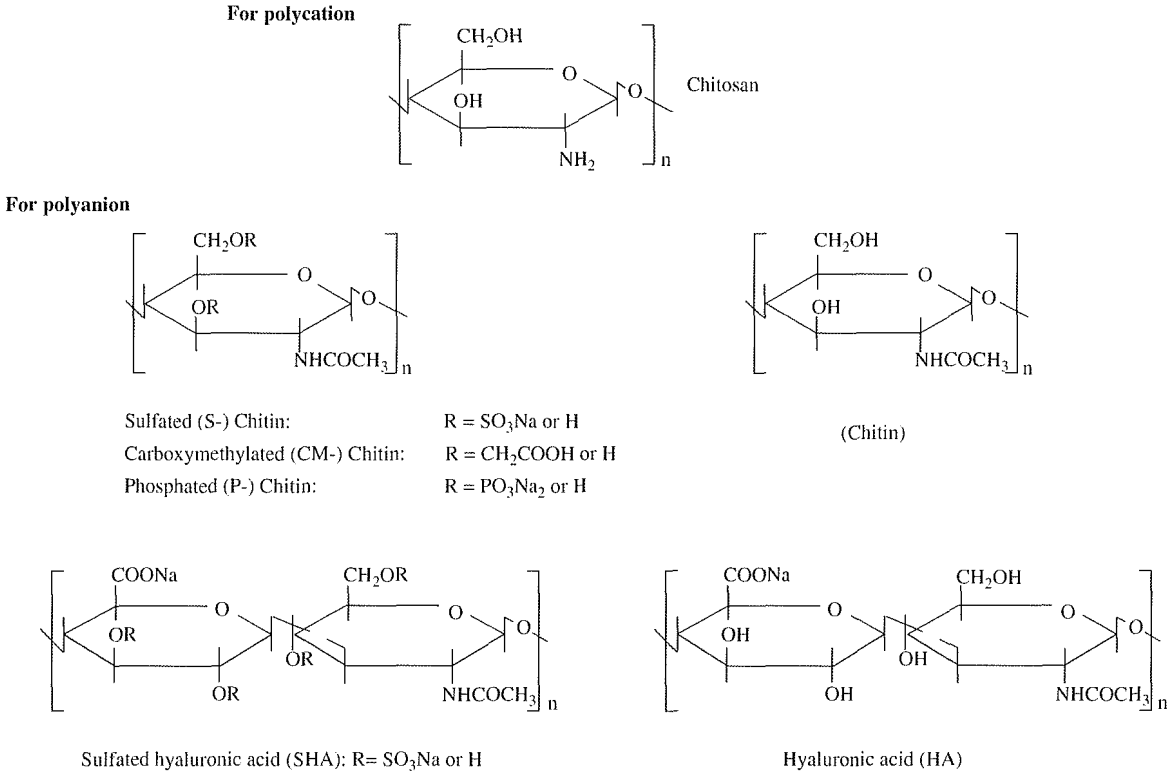

Fig. 1. Polymers for polyelectrolyte complex (PEC) in this study.

2.2. Preparation of PEC and PEC-coated dishes

Polyanions were dissolved individually in distilled water (final concentration = 5×10^{-4} mol of ionic sites/l), and the pH of the solutions was adjusted to 7.4 by adding aqueous HCl or NaOH. Chitosan was dissolved in aqueous 0.5% acetic acid solution and the pH adjusted to 6.0. The ratio of the solutions of polyanions and polycation was adjusted in each combination to neutralize the charge balance of PEC. This mixed solution (1 ml/35 mm tissue culture dish) was allowed to stand overnight at room temperature. After removing the supernatant solution, the dish was dried and annealed at 65 °C in an oven. Then, the dishes were washed with distilled water and oven-dried again to form the PEC-coated dish. This dish was sterilized for 3 min in a microwave oven. Water contact angles of PEC films were measured with the sessile drop method [23], and their zeta potentials were measured by Otsuka Electronics Co., Ltd. (Osaka, Japan).

2.3. Cell culture

NHOst were purchased from BioWhittaker Inc. (Walkersville, MD). The standard culture of NHOst was performed using alpha minimum essential medium (Gibco, Grand Island, NY) containing 20% fetal calf serum (FCS) (Kokusai Shiyaku Co., Ltd., Tokyo Japan). The cells were maintained in incubators under standard conditions (37 °C, 5%-CO_2–95%-air, saturated humidity). All assays were performed using alpha minimum essential medium containing 20% FCS, supplemented with 10 mM beta-glycerophosphate. NHOst cells (1×10^5 cells/dish/2.5 ml medium) were cultured on PEC-coated dishes to evaluate the effects of their interaction with PEC. In each experiment, the medium was changed three times before GJIC of the cells was measured and their differentiation level was evaluated after a 1-week incubation.

2.4. Estimation of differentiation level of NHOst cultured on PEC films

The proliferation of NHOst cells cultured on PEC films was estimated by Tetracolor One assay (Seikagaku Co., Tokyo, Japan), which incorporates an oxidation-reduction indicator based on detection of metabolic activity. After a 1-week incubation, 20 μl of Tetracolor One solution was added to each test dish, followed by a further 2 h incubation. The absorbance of the supernatant at 450 nm was estimated by μQuant spectrophotometer (Bio-tek Instruments, Inc., Winooski, VT). Estimation of alkaline phosphatase (ALP) activity was performed according to an original procedure by Ohyama et al. [24]. After estimating the proliferation of the NHOst cells cultured on PEC films, the cells were washed by phosphate-buffered saline (PBS(−)), followed by addition of 1 ml of 0.1 M glycine buffer (pH 10.5) containing 10 mM $MgCl_2$, 0.1 mM $ZnCl_2$ and 4 mM *p*-nitrophenylphosphate sodium salt. After incubating the cells at room temperature for 7 min, the absorbance of the glycine buffer was detected at 405 nm using μQuant to evaluate the ALP activity of the test cells. The amounts of calcium deposited by the cell during a 1-week incubation were evaluated as follows: after fixing the cells in PBS(−) containing 3% formaldehyde and washing the cells with PBS(−), 0.5 ml of 0.1 M HCl was added to each well. The amounts of calcium dissolved in HCl were estimated using a calcium detecting kit (Calcium-C test Wako, Wako, Osaka, Japan) according to manufacturer's instruction.

2.5. Measurements of GJIC activity

NHOst cultured on PEC films were subjected to fluorescence recovery after photobleaching (FRAP) analysis to estimate the inhibitory activity of these films on the GJIC. FRAP analysis was carried out according to the procedure of Wade et al. [25] with some modifications [21]. Briefly, NHOst were plated on PEC-coated dishes and incubated for 1 or 7 days. The cells were incubated for 5 min at room temperature in PBS(−) containing Ca^{2+} and Mg^{2+} (PBS(+)) and a fluorescent dye, 5,6-carboxyfluorescein diacetate. After washing off excess extracellular dye with PBS(+), the cells in PBS(+) contacting at least two other cells were subjected to FRAP analysis under a Ultima-Z confocal microscope (Meridian Instruments, Okemos, MI) with a 10 × objective lens at room temperature. The cells were photobleached with a 488 nm beam, and recovery of fluorescence intensity was subsequently monitored at 1-min intervals for a total of 4 min. The data obtained from more than seven independent cells were expressed as the average ratio of the fluorescence recovery rate to the rate obtained from NHOst cultured on a collagen-coated dish.

2.6. Statistic analysis

All data were expressed as mean values ± standard deviation of the obtained data. The Fisher–Tukey criterion was used to control for multiple comparisons and to compute the least significant difference between means.

3. Results and discussion

When NHOst were cultured on five kinds of PEC films, their morphology and attachment to the film differed with the composition of the PEC. Fig. 2 shows the morphologies of the NHOst adhering to PEC films.

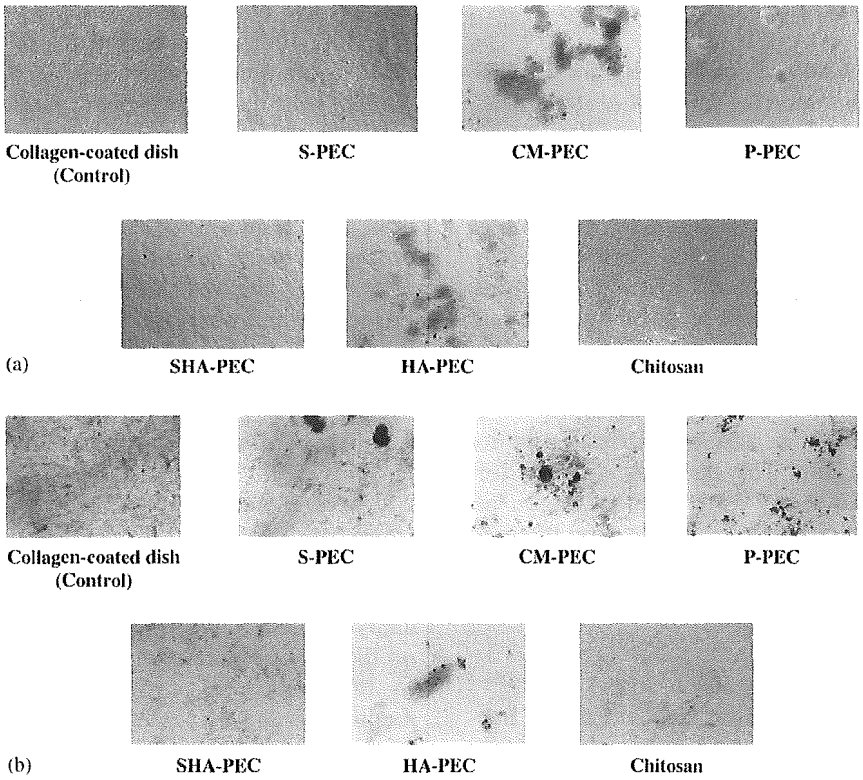

Fig. 2. Light micrographs of normal human osteoblasts (NHOst) on various PEC films after a 2-day incubation: (a) and 1-week incubation, (b). (Original magnification: × 100).

After 2-day incubation, the NHOst on PEC composed of chitosan and either sulfated chitin (S-PEC) or sulfated hyaluronan (SHA-PEC) showed morphologies similar to those on a normal culture plate. When cells were cultured on PEC of chitosan and phosphated chitin (P-PEC), some of them formed small aggregates, while the rest showed morphologies similar to those on S-PEC and SHA-PEC. On the other hand, NHOst cultured on PEC from chitosan and either carboxymethyl chitin (CM-PEC) or hyaluronan (HA-PEC) did not adhere well and showed aggregation. Similar morphologies of the cells on the PEC were observed after 1 day of incubation (data not shown). Even after 1 week of incubation, the morphologies and attachment of the cells on the PEC films did not change (Fig. 2). Only cells grown on cationic polysaccharide chitosan-coated culture dishes preserved morphology of very similar to NHOst grown on collagen-coated cultured dishes, indicating that these morphological differences are ascribable to differences in the anionic polysaccharides of which the PEC is composed.

It has been reported that cell attachment, morphology, and response are influenced by physico-chemical properties of the material surface [23,26]. To clarify what properties of PEC control the attachment and morphology of the cell, the contact angle and zeta potential of PEC films were estimated. Although their compositions are different, large differences in their contact angles were not observed (Fig. 3). On the other hand, a measurement of zeta potentials of the PEC showed interesting results (Table 1). The measurement revealed that S-PEC and SHA-PEC have negative zeta potentials, whereas PEC films made of polysaccharides

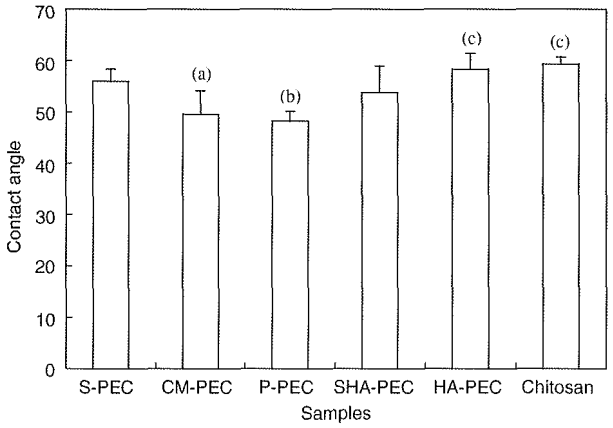

Fig. 3. Contact angles of PEC films studied: (a) $p<0.05$ against S-PEC, (b) $p<0.01$ against S-PEC, (c) $p<0.01$ against both CM-PEC and P-PEC.

Table 1
Zeta potentials of various PEC prepared on a culture dish

	Culture	S-PEC	CM-PEC	P-PEC	SHA-PEC	HA-PEC
Zeta potential (mV)	−58.7	−28.0	34.5	24.9	−5.7	29.5

Table 2
The cell number and differentiation of NHOst cultured on various PEC films after 1 week

Samples	The cell number (percent against control)	$\frac{\text{ALP activity}}{\text{The cell number}}$ (ratio)	$\frac{\text{Ca amount}}{\text{The cell number}}$ (μg/ratio)
Collagen-coated dish	100.0±17.0	1.00±0.15	3.4±0.5
S-PEC	82.2±6.1	0.98±0.11	10.7±3.6
CM-PEC	6.0±2.6*	0.05±0.08*	27.4±3.0*
P-PEC	130.4±6.3	0.02±0.01*	2.5±0.8
SHA-PEC	71.4±22.1	1.35±0.48	2.1±1.0
HA-PEC	8.1±3.0*	0.52±0.31	38.3±12.3*
Chitosan	79.5±25.0	0.93±0.13	2.7±2.0

*$p<0.01$ against collagen-coated dish.

with a carboxyl group, such as HA-PEC and CM-PEC, showed positive zeta potentials. In addition, P-PEC showed a positive potential less than that of HA-PEC. These data indicate that attachment of NHOst on surfaces with positive zeta potentials is reduced, suggesting the zeta potential of a PEC film partially controls cell attachment and morphology. Although all PEC were prepared by mixing anionic and cationic polysaccharides to neutralize their charge, zeta potential of each PEC film was ranged from −30 to 35 mV as shown in the table. This might indicate that not all anionic and cationic chemical groups were interacted to make PEC and their main chain composition and type of chemical groups may influence their side chain mobility, resulting in different surface zeta potential of each PEC. Details of surface properties of PEC films and their relationship to cell attachment will be reported in the near future.

After 1-week of incubation on various PEC films, the differentiation level of NHOst was estimated by measuring proliferation, alkaline phosphatase (ALP) activity and the amounts of calcium deposited. Table 2 shows the proliferation and ALP activity of NHOst cultured on various PEC films as well as the amounts of calcium deposited on the PEC. The proliferation of NHOst on the PEC is expressed as a percentage of proliferation of NHOst on a normal culture dish. The ALP activity was also calculated as a percentage of the control and normalized using the results of proliferation. In addition, the amount of calcium detected was normalized using the proliferation results as well. After a 1-week incubation, many dark spots, presumably calcium deposits, were observed on the collagen-coated dish and other PEC films (Fig. 2). When NHOst were cultured on CM-PEC or HA-PEC, it was observed that the NHOst aggregates were covered by the calcium deposits. It was reported that a surface with carboxyl group could induce calcium deposition after its incubation in simulated body fluid [27]. However, when the PEC were incubated in the medium without NHOst, no calcium deposition was detected. In addition, zeta potential estimation suggests less carboxyl groups are appeared on a surface of the PEC. These indicate that calcium deposition occurred only on aggregated NHOst but not on surfaces lacking NHOst. Therefore, normalization is necessary to estimate the capacity of PEC films to induce NHOst differentiation, although the raw values of deposited calcium or ALP activity are low. In fact, CM-PEC or HA-PEC films show a capacity to induce NHOst differentiation comparable to the collagen-coated dish and other PEC films, judging from the normalized values of deposited calcium shown in the table, even though the ratio of NHOst number on them was only 6–8% of that on a collagen-coated dish. Their ALP activities were, however, much lower than those on the collagen-coated dish. Incubation of the PEC films without NHOst for 1 week resulted in no calcium deposition, irrespective of their composition, suggesting that the PEC films themselves had no effect on calcium deposition. Thus, enhancement of calcium deposition on the PEC films may be ascribed to enhancement of NHOst functions related to their differentiation even though their ALP activity was suppressed. The reason for this inconsistency observed between calcium deposition and ALP activity must be investigated further.

When sulfated polysaccharides were used to prepare PEC films, proliferation of NHOst on the PEC films was 70–80% of that on a collagen-coated dish, and ALP

activity was very similar to that on the collagen-coated dish. This suggests that sulfated polysaccharide PEC does not affect NHOst functions. Actually, there were no statistical differences in the amounts of calcium deposited between NHOst on the PEC and the collagen-coated dish although NHOst on S-PEC showed higher average calcium deposition. Thus, it is suggested that the PEC films made from sulfated polysaccharides are comparable substrates to a collagen-coated dish for cell culture. When compared to a normal culture dish, it has been reported that S-PEC can induce aggregation of cultured human fibroblasts and enhance their DNA synthesis in an earlier stage of cell culture by activation of the ERK pathway [28]. Since we used a collagen-coated dish as a control in this study, it is expected that the pathway of NHOst on the dish may be already activated through integrin molecules on the NHOst membrane. Therefore, the results in this study suggest the PEC from sulfated polysaccharides have a potential to proliferate and differentiate NHOst very similar to that of collagen.

To assess the effects of PEC films on cell function, gap junctional intercellular communication (GJIC), which is an important function of cells for maintenance of homeostasis [17], of NHOst on the films were measured. As shown in Fig. 4, GJIC of NHOst on PEC films did not show statistically significant differences compared to those grown on a collagen-coated dish. Although the GJIC of NHOst on CM-PEC showed a decrease after 1 day of incubation, it had recovered after 1 week. This result suggests that most PEC films have the potential to maintain homeostasis of attached cells although they showed different influences on the number and the differentiation of NHOst. On the other hand, NHOst on chitosan, which was used as the polycation for all PEC, showed suppression of GJIC after 1 week. This suggests that chitosan disturbs homeostasis maintenance of NHOst, but improve its biocompatibility by forming PEC films with other anionic polysaccharides. Therefore, PEC might be used as a biocompatible material for medical devices and tissue engineering scaffolds.

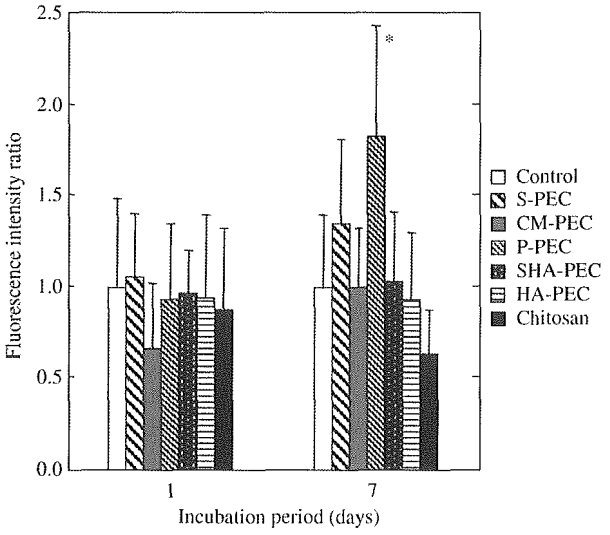

Fig. 4. Gap junctional intercellular communication activity of NHOst on various PEC films estimated by FRAP analysis technique. (*$p<0.01$ against control).

4. Conclusion

PEC films composed of various polysaccharides were prepared, and their effects on NHOst functions were evaluated. Attachment, morphology, growth and differentiation of NHOst were influenced by the composition of the PEC on which they were grown. NHOst attachment decreased and their aggregates were observed on PEC prepared from polysaccharides containing a carboxyl group (CM- and HA-PEC). ALP activity of NHOst was suppressed on these PEC films although calcium deposition was observed more frequently than on other PEC films. In addition, these PEC films strongly suppressed proliferation of NHOst. PEC prepared from phosphated chitin and chitosan (P-PEC) showed low ALP activity and calcium deposition, although the number of NHOst was highest after 1-week incubation. These indicate unsuitability of these three PEC for usage in tissue engineering. On the other hand, NHOst adhered to and proliferated well on PEC films when sulfated polysaccharides were used as the polyanion (S- and SHA-PEC). Moreover, these PEC films showed almost the same suitability as the collagen-coated dish in all cell functions studied, indicating that these PEC films, especially S-PEC can be used as a scaffold for bone regeneration. Further studies, especially in vivo studies, are needed to clarify the usefulness of PEC films for tissue engineering.

Acknowledgements

This work was partially supported by Health and Labour Sciences Research Grants for Research on Advanced Medical Technology and Risk Analysis Research on Food and Pharmaceuticals by Ministry of Health, Labour and Welfare, Health and Labour Sciences Research Grants for Research on Health Sciences focusing on Drug Innovation by the Japan Health Sciences Foundation, and Grant-in-Aid for 21st Century COE Program "Advanced Fiber Engineering" and Grant-in-Aid for Scientific Research (B), 2002 (14350495) by Ministry of Education, Science, Sports and Culture.

References

[1] Adams JC, Watt FM. Regulation of development and differentiation by the extracellular matrix. Development 1993;117:1183–98.

[2] Peterson WJ, Tachiki KH, Yamaguchi DT. Extracellular matrix alters the relationship between thymidine incorporation and proliferation of MC3T3-E1 cells during osteogenesis in vitro. Cell Prolif 2002;35:9–22.

[3] Langer R, Vacanti JP. Tissue engineering. Science 1993;260:920–6.

[4] Mooney DJ, Mikos AG. Growing new organs. Sci Am 1999;280:38–43.

[5] Hori Y, Nakamura T, Matsumoto K, Kurokawa Y, Satomi S, Shimizu Y. Tissue engineering of the small intestine by acellular collagen sponge scaffold grafting. Int J Artif Organs 2001;24:50–4.

[6] Yamamoto M, Takahashi Y, Tabata Y. Controlled release by biodegradable hydrogels enhances the ectopic bone formation of bone morphogenetic protein. Biomaterials 2003;24:4375–83.

[7] Gamez E, Ikezaki K, Fukui M, Matsuda T. Photoconstructs of nerve guidance prosthesis using photoreactive gelatin as a scaffold. Cell Transplant 2003;12:481–90.

[8] Lavik E, Teng YD, Snyder E, Langer R. Seeding neural stem cells on scaffolds of PGA, PLA, and their co-polymers. Method Mol Biol 2002;198:89–97.

[9] Lieb E, Tessmar J, Hacker M, Fischbach C, Rose D, Blunk T, Mikos AG, Gopferich A, Schulz MB. Poly(D,L-lactic acid)-poly(ethylene glycol)-monomethyl ether diblock copolymers control adhesion and osteoblastic differentiation of marrow stromal cells. Tissue Eng 2003;9:71–84.

[10] Shin M, Ishii O, Sueda T, Vacanti JP. Contractile cardiac grafts using a novel nanofibrous mesh. Biomaterials 2004;25:3717–25.

[11] Alsberg E, Anderson KW, Albeiruti A, Rowley JA, Mooney DJ. Engineering growing tissues. Proc Nat Acad Sci 2002;99:12025–30.

[12] Chang CH, Liu HC, Lin CC, Chou CH, Lin FH. Gelatin-chondroitin-hyaluronan tri-copolymer scaffold for cartilage tissue engineering. Biomaterials 2003;24:4853–8.

[13] Tsuchida E, Abe K. Interactions between macromolecules in solution and intermacromolecular complexes. Adv Polym Sci 1982;45:1–119.

[14] Hamano T, Teramoto A, Iizuka E, Abe K. Effects of polyelectrolyte complex (PEC) on human periodontal ligament fibroblasts (HPLF) function. I. Three-dimensional structure of HPLF cultured PEC. J Biomed Mater Res 1998;41:257–69.

[15] Hamano T, Teramoto A, Iizuka E, Abe K. Effects of polyelectrolyte complex (PEC) on human periodontal ligament fibroblasts (HPLF) function. II. Enhancement of HPLF differentiation and aggregation on PEC by *L*-ascorbic acid and dexamethasone. J Biomed Mater Res 1998;41:270–7.

[16] Hamano T, Chiba D, Nakatsuka K, Nagahata M, Teramoto A, Kondo Y, Hachimori A, Abe K. Evaluation of a polyelectrolyte complex (PEC) composed of chitin derivatives and chitosan, which promotes the rat calvarial osteoblast differentiation. Polym Adv Technol 2002;13:46–53.

[17] Maio AD, Vaga VL, Contreras JE. Gap junctions, homeostasis, and injury. J Cell Physiol 2002;191:269–82.

[18] Tsuchiya T, Hata H, Nakamura A. Studies on the tumor-promoting activity of biomaterials: inhibition of metabolic cooperation by polyetherurethane and silicone. J Biomed Mater Res 1995;29:113–9.

[19] Tsuchiya T, Takahara A, Cooper SL, Nakamura A. Studies on the tumor-promoting activity of polyurethanes: depletion of inhibitory action of metabolic cooperation on the surface of a polyalkyleneurethane but not a polyetherurethane. J Biomed Mater Res 1995;29:835–41.

[20] Nakaoka R, Tsuchiya T, Sakaguchi K, Nakamura A. Studies on in vitro evaluation for the biocompatibility of various biomaterials: inhibitory activity of various kinds of polymer microspheres on metabolic cooperation. J Biomed Mater Res 2001:279–84.

[21] Nakaoka R, Tsuchiya T, Nakamura A. The inhibitory mechanism of gap junctional intercellular communication induced by polyethylene and the restorative effects by surface modification with various proteins. J Biomed Mater Res 2001;57:567–74.

[22] Nagahata M, Tsuchiya T, Ishiguro T, Matsuda N, Nakatsuchi Y, Teramoto A, Hachimori A, Abe K. A novel function of *N*-cadherin and connexin 43: marked enhancement of alkaline phosphotase activity in rat calvarial osteoblast exposed to sulfated hyaluronan. Biochem Biophys Res Commun 2004;315:603–11.

[23] Tamada Y, Ikada Y. Fibroblast growth on polymer surfaces and biosynthesis of collagen. J Biomed Mater Res 1994;28:783–9.

[24] Ohyama M, Suzuki N, Yamaguchi Y, Maeno M, Otsuka K, Ito K. Effect of enamel matrix derivative on the differentiation of C2C12 cells. J Periodontol 2002;73:543–50.

[25] Wade MH, Trosko JE, Schlindler M. A fluorescence photobleaching assay of gap junctional-mediated communication between human cells. Science 1986;232:525–8.

[26] Kato S, Akagi T, Sugimura K, Kishida A, Akashi M. Evaluation of biological responses to polymeric biomaterials by RT-PCR analysis IV: study of c-myc, c-fos and p53 mRNA expression. Biomaterials 2000;21:521–7.

[27] Tanahashi M, Matsuda T. Surface functional group dependence on apatite formation on self-assembled monolayers in a simulated body fluid. J Biomed Mater Res 1997;34:305–15.

[28] Matsuda N, Horikawa M, Yoshida M, Watanabe M, Nagahata M, Teramoto A, Abe K. Enhanced DNA synthesis accompanied by constitutive phosphorylation of the ERK pathway in human fibroblasts cultured on a polyelectrolyte complex. Biomaterials 2003;24:4771–6.

Key Engineering Materials Vols. 288-289 (2005) pp. 409-412
online at http://www.scientific.net

Osteoblast Differentiation and Apatite Formation on Gamma-Irradiated PLLA Sheets

Kazuo Isama[a] and Toshie Tsuchiya[b]

Division of Medical Devices, National Institute of Health Sciences,

Kamiyoga 1-18-1, Setagaya-ku, Tokyo 158-8501, Japan

[a]isama@nihs.go.jp, [b]tsuchiya@nihs.go.jp

Keywords: Poly(L-lactide), gamma-ray irradiation, osteoblast differentiation, apatite formation.

Abstract. The effects of the γ-irradiatted PLLA on the osteoblasts and apatite formation were investigated *in vitro*. The PLLA sheet was γ-ray irradiated at the dose of 10, 25 or 50 kGy. The mouse osteoblast-like MC3T3-E1 cells and normal human osteoblast NHOst cells were micromass cultured on the PLLA sheet for 2 weeks, and then the proliferation and differentiation of the cells were determined. The proliferations of MC3T3-E1 and NHOst cells hardly changed with increasing irradiation dose. However, the differentiations of MC3T3-E1 and NHOst cells increased with irradiation dose. On the other hand, the surface of the PLLA sheet after soaking in the medium without the cells was characterized by SEM, EDX, FT-IR and XPS. The hydroxyapatite was formed on the surface of the PLLA sheet after soaking, and the amount of hydroxyapatite increased with irradiation dose. In summary, the γ-irradiated PLLA increased the differentiation of osteoblasts and also increased apatite-forming ability even without the osteoblasts. The osteoblast differentiation was enhanced well in the apatite formation on the surface of PLLA after the γ-irradiation.

Introduction

Poly(L-lactide) (PLLA) has been well reported on a good osteocompatibility *in vivo* and *in vitro*. The γ-ray sterilized PLLA sample was implanted *in vivo*, and newly bone was formed around the PLLA implant [1]. It was not clear whether there was the effect of γ-irradiation on the formation of newly bone in this result. However, it was the fact that γ-irradiation decreased the molecular weight and mechanical strength of PLLA [2]. On the other hand, PLLA fibers formed bone-like apatite in a simulated body fluid [3]. It was reported that the apatite layer formed on the bioactive glass increased the attachment and initial proliferation of osteoblasts [4]. If the apatite-forming ability of PLLA is increased by γ-irradiation, there may be a good influence on osteoblasts cultured on the irradiated PLLA. Therefore, we clarified the effects of the γ-irradiatted PLLA sheet on the osteoblasts and apatite formation *in vitro*.

Materials and Methods

Materials. PLLA sheet with 0.3 mm thickness (Shimadzu Co., Japan) was γ-ray irradiated at the dose of 10, 25 or 50 kGy using ^{60}Co as the radiation source. The weight average molecular weight (Mw) of the unirradiated PLLA was 271,000 and the Mw's of the irradiated PLLA's at the dose

of 10, 25 and 50 kGy were respectively 195,000, 142,000 and 95,000 by GPC.

Micromass Culture of Osteoblasts. Mouse osteoblast-like MC3T3-E1 cells (RIKEN Cell Bank, Japan) and normal human osteoblast NHOst cells (Clonetics Corporation, MD, USA) were grown in alpha minimum essential medium (α-MEM) supplemented with 20% fetal bovine serum. The PLLA sheet was cut into 14.0 mm diameter disk and laid in a 24-well dish. The 20 μl of cell suspension (2×10^6 cells/ml) was delivered on the disk. After the cells were attached on the disk, 1 ml of the complete medium that contained 10 mM disodium β-glycerophosphate in the culture medium was added. The complete medium was changed three times a week, and the cells cultured for 2 weeks in a 37°C humidified atmosphere of 5% CO_2.

Proliferation Assay. The number of the cells cultured on the PLLA sheet was determined by WST-8 assay [5]. Moreover, the protein and DNA contents of the cell lysate were measured by the Lowry method and the fluorescence assay using Hoechst 33258 dye, respectively [5].

Differentiation Assay. The calcium depositions of the cell cultures were stained by alizarin red S, and the areas stained dark-red were measured using the program Scion Image (Scion Co., MD, USA) [5]. The calcification was calculated as the normalized area in the cell number. Moreover, the collagen synthesis was evaluated by the hydroxyproline content of the cell lysate, and ALP activity of the cells was measured using *p*-nitrophenylphosphate as a substrate [5].

Soaking in the Medium. The PLLA sheet was cut into 14.0 mm diameter disk and laid in a 24-well dish. The complete medium of 1 ml was added without the cells. Then, the dish was stored in a 37°C humidified atmosphere of 5% CO_2, and the complete medium was changed three times a week. After soaking for 2 weeks, the PLLA disk was washed in deionized water five times quickly and dried in a silica gel desiccator.

Surface Analysis. The surface of the PLLA sheet after soaking in the complete medium without the cells was characterized by SEM, EDX, FT-IR and XPS according to the conventional methods.

Results

Proliferation of Osteoblasts Cultured on the PLLA Sheet. The cell number of MC3T3-E1 cells cultured on the PLLA sheet did not change with increasing irradiation dose (Fig. 1a). The protein and DNA contents of the cells also did not change. The other side, the cell number (Fig. 1b),

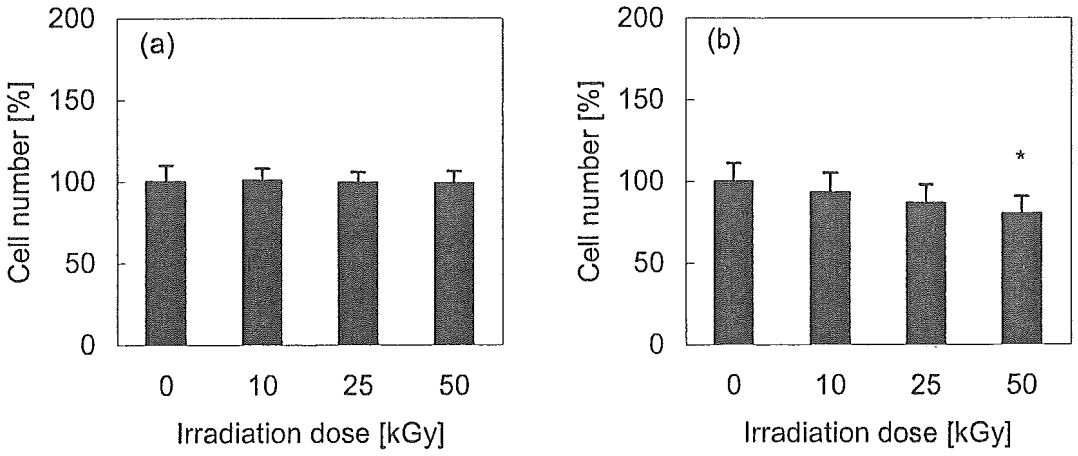

Fig. 1. The cell numbers of (a) MC3T3-E1 and (b) NHOst cells cultured on the γ-irradiated PLLA sheet.

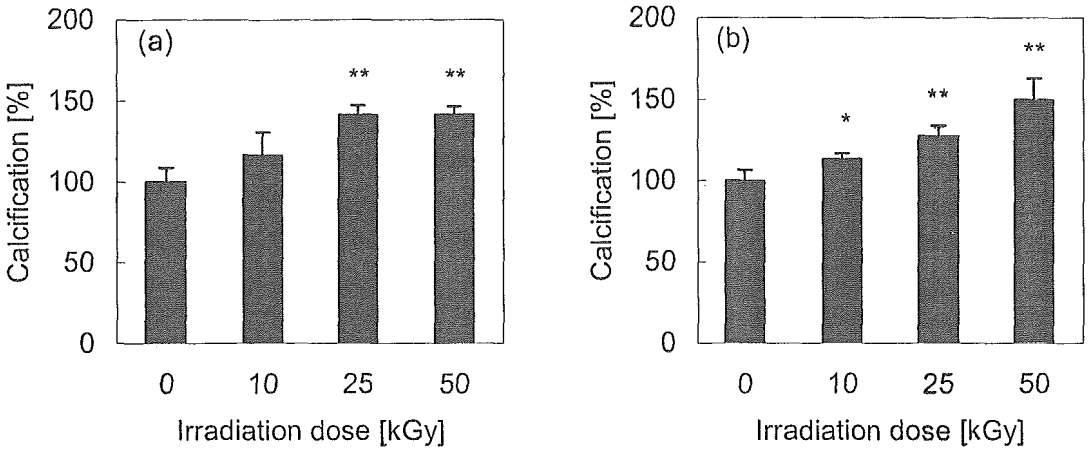

Fig. 2. The calcifications of (a) MC3T3-E1 and (b) NHOst cells cultured on the γ-irradiated PLLA sheet.

protein and DNA contents of NHOst cells cultured on the PLLA sheet slightly decreased with irradiation dose.

Differentiation of Osteoblasts Cultured on the PLLA Sheet. The calcification of MC3T3-E1 cells (Fig. 2a) and NHOst cells (Fig. 2b) remarkably increased with irradiation dose. The collagen synthesis and ALP activity of MC3T3-E1 and NHOst cells also increased as same as the calcification, respectively. The γ-irradiated PLLA remarkably promoted the differentiation of osteoblasts.

Apatite Formation on the PLLA Sheet. The SEM micrograph exhibited crystal particles on the surface of the PLLA sheet after soaking in the complete medium without the cells. The crystal particles were identified with hydroxyapatite by EDX, FT-IR and XPS spectra. The phosphate band in ATR/FT-IR spectra became strong with irradiation dose (Fig. 3). Moreover, the element rations of calcium and phosphorus increased but that of carbon decreased with irradiation dose, in XPS analysis (Fig. 4). The amount of hydroxyapatite formed on the γ-irradiated PLLA sheet increased with irradiation dose.

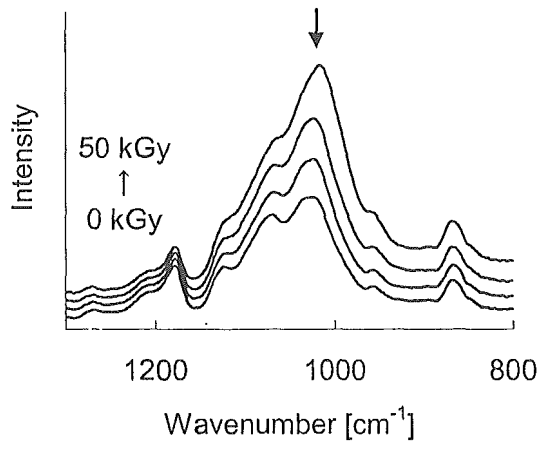

Fig. 3. The phosphate band of the γ-irradiated PLLA sheet after soaking in the medium.

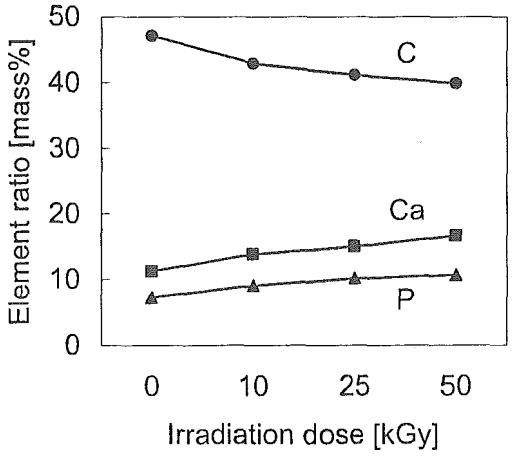

Fig. 4. The element ratios of calcium, phosphorus and carbon of the γ-irradiated PLLA sheet after soaking in the medium.

Discussion

In the present study, the γ-irradiated PLLA hardly affected the proliferation but remarkably promoted the differentiation of osteoblasts. It was expected that the low molecular weight PLLA eluted to the medium, because the molecular weight of PLLA decreased by γ-irradiation. In our recent studies, the low molecular weight PLLA enhanced the differentiation of MC3T3-E1 cells but inhibited that of NHOst cells [6, 7]. The present results, which the differentiations of MC3T3-E1 and NHOst cells both increased on the γ-irradiated PLLA sheet, would not be caused by the low molecular weight PLLA. The surface of the γ-irradiated PLLA should good influence on the differentiation of osteoblasts.

On the other hand, the γ-irradiation increased the apatite-forming ability of the PLLA sheet. Tanahashi and Matsuda reported that some negatively charged groups such as phosphate and carboxyl group strongly induced apatite formation in a simulated body fluid. They described that the apatite formation was initiated via calcium ion-absorption upon complexation with a negative surface-charged group [8]. In our study, the molecular weight of PLLA decreased with hydrolysis of ester bonds by γ-irradiation [2]. Therefore, the amount of carboxyl group of the γ-irradiated PLLA would increase with irradiation dose, and the carboxyl group would promote the apatite-forming ability of the PLLA sheet.

Fujibayashi *et al.* compared *in vivo* bone ingrowth and *in vitro* apatite formation on Na_2O-CaO-SiO_2 glasses. The quantities of newly bone formed on the glasses correlated with their apatite-forming abilities in simulated body fluid. They propose to evaluate the apatite-forming ability in order to confirm the *in vivo* bioactibity of biomaterials [9]. In our present study, the γ-irradiation enhanced the apatite-forming ability of the PLLA sheet, and then the γ-irradiated PLLA sheet promoted the differentiation of osteoblasts. The osteoblast differentiation should connect with the apatite formation on the γ-irradiated PLLA sheet.

In conclusion, the γ-irradiated PLLA hardly affected the proliferation but promoted the differentiation of osteoblasts with increasing irradiation dose. On the other hand, the hydroxyapatite was formed on the PLLA sheet in the medium, and the γ-irradiation enhanced apatite-forming ability of the PLLA. It was suggested that the connection between the osteoblast differentiation and apatite formation on the γ-irradiated PLLA sheets.

References

[1] T.E. Otto, P. Patka, H.J.Th.M. Haarman, C.P.A.T. Klein and R. Vriesde: J. Mater. Sci. Mater. Med. 5 (1994), 407-410.

[2] K. Isama and T. Tsuchiya: Bull. Natl. Inst. Health Sci. 119 (2001), 61-64.

[3] X. Yuan, A.F.T. Mak and J. Li: J. Biomed. Mater. Res. 57 (2001), 140-150.

[4] N. Olmo, A.I. Martin, A.J. Salinas, J. Turnay, M. Vallet-Regi and M.A. Lizarbe: Biomaterials 24 (2003), 3383-3393.

[5] K. Isama and T. Tsuchiya: J. Biomater. Sci. Polymer Edn. 13 (2002), 153-166.

[6] K. Isama and T. Tsuchiya: Biomaterials 24 (2003), 3303-3309.

[7] K. Isama, Y. Ikarashi and T. Tsuchiya: BIO INDUSTRY 19 (2002), 21-29.

[8] M. Tanahashi and T. Matsuda: J. Biomed. Mater. Res. 34 (1997), 305-315.

[9] S. Fujibayashi, M. Neo, H.M. Kim, T. Kokubo and T. Nakamura: Biomaterials 24 (2003), 1349-1356.

再生医療・繊維工学・人工臓器に使用される医療用材料の安全性・有効性に関する基本的考え方

Standpoints and Principles for the Evaluation of Safety and Officacy of Biomaterials Applied for Tissue Engineered Products

土屋利江

1. はじめに

国内外共に開発競争が盛んな医療材料の一つとして生分解性材料があげられる。限られた紙面の都合上、ここでは、注目されている生分解性材料において、現在、安全性・有効性において問題になっている点を中心に述べる。生分解性材料は、やがては生体内で消失し、残存しないという長所があるものの、吸収性材料であるが故に、クラスⅣに分類されるハイリスク医療材料である。最近の不具合報告や前臨床試験研究からも、解決すべきいくつかのポイントがあるので、現在考えられる基本的考え方について述べる。

2. 安全性

各種モノマーから合成される高分子系では、使用される触媒の選択が安全性・有効性を考える上で第1のキーポイントとなる。環境および生体ハザードとして有名な触媒を使用している例がある。生分解性材料を医療材料として使用した場合、生体内で吸収される。使用される材料のトータル量は医療材料の使用目的によって数mgから数g程度まで異なっている。量が多くなると安全性上のリスクも高くなる。安全性上問題はないのか最新の知見を十分検討して合成のスキームを描いていただきたい。また、医療材料として使用する場合、使用する部位、使用する量、分解速度によって安全性に関するリスクレベルは異なる。慎重な検討を御願いしたい。

3. 経済性と安全性

医療材料も、エコマテリアルであることが、理想的であるが、通常、工業製品用のエコマテリアルとなると数百から数千トン単位で合成され、地球環境系へ放出される。また、使用量も多く、できるだけ低コストで生産されることになる。工業界では、安価な触媒で効率よく重合できる製法が望まれる。安全性に対する検討事項もあるが、医療材料として使用される場合とは試験選択項目が異なる。分解性という点でエコマテリアルの候補と考えられるポリグリコール酸には、環境ハザードで有名な有機錫で合成された製品が販売されている例がある。有機錫は、ppbあるいはそれ以下のオーダーで神経毒性を示す触媒である。作用域が低濃度であるため、残留物の分析では、一般化学分析では、同定・定量することは容易ではない。製造工程が示してあれば、容易に知ることが可能であるが、カタログ掲載の製品では、使用されている触媒が、明示されていないし、たづねても回答がかえってこないケースが多い。世の中に登場しているエコマテリアルと称される材料が、安全性上、危惧される点が解決できた製造工程で合成されているか確認する必要がある。環境中に放出されれば、触媒は、土壌中に残留することになり、海にも流れ、やがては、魚貝類に蓄積し、食物連鎖によりやがてはヒトの健康への悪影響が懸念される。これらの点は、逆の発想をすれば、有効性の高い材料開発のアイデアともなりうる。すなわち、毒性のある触媒の代わりに、有効性の高い触媒を使用すれば、安全で有効性の高い材料開発の創製となるかもしれない。

海外では、すでに無触媒条件下で分子量100万のレベルまで重合した生分解製材料を高価な価格で販売しているという。この分野の国際情報の流通がわるい。

4. 生体適合性

第2のポイントは、生体内に埋植すると炎症反応を惹起しやすい性質を示す材料がある。炎症反応が起これば、例えば、バイオ軟骨において、動物に細胞組み込み型生分解性スキャホールドを埋植し、炎症がおきれば、スキャホールドに播種した軟骨細胞等の分化発現や機能維持の目的を達成できない。従って、このような材料に起因した炎症反応を回避あるいは消失させるための創意工夫をしていただきたい。炎症反応惹起の有無はin vivoで確認する必要がある。ある生分解性高分子からなる材料に軟骨細胞を播種してin vitro培養すると分化を非常によく促進する。次に、動物の軟骨欠損部分に移植したところ、組織再生がうまくいかない、材料や細胞組み込み型材料を埋植すると組織再生は遅延し、それらをまったく埋植しない軟骨欠損のまま

TOSHIE TSUCHIYA
国立医薬品食品衛生研究所　療品部長
〒158-8501　東京都世田谷区上用賀1-18-1
Tel : 03-3700-9196　Fax : 03-3700-9196
E-mail : tsuchiya@nihs.go.jp
〈専門〉繊維工学、医療材料の安全性と生体適合性

の群の自然治癒率がもっとも優れていたということも実際ありうる。Tissue Engineering関連の論文で掲載されたデータで、in vitroでどんなに成績が良くても、生体内で、同じように作用するか否かは、別の問題となる。in vitroで優れた成績を示している必要性はあるが、必ずしもin vivoで成功するとは限らない。生体内は、複数の細胞・組織のネットワークで営まれております。in vitro系は、特定の細胞の反応をみていること、さらに、検出している指標のみを解析しているにすぎないことを、いつも考慮しておく必要がある。

5. 更なる生体適合性

第3のポイントは、合成高分子には、ゲッシ類で比較的高い腫瘍発生率を示す材料があることに留意して、新たな材料開発を行うべきである。たとえば、京都大学の研究成果では、ポリグリコール酸は、フィルムをラット皮下に長期間埋植しても、腫瘍の発生を認めなかったが、ポリ乳酸フィルムを、ラット皮下に埋植した結果、埋植ラットの40%に腫瘍発生を認めている。ポリカプロラクトンとの共重合体では、50%の腫瘍発生頻度であった。一方、材料発癌では、20年以上昔の現象から、フィルム状のものをゲッシ類の皮下に埋植すると、腫瘍を発生する。との古典的な説がある。フィルムがすべて同程度腫瘍化するわけではない。シリコンフィルムでは、手術群に近く、低発生率である。フィルムで腫瘍発生する生分解性材料を、粒子状にして埋植した結果、やはり腫瘍を発生し、発生率は、埋植量に比例した。私は、埋植材料の化学組成、物理学的性質、残留性(分解速度)、血管系の有無などがサイトカイン産生、コネキシン機能変化、炎症反応による活性酸素産生、修復能などに影響を与え、腫瘍発生率に関係すると考えている。

医療材料の安全性評価においては、ガイドラインにある一通りの試験項目について受託機関等により試験し、すべて陰性結果を得ると、安全であると考えやすいが、その試験が適切な抽出やサンプル適用方法でおこなわれていない限り、意味のない試験結果となることを強調しておく。生分解性材料の場合に、そのオリゴマーの安全性についても評価することが、長期予測をおこなう上で重要である。

第10回高分子分析討論会(高分子の分析及びキャラクタリゼーション)－研究発表募集－

主　催：日本分析化学会高分子分析研究懇談会　　**協　賛**：㈳繊維学会　　**日　時**：平成17年10月27日(木)・28日(金)
会　場：工学院大学新宿校舎〔東京都新宿区西新宿1-24-2、交通：JR(山手線・中央線・埼京線)、京王線、小田急線、地下鉄(丸の内線・都営新宿線)「新宿」駅下車西口より徒歩5分。大江戸線「都庁前」駅直結〕
http://www.kogakuin.ac.jp/mnp/shinjuku/map_shinjuku.pdf
発表申込締切：7月1日(金)　　**発表要旨締切**：10月7日(金)
研究発表申込先・問合先：〒305-8565　茨城県つくば市東1-1-1　つくば中央5
(独)産業技術総合研究所　計測標準研究部門　有機分析科　高分子標準研究室　松山重倫
TEL：029-861-4617、FAX：029-861-4618、E-mail：polymer@m.aist.go.jp

---

第41回X線分析討論会－講演募集－

主　催：日本分析化学会X線分析研究懇談会　　**協　賛**：㈳繊維学会ほか
日　時：平成17年10月21日(金)・22日(土)
会　場：京都大学福井謙一記念研究センター(京都市左京区高野西開町34-4)
講演申込締切日：8月10日(水)　　**講演要旨締切日**：9月16日(金)
詳細については下記にお問い合せください。
〒141-0031　品川区西五反田1-26-2　五反田サンハイツ304号　日本分析化学会X線分析研究懇談会
TEL：03-3490-3351　FAX：03-3490-3572　E-mail：ktanaka@jsac.or.jp

「高分子材料の耐久性評価」に関する講習会

主　催：日本材料学会　　**協　賛**：㈳繊維学会ほか　　**日　時**：平成17年7月22日(金)　9：30～14：40
会　場：工学院大学新宿校舎28階第1会議室　〒163-8677　東京都新宿区西新宿1-24-2(TEL：03-3342-1211)
プログラム、参加申込の詳細については、下記にお問い合せください。
〒606-8301　京都市左京区吉田泉殿町1-101　日本材料学会「高分子材料の耐久性評価」講習会係
TEL：075-761-5321　FAX：075-761-5325　E-mail：jimu@jsms.jp