

Fig. 3. Insulin content of HIT-T15 cells incubated with HA-coated (□) and HA-added (■). Cells were incubated in the presence of different HA concentrations (0.1–0.5 mg/dish) for 24 h and then stimulated for 60 min with 11.1 mM glucose. The insulin content in the extracts was determined by ELISA insulin kit. Each value denotes the mean \pm S.D. of three separate experiments. ** $P \leq 0.01$ and *** $P \leq 0.001$ compared to control.

after microinjection. Fig. 4A shows the patterns of dye transfer in HIT-T15 cells treated with HA-coating (0.1, 0.25, and 0.5 mg/dish) for 24 h. Most microinjections led to the intercellular transfer of Lucifer yellow, indicating the frequent coupling of HIT-T15 cells. Under control conditions, microinjection experiments revealed that 47.1% of HIT-T15 cells transferred Lucifer yellow with a limited number (1.5 ± 0.6) of microinjection cells. In HA-coated conditions, almost injected cells (95%) showed Lucifer yellow dye transfer, the number of Lucifer yellow-transferred cells (3.2 ± 1.3 , 4.4 ± 1.9 , and 4.1 ± 1.9 , respectively) was more than that of the control condition ($P < 0.001$) (Fig. 4B), which indicated that GJIC function was activated by the HA-coating.

3.4. Cx43 expression

Cx43 is the 43-kDa member of a conserved family of membrane spanning gap-junction proteins. To provide further evidence that the HA-coating increased the function of GJIC, relative to the levels of actin, comparable levels of immunolabeled Cx43 was detected in 0.1, 0.25, and 0.5 mg/dish of HA-coating cells. Whole cell lysates from HA-coated dish were subjected to SDS-PAGE. Immunoblot analysis was performed with an antibody that specifically recognized Cx43 or β -actin. A Western blot analysis revealed that Cx43 proteins are present in cultured HIT-T15 cells in three forms at 43 kDa region, consisting of a nonphosphorylated form and phosphorylated forms (P1 and P2). HA-coating appeared to induce a

greater concentration-dependent increase in all three Cx43 protein levels than control. However, the protein level of β -actin was no different from them (Fig. 5), indicating HA-coating increases the function of GJIC via the expression of Cx43. To account for differences in loading, proteins were both stained with Coomassie blue and immunolabeled for β -actin. The latter staining, which did not change in our experiments relative to that of Coomassie blue (data not shown), was used as an internal standard. These results suggested that HA-coating specifically increased the Cx43 protein but not all cell proteins of HIT-T15 cells.

4. Discussion

The transplantation strategy of bioartificial pancreas is to construct bioartificial tissues in vitro from cells or islets and a support matrix and implant the construct into the body in place of the original. The support matrix must be able to maintain the functions of differentiated cells or contain and/or be able to release appropriate biological signaling information to promote and maintain cell adhesion and differentiation. HA is a high-molecular-mass polysaccharide of support matrix in the body, which is believed to play roles in maintaining various physiological functions including water and plasma protein homeostasis, cell proliferation, cell locomotion, and migration [3]. HA is plentiful, easy to extract and mold into a variety of shape, and biodegradable. It is thus widely used matrix biomaterial for bioartificial tissues [10]. In this study, we investigated whether administration of various concentration of HMW HA influences the viability, GJIC, and insulin secretion of pancreatic β -cells as a matrix biomaterial of bioartificial pancreatic constructs.

Previous study has shown that HMW (310 and 800 kDa) HA-coating (2.0 mg/dish) resulted in low adhesiveness to the cells and the decrease of viability in normal human dermal fibroblasts, because of the change in GJIC functions and induction of various genes including cytokines, adhesion molecules, and growth factors [6,11,12]. In the present study, similar results were obtained. After 12 h, the HIT-T15 cells grown into low concentration HA-coated dishes (0.1, 0.25, and 0.5 mg/dish) and control cells already had attached and confluent but not high concentration HA-coated dishes (≥ 1.0 mg/dish). We showed that treatment with high concentration of HMW (1680 kDa) HA-coated dose dependently inhibited the viability of HIT-T15 cells. In contrast, there was no difference in viability of HIT-T15 cells between the control and HA-added dishes. These results indicated that among the individual qualities of ECM, the viscosity plays a decisive role. The changes of cell viability by HA-treatment may depend on the cell attachment activity. The difference in cell attachment activity may depend on the surface structure of the coated HA, because the HMW HA-coated surface provides a stable anionic surface that prevents cells attachment at the early time [13]. This result suggests that the molecular-weight size of HA and its

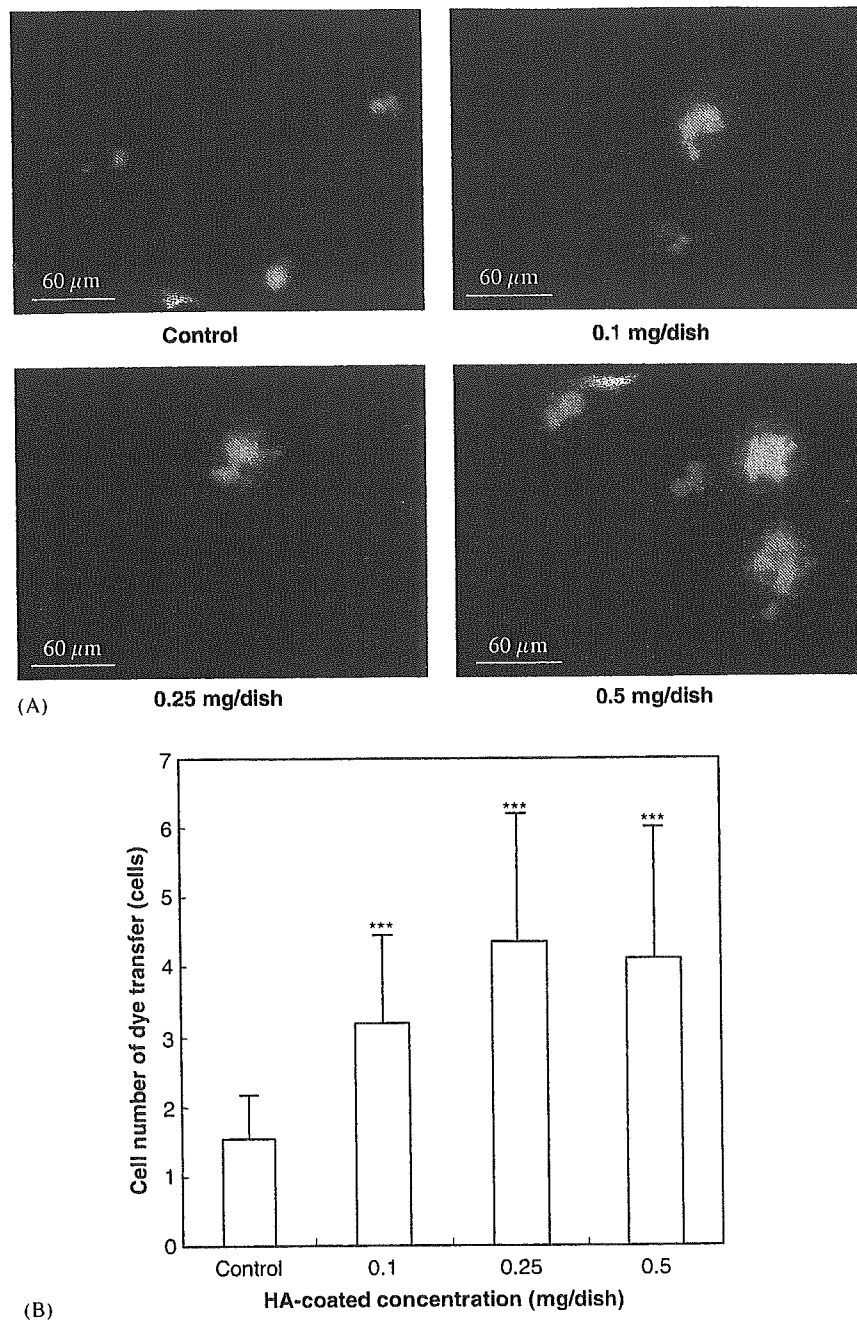


Fig. 4. Concentration-dependent effects of HA-coating on dye transfer in HIT-T15 cells. Cell adherent to glass coverslips were microinjected with 4% Lucifer yellow. Transfer of dye to neighboring cells was assessed by epifluorescence microscopy 2 min later. This is a representative expression of 18 injections per group (A). The number of neighboring cells that received dye was quantified (B). Each value expressed as the mean \pm S.D. ($n = 18$). *** $P \leq 0.001$ compared to control.

application method and concentration are important factors for generating biocompatible tissue-engineered products.

It has been reported that single β -cells (which cannot form gap junctions) show alterations in both basal and stimulated release of insulin, in protein biosynthesis, and in the expression of the insulin gene. The sustained stimulation of insulin release is associated with an increase in β -cells coupling, in the expression of gap junctions by a

unique mechanism for direct equilibration of ionic and molecular gradients between nearby cells [14–16]. In this study, we found that the insulin release and insulin content are increased and GJIC activity was enhanced in cultured HIT-T15 cells by low concentration HMW HA-coating in spite of the inhibitory effects on the cell viability in high concentration HA-coating dishes. This finding was consistent with previous reports. The effect of HA may be influenced by the viscosity of HA, the concentration of

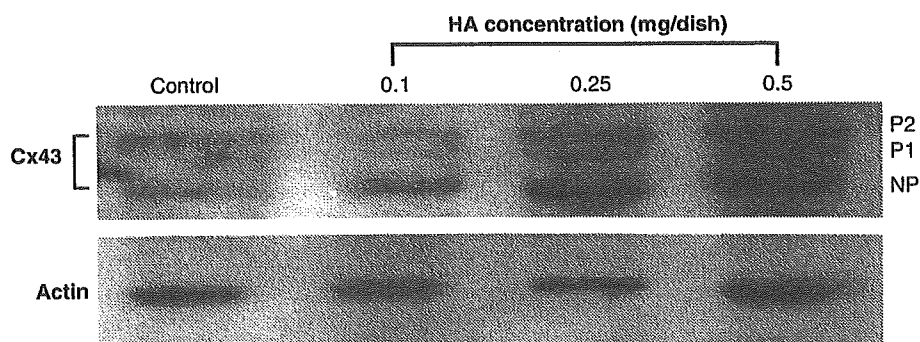


Fig. 5. Identification of Cx43 in HIT-T15 cells grown on the HA-coating dish by Western blot analysis. After HIT-T15 cells were incubated into HA-coated dish for 24 h, cells were lysed and proteins (20 μ g) were separated by SDS-PAGE followed by Western blotting using rabbit anti-Cx43 antibody. Actin immunostaining was used to assess equivalent protein loading. This is a representative autoradiogram of three experiments.

FBS and the nutrients in media such as hormone, growth factor (FGF, etc.), cell adhesion molecule (N-CAM and cadherins), and transportation protein [6,17]. As a result, the HIT-T15 cells can use these nutrients and the nutrient-enriched substrata (e.g. natural ECMs) by ionic interaction and the binding of HMW HA to various kinds of cytokines, to change the cell aggregations, resulting in the increase of GJIC. With the evidence above, the enhancement of GJIC activity induced by HA-coating participated in the regulation of insulin release and insulin biosynthesis. On the other hand, the glucose stimulus-secretion coupling in β -cells generated several signals, including a signal to secrete preformed insulin stored in secretory vesicles, a signal, which may be the same or different, to secrete newly made insulin, and a signal to synthesize more insulin. The mechanism of glucose-induced insulin secretion is distinct from that of glucose-induced proinsulin biosynthesis and insulin gene transcription [18]. Moreover, the qualities of ECM affect the insulin release [19]. Therefore, it is possible that HA-coated dishes promoted a large increase in insulin synthesis but only a modest increase in insulin release. The detailed action mechanism should be investigated in the next study.

In native and tumoral insulin-producing pancreatic β -cells, gap-junction protein Cx43 has been identified. Furthermore, the stable transfection of the gene coding for Cx43 induces the expression of functional gap-junction channels and improves both the biosynthetic and secretory defects of the cells. Cx43-transfection and incidence of junctional coupling also secrete more insulin than wild-type and noncommunicating cells, the absence of Cx43 implicated in the loss of β -cell-specific functions in vitro and in vivo [9,14]. In this study, HA-coating expressing high levels of the Cx43, gap junctions, and coupling, showed the striking enhancement of the amounts of stored hormone in HIT-T15 cells and promoted the glucose-induced insulin release, indicating that adequate levels of Cx43 and coupling are required for proper insulin production. These results provide further evidence that HA-coating increases the pancreatic β -cells function by enhancing the function of Cx43-mediated GJIC.

5. Conclusion

In conclusion, the function of GJIC is considered to be a useful marker for evaluating tissue-engineered products. The data obtained in this study show that gap junctions contribute to regulating some still-unknown mechanism to couple the stimulus-secretion of HIT-T15 cells under the condition of low concentration HA-coating. The growth regulation with a bioartificial pancreatic construct using HA is achievable. These results give useful information on design biocompatibility of HA when the HA is used as a biomaterial for bioartificial pancreas. HA-coating may be a new technique for constructing three-dimensional bioartificial pancreas in tissue engineering.

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Hydroxy apatite microspheres enhance gap junctional intercellular communication of human osteoblasts composed of connexin 43 and 45

Ryusuke Nakaoka, Saifuddin Ahmed, Toshie Tsuchiya

Division of Medical Devices, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan

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Abstract: The aseptic loosening of artificial joints with associated periprosthetic bone resorption may be partly due to the suppression of osteoblast function to form new bone by wear debris from the joint. To assess the effect of wear debris on osteoblasts, effects of model wear debris on gap junctional intercellular communication (GJIC) of normal human osteoblasts were estimated. The GJIC activity of the osteoblasts after a 1-day incubation with the microspheres was similar to that of normal osteoblasts. However, hydroxy apatite particles, which have been reported to enhance the differentiation of osteoblasts in contact with them, enhanced the GJIC function of the osteoblasts. From RT-PCR studies, not only connexin 43 but also connexin 45 is suggested to play a role in the GJIC of the osteoblasts in an early stage of

coculture with the microspheres, although it is still unclear how these connexins work and are regulated in the GJIC and differentiation. However, this study suggests that there is a relationship between the early levels of GJIC and the differentiation of the cells. Therefore, estimating the effect of biomaterials, even in the microsphere form, on the GJIC of model cells, with which the biomaterials may be in contact *in vivo*, can provide important information about their biocompatibility. © 2005 Wiley Periodicals, Inc. *J Biomed Mater Res* 74A: 181–186, 2005

Key words: gap junctional intercellular communication; human osteoblasts; microspheres; hydroxy apatite; connexin

INTRODUCTION

Biomaterials implanted into the harsh environment of the body cannot maintain their original shape, or even their desired function, sometimes resulting in undesirable side effects. One well-known example is the aseptic loosening of artificial joints observed in many patients who underwent a total joint replacement 5 to 25 years ago. It has already been reported that aseptic loosening with associated periprosthetic bone resorption is partly due to the activation of macrophages and osteoclasts by wear debris from the artificial joint.^{1–14} Macrophages stimulated by wear debris *in vitro* release significant amounts of inflammatory mediators such as interleukin-1, interleukin-6, prostaglandin E2, collagenase, and tumor necrosis factor.^{6–14} In addition, the biological effects of wear debris may depend on the type of material used as well

as the shape, size, and amount of the debris.^{4–11} Therefore, it is important to estimate the biocompatibility of biomaterials with not only their original shape but also possible transformed shapes after their usage.

During the last decade, we have been researching the inhibitory potential of many kinds of biomaterials on gap junctional intercellular communication (GJIC) as an index for their biocompatibility.^{15–18} GJIC is a function that plays an important role in maintaining cell and tissue homeostasis by exchanging low molecular weight molecules, which results in regulating cell growth, development, and differentiation of cells.^{19,20} Therefore, it is reasonable that disruption of this function is the cause of many kinds of diseases. In a previous report,¹⁸ we examined the inhibitory activity of polymer microspheres, which were used as model wear debris from biomedical polymer *in vivo*, on the GJIC of rodent-derived fibroblasts. We concluded that estimating the inhibitory activity of the microspheres on the GJIC might be useful for considering their side effects in the body. In other words, it may be possible to predict whether wear debris causes aseptic loosening of artificial joints by estimating their effect on GJIC function.

No benefit of any kind will be received either directly or indirectly by the authors

Correspondence to: R. Nakaoka; e-mail: nakoaka@nihs.go.jp

However, it must be noted that the effects of the microspheres may be different when the effects on the GJIC of human-derived cells are estimated. Osteoblasts have been reported to communicate with one another via GJIC function, and the function is believed to be critical to the coordinated cell behavior necessary in bone tissue development.^{21,22} Therefore, the question is raised whether wear debris has an inhibitory effect on the GJIC and the GJIC inhibition has a relation with the aseptic loosening of artificial joints. Because we have already observed some precoated polymer microspheres around 5 μm in diameter showed the potential to inhibit GJIC of fibroblasts contacting with them,²³ we estimated effects of various microspheres around 5 μm in diameter on GJIC function using normal human osteoblasts to discuss the relationship between the GJIC and the differentiation of osteoblasts. In this study, we employed fluorescence recovery after photobleaching (FRAP) analysis for estimating the GJIC function,¹⁷ and assessed the potential effect of many kinds of microspheres on the GJIC.

MATERIALS AND METHODS

Microspheres

Monodispersed polystyrene (PS) microspheres (5 μm in diameter) were purchased from Japan Synthetic Rubber Co., Ltd. (Tokyo, Japan). Low-density polyethylene (PE) microspheres were generously supplied by Sumitomo Seika chemicals Co., Ltd. (Tokyo, Japan). Alumina (Al_2O_3) microspheres were obtained from the Association of Powder Process Industry and Engineering. Sintered hydroxy apatite microspheres (HA, 7.2 μm in diameter) were prepared and supplied by Ube Material Industries, Ltd. A Multisizer II (Coulter Electronics Inc., Hialeah, FL) was used to determine the average diameter of PE and alumina microspheres: 6.4 and 5.1 μm , respectively. Microspheres were sterilized by dispersing them in a 70% ethanol solution, followed by centrifugation in sterile conditions to remove the ethanol solution. The microspheres were dispersed in sterile methanol for cell differentiation tests at specified concentrations. The suspension of microspheres in methanol was added to 35-mm type I collagen-coated cell culture dishes (Asahi techno glass, Chiba, Japan), and the plates dried overnight at room temperature. The obtained microsphere-coated dishes (100 $\mu\text{g}/\text{dish}$) were subjected to the assays.

Cell culture

Normal human osteoblasts (NH₂Ost) were purchased from BioWhittaker Inc. (Walkersville, MD). The standard culture of NH₂Ost was performed using alpha minimum essential medium (Gibco) 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. NH₂Ost (1×10^5 cells/dish/2.5 mL medium) were cultured on microsphere-coated dishes for estimating the effect of the microspheres interacted from the bottom of the cells. To estimate the effect of microspheres on cells adhered to the culture plates, the NH₂Ost cells were cultured with microsphere-containing medium (100 $\mu\text{g}/2.5$ mL medium) after they had adhered to the collagen-coated dishes. The test cells were cultured while changing the medium three times when the measurement of GJIC was performed after a 7-day incubation.

Measurement of GJIC activities

NH₂Ost cultured with microspheres were subjected to fluorescence recovery after photobleaching (FRAP) analysis to estimate the inhibitory activity of these microspheres toward the GJIC. FRAP analysis was carried out according to an original procedure by Wade et al.,²⁴ with some modifications.¹⁷ Briefly, NH₂Ost were plated on microsphere-coated dishes and incubated for 1 or 7 days. After a wash with phosphate buffer saline (PBS) containing MgCl_2 and CaCl_2 [PBS(+)], the cells were incubated for 5 min at room temperature in PBS(+) containing 5,6-carboxyfluorescein diacetate (7 $\mu\text{g}/\text{mL}$, excitation 488 nm and emission 515 nm). After the washing off of excess extracellular dye with PBS(+), the cells in the test dishes in PBS(+) were subjected to the FRAP analysis. In the control experiment, cells were inoculated on an untreated glass bottom dish and treated with the same procedure as the tested cells. Cells in contact with test microspheres and at least two other cells were subjected to FRAP analysis under an Ultima-Z confocal microscope (Meridian Instrument, Okemos, MI) with a 10 \times objective lens at room temperature. The cells were photobleached with a 488-nm beam and the recovery of fluorescence intensity was subsequently monitored at 1-min intervals for a total period of 4 min. The data obtained from more than seven independent cells were expressed as the average of fluorescence recovery rate in comparison to the rate obtained from NH₂Ost cultured without microspheres.

Effect of microspheres on calcium deposition by NH₂Ost

The amount of calcium deposited during a 7-day incubation of the cells were evaluated as follows: NH₂Ost were cocultured with either precoated or added microspheres in 24-well collagen-coated culture plates (Asahi techno glass, Chiba, Japan) for 1 week (2×10^4 cells/20 μg microspheres/well/500 μL medium). After the cells were fixed in formaldehyde, 0.5 mL of 0.1 M HCl was added to each well after washing the cells with PBS. The amounts of calcium dissolved in HCl were estimated using a Calcium detecting kit (Calcium-C test Wako, Wako, Osaka, Japan) according to the manufacturer's direction.

RT-PCR for estimating expression of connexins

According to the method reported by Ichikawa et al.,²⁵ RT-PCR was performed to detect the expression of connexin mRNA in NHOst. After culturing NHOst with microspheres for a scheduled time, total RNA was extracted from the NHOst using TRIZOL[®] reagent (Invitrogen Corp., Carlsbad, CA) according to the manufacturer's instructions. After dissolving the RNA in diethylpyrocarbonate-treated water, the total RNA concentration was measured spectrophotometrically using Genequant (Amersham Biosciences Corp., Piscataway, NJ). RNA samples were adjusted to a minimum concentration among collected samples in each experiment and reversibly transcribed to cDNA using Superscript[™] II (Invitrogen Corp.). For PCR amplification of human connexin 45, Takara Ex-Taq[™] (Takara Shuzo Co., Ltd., Shiga, Japan) was used with Ex-Taq[™] buffer consisting of 20 pmol each of two human connexin-45 specific primers (forward 5'GTGGCAACTCCCTCTGTGAT3' and reverse 5'GGATCCTCAAGTTCCTCCT3'). For PCR amplification of human connexin 26, 32, and 43, Takara LA-Taq[™] (Takara Shuzo Co., Ltd.) was used with Ex-Taq[™] buffer consisting of 6 pmol each of the human connexin-specific primers (for connexin 26, forward 5'ATGGATTGGGGCACGC3' and reverse 5'TTAAACTGGCTTTTTGACTTCCC3'. For connexin 32, forward 5'ATGAACTGGACAGGTTTGTACACCTTGCTC3' and reverse 5'TCAGCAGGCCGAGCAGCGG3'. For connexin 43, forward 5'ATGGGTGACTGGAGCCCTTAGGC3' and reverse 5'CTAGATCTCCAGGTCATCAGGCCG3'). The PCR profile for connexin 45 involved pretreatment at 95°C for 2 min, followed by 35 cycles of denaturation at 95°C for 45 s, annealing at 54°C for 45 s, and extension at 72°C for 90 s. The PCR profile for connexin 26, 32, and 43 (35 times) was as follows: pretreatment at 95°C for 2 min, denaturation at 95°C for 30 s, annealing at 54°C for 30 s, and extension at 72°C for 120 s. Reaction products were analyzed by electrophoresis in 1.5% (w/v) agarose gel, followed by staining of

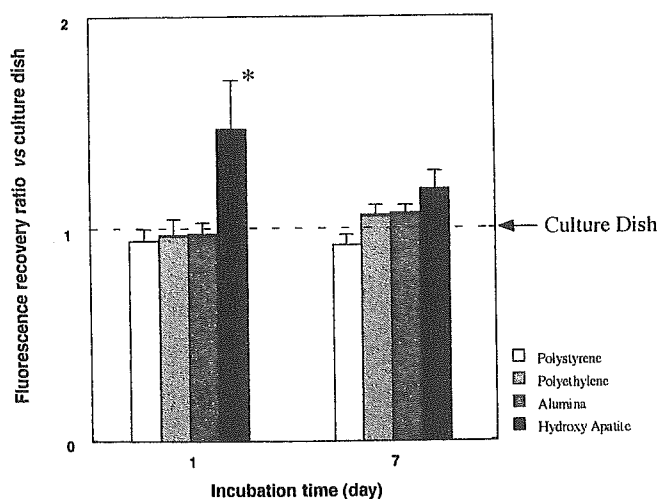


Figure 1. Effect of precoated microspheres on gap junctional intercellular communication of NHOst estimated from fluorescence recovery rates of target cells. The recovery rates of the cells on untreated culture dishes on days 1 and 7 were used as standards of all obtained data, respectively. (* $p < 0.01$ against culture dish).

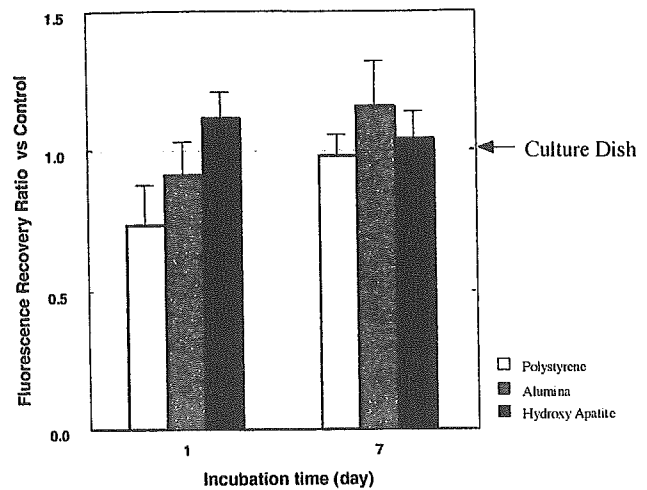


Figure 2. Effect of added microspheres on gap junctional intercellular communication of NHOst estimated from fluorescence recovery rates of target cells. The recovery rates of the cells on untreated culture dishes on days 1 and 7 were used as standards of all obtained data, respectively.

the products by SYBR[®] Green I (Takara Shuzo Co., Ltd.) and detection of a 566-bp (connexin 45), 671-bp (connexin 26), 852-bp (connexin 32), and 1149-bp (connexin 43) band, respectively. For the standardization of connexin cDNA, PCR amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA in each sample was performed using GAPDH-specific primers (forward 5'CCCATCACCATCTTCCAGGAGCGAGA3' and reverse 5'TAAGTAGGACAA-CAAGGAGGTCGTGACGACGC3'; product size 578-bp). All reactions included negative controls without cDNA.

Statistical analysis

All data were expressed as the mean value \pm the standard error of the means of the obtained data and treated statistically with Student's *t* test.

RESULTS

Figure 1 shows effects of various microspheres on GJIC of NHOst in contact with the microspheres for 1 and 7 days. The microspheres were precoated on 35-mm culture dishes before cell seeding. When the NHOst were cultured with precoated PS, PE, and alumina microspheres, their GJIC level was similar to that in NHOst cultured on a normal culture dish. On the other hand, the GJIC level was 1.5 times that of NHOst on the normal dish when they were cultured with precoated hydroxy apatite microspheres. After 7 days, the GJIC of NHOst in contact with microspheres became similar to that of normal NHOst, irrespective of the type of microsphere. The change in GJIC of NHOst in contact with added microspheres is shown in Figure 2. As seen in Figure 1, hy-

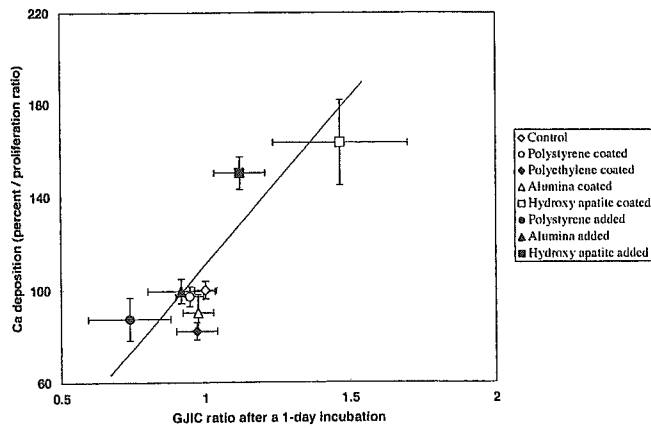


Figure 3. Relationship between GJIC on day 1 and calcium deposition ratio after 7-day coculture of NHOst with various microspheres ($r^2 = 0.74$).

droxy apatite microspheres enhanced their GJIC after a 1-day culture compared to cells on a normal plate. The degree of enhancement of GJIC is, however, smaller than that seen in NHOst in Figure 1, and no significant difference was observed between NHOst in contact with the hydroxy apatite microspheres and those cultured without microspheres. In addition, Figure 2 indicates that addition of PS microspheres into a culture of NHOst inhibited GJIC.

To consider the effects of tested microspheres on not only GJIC but also the differentiation of NHOst, changes in the amount of calcium deposited after a 1-week coculture of NHOst with various microspheres were estimated. From Figure 3, it is suggested that there is the possible relation between the GJIC of NHOst cocultured with microspheres for 1 day and

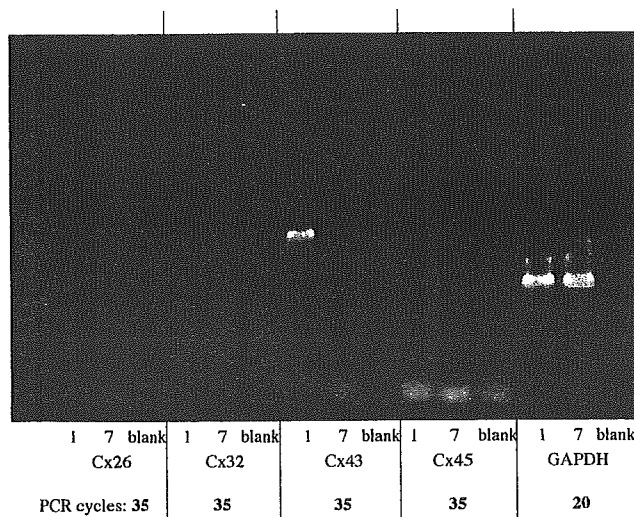


Figure 4. Expression of mRNA of various connexins (Cx) in NHOst cultured for 1 and 7 days. The number of NHOst cultured on 35-mm collagen-coated culture dishes was 2×10^5 . RT-PCR cycles of each lane are expressed at the bottom of the figure.

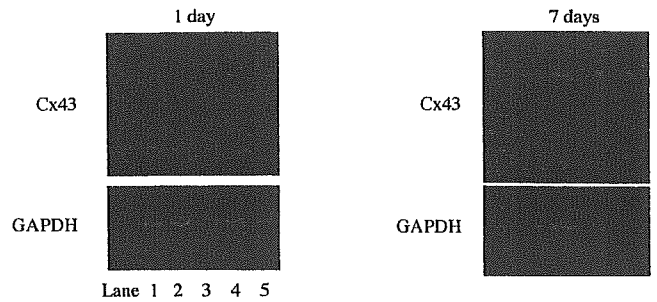


Figure 5. Expression of connexin 43 (Cx 43) mRNA in NHOst cultured with various precoated microspheres. The number of PCR cycles for connexin 43 and GAPDH is 35 and 20, respectively. Lane 1: without microspheres; lane 2: with PS microspheres; lane 3: with PE microspheres; lane 4: with alumina microspheres; lane 5: with HA microspheres.

the amount of calcium deposited after a 1-week coculture with the same microspheres.

To clarify which connexins exist in NHOst, we performed RT-PCR to detect mRNA of connexin 26, 32, 43, and 45 in NHOst cultured on a normal culture dish. Figure 4 shows the result of RT-PCR to amplify the mRNA from whole RNA collected from NHOst cultured for 1 and 7 days. As shown in the figure, only connexin 43 and 45 were detected in NHOst. When cells were cultured for 7 days, connexin 43 was detected at a lower level than that detected after the 1-day culture, while connexin 45 was not detected.

Figures 5 and 6 show the results of RT-PCR to amplify mRNA of connexin 43 and 45 in NHOst cultured with various precoated microspheres. The NHOst cultured with microspheres did not express mRNA of connexin 43, except those with PE microspheres. After 7 days, the expression was suppressed in the normal NHOst while the expression was observed in NHOst cultured with microspheres, irrespective of kind of the microsphere. On the other hand, mRNA expression of connexin 45 was suppressed after a 1-day culture of NHOst only with alumina microspheres, followed by a decrease in expression of the mRNA after their 7-day culture.

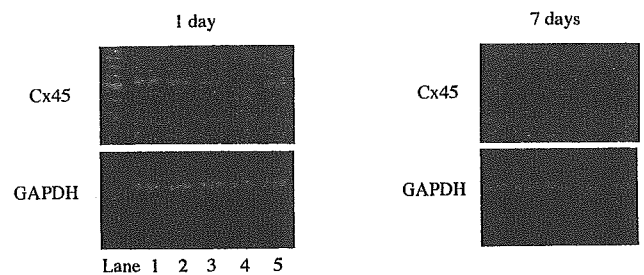


Figure 6. Expression of connexin 45 (Cx 45) mRNA in NHOst cultured with various precoated microspheres. The number of PCR cycles for connexin 45 and GAPDH is 40 and 20, respectively. Lane 1: without microspheres; lane 2: with PS microspheres; lane 3: with PE microspheres; lane 4: with alumina microspheres; lane 5: with HA microspheres.

DISCUSSION

As shown in Figure 3, normal human osteoblasts (NHOst) in contact with microspheres showed different levels of calcium deposition only after a 1-week culture, suggesting composition of the microspheres affects NHOst differentiation level. The differentiation was suppressed by the contact with PS, PE, and alumina microspheres, while HA microspheres showed the potential to enhance the differentiation. It has been reported that GJIC plays an important role in not only the homeostasis of cells but also their differentiation.¹⁹⁻²² In addition, GJIC is affected by the microsphere's composition, as has been reported using a fibroblast cell line.¹⁸ Therefore, the results shown in Figures 1 and 2 suggest that the enhanced differentiation of NHOst relates to GJIC enhancement on a 1-day culture in contact with HA microspheres, especially the precoated microspheres. In addition, on coculture with other microspheres, GJIC was slightly suppressed at 1 day, although no significant difference compared to control NHOst was observed. We have already studied effects of the microspheres on NHOst differentiation, and enhancement of calcium deposition by coculture with the hydroxy apatite microspheres was observed. Figure 3 suggests a relationship between the calcium deposition and GJIC on day 1. This also indicates that GJIC of the NHOst, in contact with materials in the microsphere form, in the early stage may be one factor affecting their differentiation.

It has been reported that GJIC of cells derived from human osteoblasts is mainly composed of connexin 43 and 45.^{22,26,27} In this study, it is also indicated that GJIC of NHOst is composed of connexin 43 and 45 (Fig. 4). Therefore, it is possible that changes in the level of their GJIC is ascribed to the change in mRNA expression level of connexin 43 and 45 and their expression ratio. From Figures 5 and 6, mRNA of connexin 43 was expressed only in normal NHOst and those cultured with PE microspheres, while it was slightly expressed in NHOst cocultured with HA. On the other hand, mRNA of connexin 45 was expressed in NHOst in all conditions, except those cocultured with alumina microspheres. Because HA was observed to enhance GJIC of NHOst, this suggests that connexin 45 may play a role in GJIC at an early stage. This also suggests that a higher level of connexin 45 than that of connexin 43 may be important in the enhancement of GJIC. However, although the mRNA expression of neither connexin 43 nor 45 was observed in NHOst cocultured with alumina microspheres, their GJIC was similar to that of normal NHOst. Moreover, it has reported that gap junctions formed by connexin 43 are more permeable to negatively charged dyes such as lucifer yellow, calcein, and carboxyfluorescein used in this study, more than those formed by

connexin 45, and an increase of connexin 43 expression and GJIC function parallel osteoblast differentiation.^{22,28} These are inconsistent with our findings and indicate that not high expression, but a rapid decrease of connexin 45 mRNA is probably very important for GJIC change and differentiation of the osteoblasts. Therefore, even though connexin 45 may play an important role in the early stage of GJIC in NHOst, it is probable that another connexins or other mechanisms of GJIC play a role in the GJIC of NHOst.

Because many proteins are involved in GJIC formation,²⁸ other mechanisms or proteins may be important in the GJIC change induced by the contact with the microspheres. It has reported that cadherins, which are important proteins for form tight junction between cells, control connexin 43-mediated GJIC.^{29,30} In addition, a microtubule network inside a cell has been reported to play an important role as guidance for delivery of connexons, which are composed of six connexin molecules, to the cell membrane to make gap junctions.³¹ Usually, surface characteristics of materials affect cell attachment as well as cell morphology, suggesting signal cascades of cell attachment and cytoskeleton rearrangement in the cell were influenced by the characteristics. Therefore, it is probable that a surface characteristic of the microspheres affect these molecules in NHOst, resulting in changes of GJIC activities. Further studies on changes in not only connexin molecules but also other molecules such as cadherin, actin, and microtubule in NHOst, is necessary to clarify the mechanism of GJIC. In the future, we will study the above, and find another molecules participating in the GJIC of NHOst and the mechanisms regulating the connexins in NHOst.

In conclusion, the GJIC level of NHOst changes on contact with microspheres, and is affected by the composition of the microspheres. The GJIC level in the early stage might be important in the differentiation control of NHOst and the level may be controlled partly by expression of connexin 43, connexin 45, and unclarified connexins in addition to other mechanisms regulating GJIC function. Detecting a biomaterial's effect on the GJIC of human cells may be one useful method for estimating its biocompatibility.

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The response of normal human osteoblasts to anionic polysaccharide polyelectrolyte complexes

Misao Nagahata^{a,b,1}, Ryusuke Nakaoka^{a,1,*}, Akira Teramoto^b,
Koji Abe^b, Toshie Tsuchiya^a

^aDivision of Medical Devices, National Institute of Health Sciences, 1-81-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan

^bDepartment of Functional Polymer Science, Faculty of Textile Science and Technology, Shinshu University, Ueda 386-8567, Japan

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Abstract

Polyelectrolyte complexes (PEC) were prepared from chitosan as the polycation and several synthesized functional anion polysaccharides, and their effects on cell attachment, morphology, proliferation and differentiation were estimated using normal human osteoblasts (NH₂Ost). After a 1-week incubation, PEC made from polysaccharides having carboxyl groups as polyanions showed low viability of NH₂Ost on it although the NH₂Ost on it showed an enhancement in their differentiation level. On the other hand, NH₂Ost on PEC made from sulfated or phosphated polysaccharides showed similar attachment and morphology to those on the collagen-coated dish. When the number of NH₂Ost was estimated after 1 week, the number on the PEC was ranged from 70% to 130% of those on the collagen-coated dish, indicating few effects of these PEC on cell proliferation. In addition, NH₂Ost on PEC films made from sulfated polysaccharides differentiated to a level very similar to that observed on the collagen-coated dish, indicating that these PEC films maintain the normal potential of NH₂Ost to both proliferate and differentiate. Measurement of gap junctional intercellular communication of NH₂Ost on PEC revealed that PEC did not inhibit communication, suggesting that PEC films have few effects on cell homeostasis. Thus, PEC made from the sulfated polysaccharide may be a useful material as a new scaffold for bone regeneration.

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Keywords: Polyelectrolyte complex; Normal human osteoblasts; Cell proliferation; Cell differentiation; Gap junctional intercellular communication

1. Introduction

The extracellular matrix (ECM) provides an essential three-dimensional (3D) environment for cells to construct several kinds of tissues. The ECM, consisting of numerous kinds of molecules such as proteins, polysaccharides and proteoglycans regulates the behavior of surrounding cells to form tissues and organs precisely [1,2]. For tissue regeneration trials using *in vitro*

techniques, therefore, it is indispensable to develop a synthetic ECM scaffold that functions similarly to the native ECM. For more than a decade, engineering of new tissues by using selective cell transplantation on polymer scaffolds as an artificial ECM instead of tissue transplantation to other living bodies has been studied [3,4]. Recently, many studies on developing a scaffold for tissue regeneration have been done using ECM proteins such as collagen and gelatin [5–7], biodegradable synthetic polymers [8–10] and polysaccharides [11,12]. Because proteins derived from human tissues have many problems such as antigenicity or potential for infection, a biocompatible synthetic polymer or polysaccharide may be preferable for tissue regeneration.

*Corresponding author. Division of Medical Devices, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan. Tel.: +81 3 3700 9264; fax: +81 3 3707 6950.

E-mail addresses: nagahata@nihs.go.jp (M. Nagahata), nakaoka@nihs.go.jp (R. Nakaoka).

¹These authors contributed equally to this work.

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].

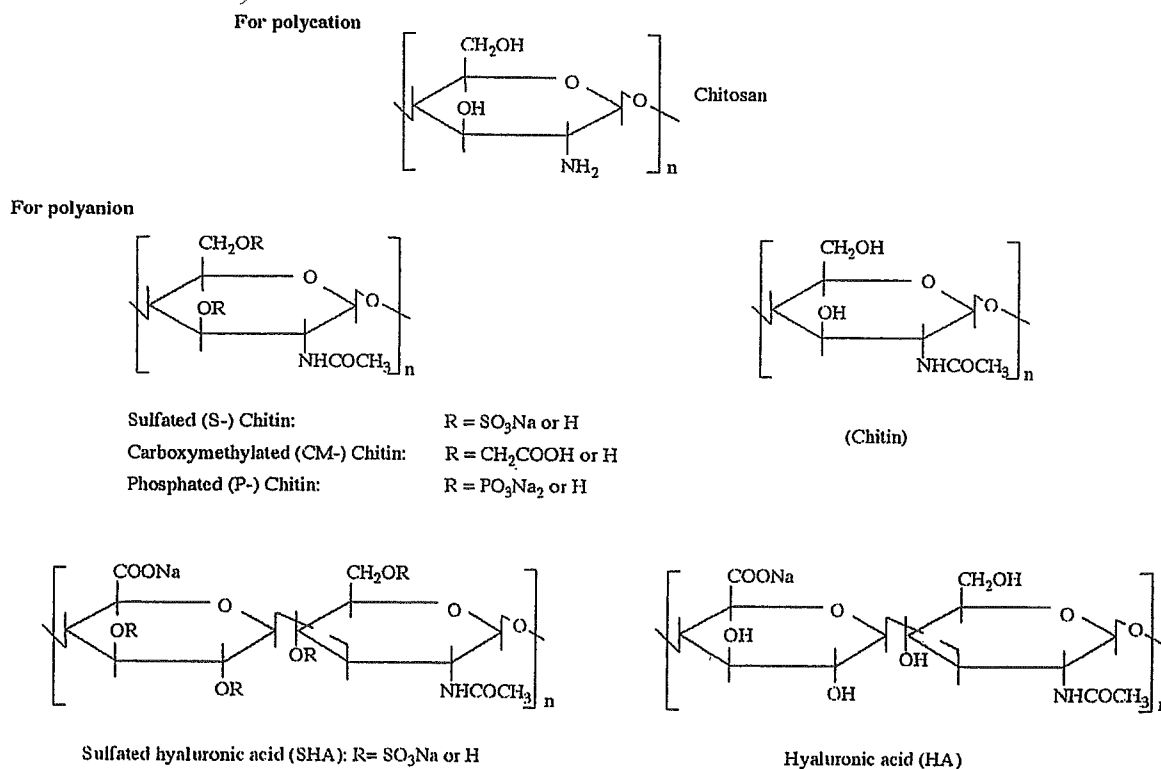


Fig. 1. Polymers for polyelectrolyte complex (PEC) in this study.

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% CO₂–95% 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 MgCl₂, 0.1 mM ZnCl₂ 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 Ca²⁺ and Mg²⁺ (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.

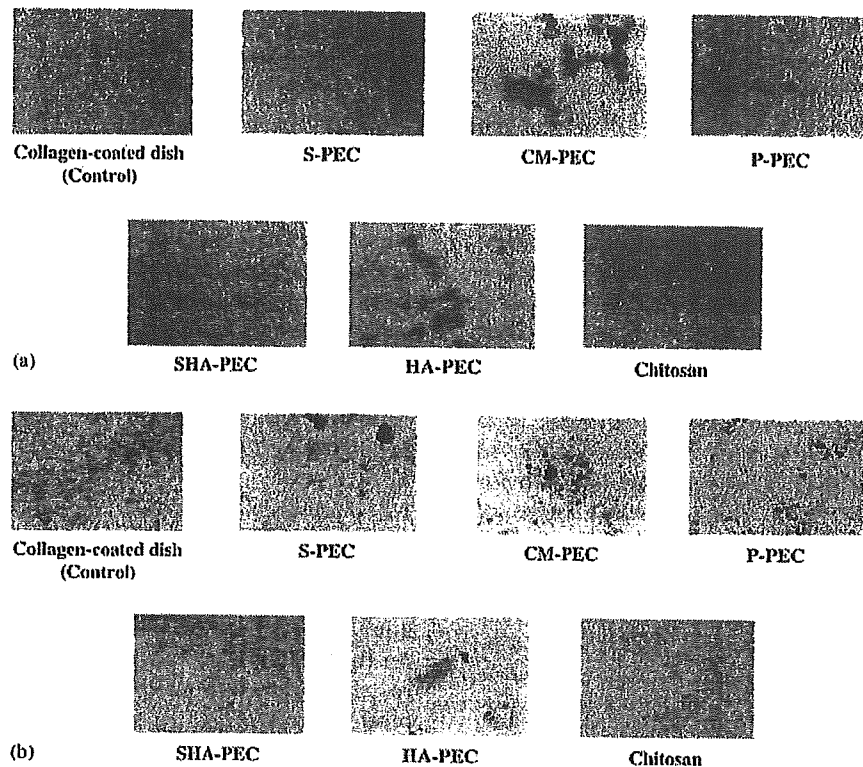


Fig. 2. Light micrographs of normal human osteoblasts (NHOst) on various PEC films after a 2-day incubation: (a) and 1-week incubation, (b). (Original magnification: $\times 100$).

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

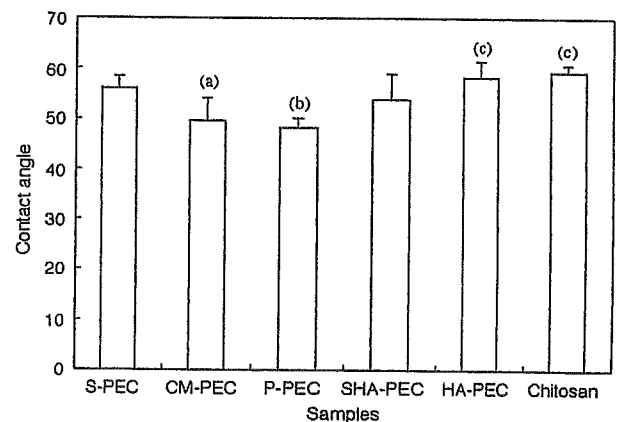


Fig. 3. Contact angles of PEC films studied: (a) $p < 0.05$ against S-PEC, (b) $p < 0.01$ against S-PEC, (c) $p < 0.01$ against both CM-PEC and P-PEC.

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

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

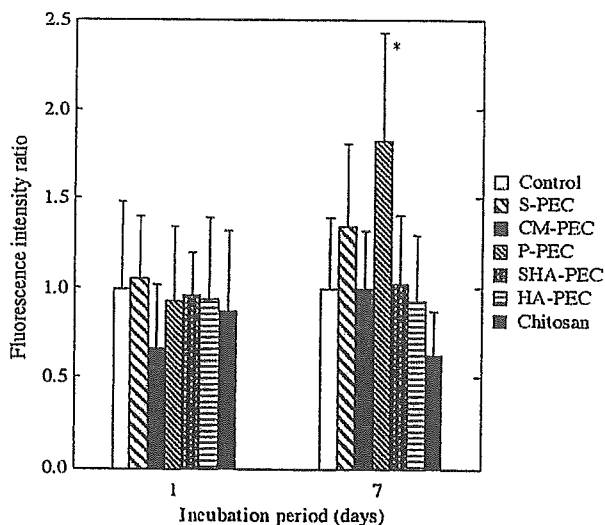


Fig. 4. Gap junctional intercellular communication activity of NHOst on various PEC films estimated by FRAP analysis technique. (* $p < 0.01$ against control).

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.

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

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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
^aisama@nihs.go.jp, ^btsuchiya@nihs.go.jp

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

Abstract. The effects of the γ -irradiated 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 -irradiated 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 ⁶⁰Co 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 μ l 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% CO₂.

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% CO₂, 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),

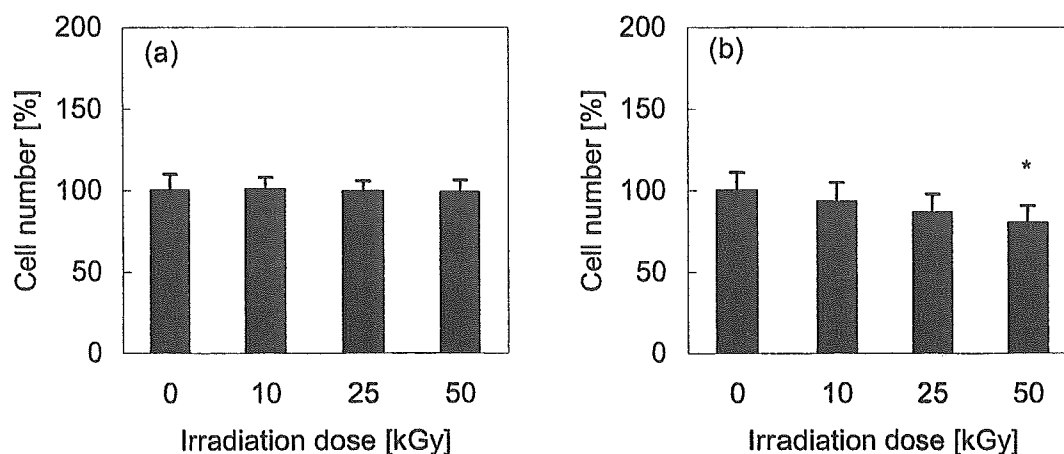


Fig. 1. The cell numbers of (a) MC3T3-E1 and (b) NHOst cells cultured on the γ -irradiated PLLA sheet.

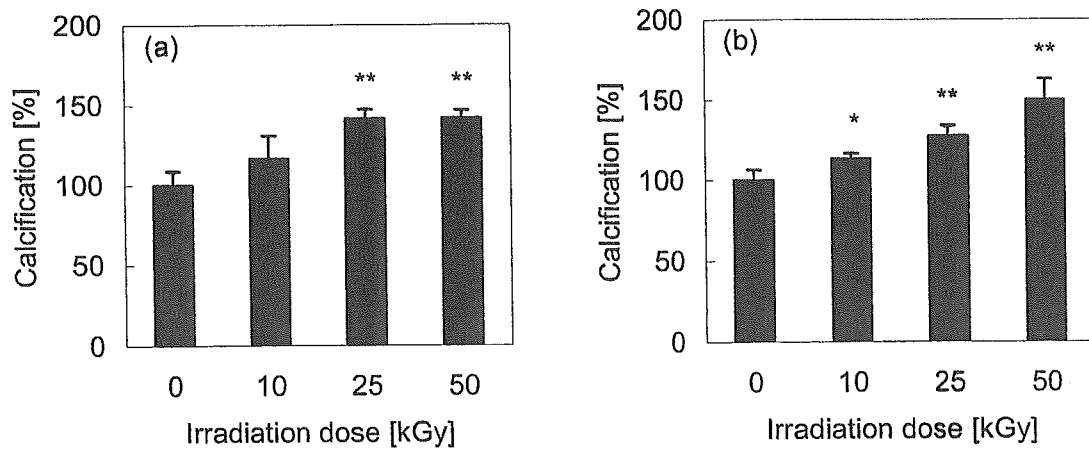


Fig. 2. The calcifications of (a) MC3T3-E1 and (b) NHOst cells cultured on the γ -irradiated PLLA sheet.

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 ratios 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.

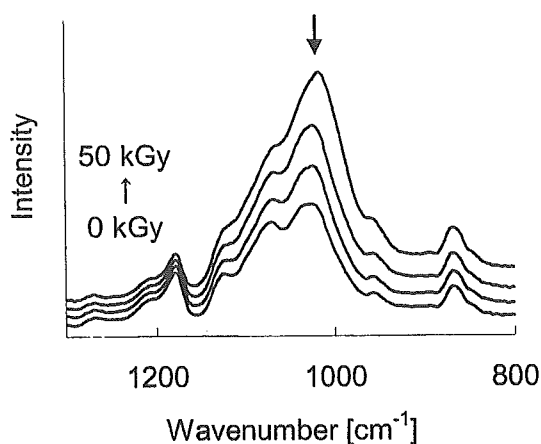


Fig. 3. The phosphate band of the γ -irradiated PLLA sheet after soaking in the medium.

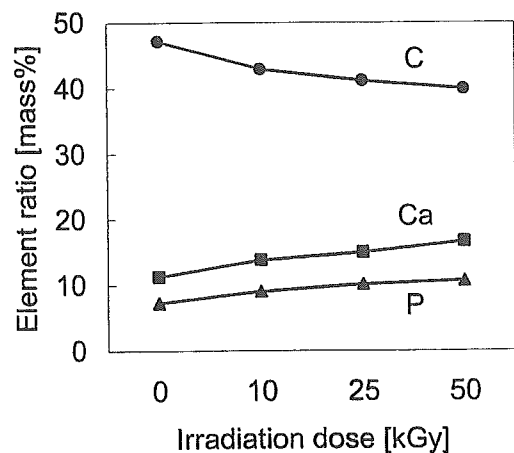


Fig. 4. The element ratios of calcium, phosphorus and carbon of the γ -irradiated PLLA sheet after soaking in the medium.