Biocompatibility of Various Kinds of Polymer Microspheres Estimated from Their Effect on Gap Junctional Intercellular Communication of Fibroblasts

Ryusuke Nakaoka * and Toshie Tsuchiya

Division of Medical Devices, National Institute of Health Sciences, Tokyo 158-8501, Japan

Gap junctional intercellular communication is a function that plays an important role in maintaining cell and tissue homeostasis and in regulating cell growth, development, and differentiation. Change in this function when contacting fibroblasts with various polymer microspheres was estimated using the fluorescence recovery after photobleaching (FRAP) assay system. When the cells were in contact on test dishes, the inhibition level increased as the diameters of polystyrene microspheres decreased, except for a microsphere with 0.5 µm diameter. The function was inclined to be recovered with the increase of the incubation time, while it was not recovered when the cells were cultured with pre-coated polystyrene microspheres. As well as inhibitory activities of the function, cytotoxicity potentials of tested microspheres depended on their diameter and their composition. These findings suggest that the size and the physico-chemical character of polymer microspheres, and how cells recognize them plays important roles in causing influences of the microspheres on both gap junctional intercellular communication and their cytotoxicity. Therefore, estimating the inhibitory effect of biomaterials on the gap junctional intercellular communication will provide valuable information about the biocompatibility of materials even in the form of particles.

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1. Introduction

It is important for biomaterials to make them safer and more effective in order to use them for not only for tissue engineering studies but also studies of artificial organs or orthopedic devices. It is indispensable for biomaterial development to estimate their toxicity and dysfunction on cellular level as well as human body.

It is well known that biomaterials implanted into body, due to a severe environment of inside of body, cannot maintain their original shape, and even their desired function in the body, sometimes resulting in undesirable side effects. The most famous case is aseptic loosening of artificial joint observed in many patients who underwent operation of total joint replacement 5 to 25 years ago. Many researchers have reported that the aseptic loosening with associated periprosthetic bone resorption is partly due to activation of macrophages and osteoclasts by wear debris from artificial joint.1-14) Macrophages stimulated with 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) The biological effect of wear debris may depend on the type of materials used as well as the shape, size, and the amounts of the debris.4-11) Therefore, it is very important to estimate 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) to use the potential 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, that 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 the previous report,18) we have estimated an inhibitory activity of polymer microspheres, which were used as model wear debris from biomedical polymer in vivo, on the GJIC using a metabolic cooperation assay system that is a method for estimating the GJIC function of Chinese hamster-derived fibroblasts. We concluded that estimating the inhibitory activity of the microspheres on the GJIC might be useful for considering their side effects in body. In other words, it may be possible to predict whether wear debris causes aseptic loosening of artificial joint from estimating their effect on GJIC function. However, it must be noted that a distribution of coated microspheres in each test wells may be different in spite of the same amounts of coated microsphere, because the method in previous study was carried out without any special equipments useful for homogeneous coating. Different distribution of coated microspheres makes reproducibility of experiments poor because the probability of contacting cell and microspheres will be affected by the distribution. To solve this problem, we have employed fluorescence recovery after photobleaching (FRAP) analysis technique for estimating the GJIC function.21) Using the FRAP analysis, the inhibitory potential of many kinds of polymer microspheres, as model wear debris, on the GJIC was assessed, and effects of characteristics of the microspheres and conditions of interaction between cells and microspheres on the GJIC were discussed.

2. Materials and Methods

2.1 Microspheres

Monodispersed polystyrene (PS) microspheres with different diameter were purchased and kindly supplied from Japan Synthetic Rubber Co., Ltd. (Tokyo, JAPAN). Low-density polyethylene (PE) and ethylene-acrylic acid copolymer (EA) microspheres were kindly supplied from Sumitomo Seika

*Corresponding author: E-mail: nakaoka@nihs.go.jp
2.2 Cell culture

Balb/3T3 clone A31-1-1 cells were kindly provided by Dr. T. Kuroki, University of Tokyo. The standard cell culture, cytotoxic assay and GJIC assay were performed using Eagle’s MEM (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) containing 10% fetal calf serum (FCS) (Kokusai Shiyaku Co., Ltd., Tokyo Japan) in incubators under standard conditions (37°C, 5%-CO₂-95%-air, saturated humidity).

2.3 Cytotoxicity of microspheres

To estimate the effect of microspheres on cell function in detail, microspheres contacted cells with those upper or bottom side, as in the previous report. Briefly, polymer microspheres were sterilized by dispersing in 70% ethanol solution, followed by centrifugation in sterile condition to remove ethanol. The microspheres were dispersed in sterile methanol or test medium at specified concentration. To contact microspheres from bottom of cells, suspension of microspheres in methanol was added into glass bottom microwell dish (MatTek Co., Ashland, MA, USA), followed by drying the plates for overnight at room temperature. The obtained microsphere-coated dishes were subjected to the assays. For estimating effects of microspheres contacting upper side of cell on cytotoxicity and the GJIC function, the suspension in test medium was added to the dishes after adhesion of test cells.

Cytotoxicity of the microspheres was estimated by staining the cells in contact with the microspheres using crystal violet dye. A31-1-1 cells (1 × 10⁴/100 µL Eagle’s MEM-10% FCS) were cultured on microsphere-coated 96 well culture plates, or with microspheres after their adherence onto the non-coated plates. After 3-day incubation, aliquots of 100 µL methanol containing 4% crystal violet dye were added to each well to stain survived cells. After extraction of the dye from the stained cells into methanol, the absorbance of the extract at 600 nm was measured with a TiterTek Multispec Plus MK II (Flow Laboratories Inc., McLean, VA, USA) and the absorbance of subjected extracts were compared with that of extracts without microspheres.

2.4 Measurement of GJIC activities

The obtained dishes were subjected to fluorescence recovery after photobleaching (FRAP) analysis in order to estimate inhibitory activity of microspheres on the GJIC. FRAP analysis was carried out according to a procedure reported by Wade et al. with some modifications. Briefly, A31-1-1 cells (2.5 × 10⁴/2.5 mL Eagle’s MEM-10% FCS) were plated on untreated and microsphere-coated 35 mm glass bottom dishes. After cell attachment, microsphere suspension in the medium was added to the untreated dishes for estimating the effect of microsphere addition over test cells, and the dishes were incubated for 1 to 3 days. After washing with phosphate buffer saline solution containing MgCl₂ and CaCl₂ (PBS(+)), the cells were incubated for 10 min at room temperature in PBS(+) containing 5.6-carboxyfluorescein diacetate (7 µg/mL, excitation 488 nm and emission 515 nm). After washing excess extracellular dye by PBS(+), the cells in the test dishes in PBS(+) were subjected to the FRAP analysis. For a control experiment, cells were inoculated on an untreated glass bottom dish and treated with the same procedure with the tested cells. Cells contacting with test microspheres and at least two other cells were subjected to FRAP analysis under Ultima-Z confocal microscopy (Meridian Instrument, Okemos, MI) with a 10 times objective lens at room temperature. The cells were photobleached with 488 nm beam and recovery of their fluorescence intensity was subsequently monitored at 1-minute intervals for a total period of 4 minutes. The results were calculated from obtained data from more than 7 independent cells and expressed as the average of fluorescence recovery rate ± their standard deviation. The results were treated statistically with Student’s t-test.

2.5 Western blotting of connexin 43

Immunoblots of connexin 43, which A31-1-1 cells express as gap junction protein, were performed according to the method proposed by Hayashi et al. The cells on the test film were lysed by treatment with 20% SDS aqueous solution containing 2 mM phenylmethylsulfonyl fluoride (PMSF), 1 µM leupeptin, 1 µM antipain, 10 mM iodoacetamide, 0.1 mM sodium orthovanadate and 5 mM sodium fluoride. After sonication of the lysates with 10 seconds pulse for three times using a probe sonicator (Sonics and Materials Inc., Danbury, CT, USA), their protein concentrations were determined by using BCA protein assay reagent (Pierce, Rockford, IL, USA). The lysate was mixed with equal volume of Laemmli sample buffer and proteins were separated on 7.5% SDS polyacrylamide gels and transferred to PVDF membranes. Connexin 43 was detected using rabbit polyclonal anti-connexin 43 antibody (Zymed, San Francisco, CA, USA), followed by incubation with secondary horseradish peroxidase-conjugated antibody and detection with the ECL chemiluminescent detection reagent (Amersham Pharmacia Biotech UK Ltd., Buckinghamshire, UK).

3. Results

3.1 Cytotoxicity of microspheres

Figures 1 and 2 showed cytotoxicity of three kinds of PS microspheres after 3-day incubation when they were added to A31-1-1 wells after cell adhesion or coated onto test wells before cell seeding. When cells were attached to the microspheres after adhering to the bottom of the wells, it was obvious that microspheres’ cytotoxicity depended on their amounts and sizes. An increase in the amounts of microspheres added resulted in an enhancement of their cytotoxicity. In addition, their cytotoxicity also became stronger if their diameter was decreased. On the other hand, when the microspheres were coated onto test wells, their cytotoxicity did not depend on their amounts when their diameter was larger than 1 µm. When the microspheres with 10 µm diameter were precoated, the cell viability increased slightly. Other kinds of microspheres, PE, EA and PLLA, showed less cytotoxicity compared with the PS microspheres of comparable diameters.
Fig. 1 Cytotoxicity of added PS microspheres on A31-1-1 cells detected after 3-day incubation using 96 well culture plates. The diameter of tested microspheres was 0.1 (circle), 1 (triangle) and 10 µm (square), respectively.

Fig. 2 Cytotoxicity of pre-coated PS microspheres on A31-1-1 cells detected after 3-days incubation using 96 well culture plates. The diameter of tested microspheres was 0.1 (circle), 1 (triangle) and 10 µm (square), respectively.

(data not shown). Calculating from these results, we adopted 0.0001 kg/m² (0.1 mg/35 mm glass bottom dish) as an amount of microspheres for FRAP assay, in which amount more than 70% of the cells in contact with the microspheres might survive during the assay.

3.2 Effect of microspheres on GJIC function

By using the FRAP technique, a slight inhibitory effect on the GJIC was observed after 1-day incubation when PS microspheres of 1 µm diameter were added directly into the medium after cell adhesion, although the activity disappeared after 3-day incubation, as shown in Fig. 3. When test cells were cultured on wells pre-coated by PS microspheres with various diameters, the GJIC function was inhibited, as shown in Fig. 4. This figure indicates that the inhibitory activity of the microspheres decrease as their diameter increase after 1-day incubation, except for 0.5 µm PS microsphere. After incubation for 3 days, the microspheres inhibited the GJIC function, irrespective to their diameter.

Figure 5 shows the results of the GJIC inhibition induced by various kinds of pre-coated microspheres with diameter ranging from 5 to 9 µm. After 1-day incubation, only PLLA microspheres showed inhibitory activity on the GJIC function. After 3-day incubation, while PS and PE microspheres showed the inhibitory activity, the GJIC function inhibited by
PLLAs was recovered to a normal level. On the other hand, EA microspheres did not show the inhibitory activity after 1-day incubation and were likely to enhance the GJIC function after 3-day incubation.

Figure 6 shows the effect of PLLA microspheres added to adherence cells on the GJIC function. After 1-day incubation, the GJIC function was suppressed to about 70% of normal cells. However, after 3-day incubation, the function was enhanced rather than inhibited.

### 3.3 Expression of connexin 43 in A31-1-1 cells contacting with various microspheres

Figure 7 shows an expression of connexin 43 protein in A31-1-1 cells interacted with various pre-coated PS microspheres for 1 to 3 days. The expression levels of connexin 43 in the cells contacting with 0.1 µm PS microspheres, of which inhibitory activity on the GJIC was found the strongest, were decreased after 1-day incubation. Although differences in the inhibitory activity of 5 kinds of the PS microspheres on the GJIC were observed after 1-day incubation in Fig. 4, phosphorylated states of expressed connexin, which are related with its function in the GJIC, were almost similar after 1-day incubation. On the other hand, after 3-day incubation, inhibitory level on the GJIC function was the strongest with 0.5 µm PS microspheres as shown in Fig. 4. However, differences of connexin 43 expression in the cells contacting with polystyrene microspheres with different diameters could not be observed.

### 4. Discussion

Because a coating method in this study was carried out without any special equipments, there may be differences in microsphere distribution on test wells that affect a reproducibility of experimental results of the GJIC. We applied the FRAP analysis in this study for estimating the GJIC function of cells, because the FRAP analysis can choose the cell contacting with microspheres and can be used to estimate time profiles of the GJIC changes, as shown in this study. Before adapting this analysis, we checked cytotoxicity of microspheres in order to determine the adequate amounts of coating for the analysis. In this study, we found out that a cytotoxicity of PS microspheres with 10 µm diameter decreased as the pre-coated amounts increased. Microscopic observation suggested that the microsphere with 10 µm diameter could not be phagocytosed by the cell, resulting in an increase of surface area where the cell could adhere. From these results, we employed 0.1 mg of microspheres per 35 mm glass bottom dish as the amounts for estimating their inhibitory activity on the GJIC function without any cytotoxic effects.

The PS microspheres showed inhibitory activities on the GJIC function when they were pre-coated onto a test dish although their cytotoxicity was not observed at this amount. The result obtained after 1-day incubation shown in Fig. 4 is consistent with our previous study. However, the expression status of connexin 43 in the cells contacted with PS microspheres for 1 day was almost similar in all cases, inconsistent with their inhibitory activities on the GJIC function. In addition, a change in the phosphorylated status of the connexin was hardly indicated after 3-day incubation with PS microspheres. It suggests that inhibitory action of PS microspheres is not due to the change in the connexin status. From our previous study, the expression of connexin 43 in fibroblasts cultured on a PE film was apparently different from that on a normal culture dish, suggesting that an interaction between the cell and PE surface resulted in irregular intracellular signal transduction, followed by abnormal expression of connexin. It was reported that the phosphorylated state of connexin 43 was important for its accurate assembly and distribution inside the cell. Why the different expression status could not be observed from Fig. 7, as observed in the previous study, may be due to a small contact area of the microspheres with the fibroblasts, compared to the polymer film. This small contact area might induce a small change in the connexin status that is hardly observed by western blotting. However, it is probable that another mechanism including other protein molecules has a responsibility for the inhibition of the GJIC in contact with the microspheres. To discuss the effect of their diameter on the GJIC function, we estimated the change in an activity of A31-1-1 cells contacting with the PS microspheres with various sizes using Alamar Blue™ reagent (BioSource International, Inc., Camarillo, CA, USA), which incorporates an oxidation-reduction indicator based on detection of a mitochondrial activity in a cell. Compared with the results from cytotoxic study of the microspheres shown in Figs. 1 and 2, the mitochondrial activity of each cell was enhanced when the cell contacts with microspheres for 3 days that is suggested from the results shown in Figs. 8 and 9. Especially, when the microspheres were pre-coated, their effect on this mitochondrial activity was enhanced. When the microspheres were pre-coated, a decrease in microsphere diameter enhanced the metabolic activity, while cells in contact with 0.1 µm added PS microspheres showed little change in the activity per cell. In addition, it is known that the size of the microspheres plays an important role in phagocytosis and fibroblasts can phagocyte the small substances like particles. Therefore, this size effect may also play an important role in different GJIC inhibition induced by pre-coated microspheres of different sizes. The synergistic effect of their size effect on phagocytosis and the mitochondrial activity may result in lower GJIC inhibition of 0.5 µm PS microspheres.

It is interesting to note that increase in interacting time of cells with microspheres may influence microsphere effects on
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Fig. 7 Effect of pre-coated PS microspheres' diameter on the expression of connexin 43 in A31-1-1 cells. Non-phosphorylated connexin is expressed as P0 and phosphorylated connexin is expressed as P1 or P2.

Fig. 8 Effect of added PS microspheres on a mitochondrial activity of A31-1-1 cells detected after 3-day incubation. The diameter of tested microspheres was 0.1 (circle), 1 (triangle) and 10 µm (square), respectively.

Fig. 9 Effect of pre-coated PS microspheres on a mitochondrial activity of A31-1-1 cells detected after 3-day incubation. The diameter of tested microspheres was 0.1 (circle), 1 (triangle) and 10 µm (square), respectively.

Cell function. Recovery of the inhibitory activity induced by PLLA and EA microspheres after 3-day incubation indicates that interacting time of cells with microspheres is an important factor on the GJIC function. On the other hand, PE and PS increase the inhibitory activity as the interacting time increases. These findings suggest that a characteristic of microspheres, as well as the interacting time, affects the GJIC inhibition induced by contact with the microspheres. In addition, this study indicated that added microspheres showed different inhibitory activity from pre-coated microspheres on the GJIC function although other experimental factors were the same.

This suggests that the condition of cellular contact to the materials is also one of the important factors to arise the effect of the microspheres on cell function. In our previous study, the inhibitory activity of added PLLA microspheres could not be detected by metabolic cooperation (MC) assay system. Moreover, the inhibitory activity of microspheres could not be found out when the microspheres were added after cell attachment on test dishes by the MC assay system. However, the inhibitory activity was detected using the FRAP analysis in this study. These suggest that the FRAP analysis is a suit-
able method for detecting the GJIC changes of test cells in contact with microspheres.

Osteoclastic cells are not only important cells in bone resorption as they play an important role in bone formation, but also an essential role for differentiation of precursor cells into osteoclasts, which play an important role in bone resorption induced by wear debris from artificial hip joint. To consider the bone resorption induced by wear debris, it is natural to study not only macrophages and osteoclasts but also osteoblastic cells. It has been reported that osteoblastic cells have the GJIC function and the function is believed to be critical for the coordinated cell behavior necessary in bone tissue development. It was reported that enhancing the GJIC function of osteoblasts by connexin gene transfection induced up-regulation of osteocalcin and bone sialoprotein gene transcription. In addition, wear debris particles have been reported to suppress collagen and glycosaminoglycan synthesis from osteoblasts when the particles are included in the osteoblasts. Therefore, it should be important to discuss effects of wear debris on the GJIC function using microspheres as model wear debris and fibroblasts as model cells which make gap junction of connexin 43 with neighbor cells. Recently, we have been studying effects of microspheres made from many biomaterials on the GJIC and differentiation of normal human osteoblastic cells. The study has suggested that effects of the microspheres on the GJIC within 1-day contact have more critical influences on the differentiation of the osteoblastic cells than those on the GJIC during the later contact period. This indicates that estimating the effects of biomaterials on the GJIC of the osteoblastic cells is very useful to determine the biocompatibility, including the effect on cell differentiation, of biomaterials, even in particle form. In the near future, we will report and discuss about these results.

5. Conclusion

The size and the composition of polymer microspheres, and their contact condition with cells affects the GJIC function of the cells, which plays an important role in maintaining cell and tissue homeostasis and regulating cell growth, development and differentiation. This finding suggests the possibility that biocompatibility of biomaterials, even in a particle form, can be estimated by analyzing their effect on the GJIC function of cells.

REFERENCES