Electronics and Communications in Japan, Vol. 93, No. 1, 2010
Translated from Denki Gakkai Ronbunshi, Vol. 128-C, No. 7, July 2008, pp. 1029–1035

Cytotoxicity Test and Mass Spectrometry of IPMC

KAZUTO TAKASHIMA,[1] NORIHIRO KAMAMICHI,[1,2] TOHRU YAGI,[1,3] KINJI ASAKA,[1,4] and TOSHIHARU MUKAI[1]

[1]Bio-Mimetic Control Research Center, RIKEN, Japan
(present affiliation: RIKEN-TRI Collaboration Center for Human-Interactive Robot Research, Advanced Science Institute, RIKEN, Japan)
[2]School of Science and Technology for Future Life, Tokyo Denki University, Japan
[3]Graduate School of Information Science and Engineering, Tokyo Institute of Technology, Japan
[4]Research Institute for Cell Engineering, AIST, Japan

SUMMARY

Ionic polymer-metal composites (IPMC) are promising materials in biomedical actuators and sensors, as they are soft and flexible, thus assuring the safety of the device itself. The purpose of this study is to investigate the biocompatibility of IPMC in *in vitro* experiments in order to evaluate their applicability in biomedical fields. In addition to an IPMC specimen prepared by the conventional "impregnation-reduction method" using cationic gold complexes and reducing agents, two specimens were prepared by additional processes: one specimen was reduced in Na_2SO_3 solution and the other was cleaned in H_2O_2 solution. A colony-forming test using Chinese hamster V79 cells showed high cytotoxicity for all IPMC specimens. Examination by direct inlet mass spectrometry (DI-MS) revealed that the peak intensity of the gold complex (particularly, $m/z = 180$) was different from that of Nafion film. Monitoring the peak at $m/z = 180$ showed a remnant with the structure of phenanthroline in IPMC specimens that were not cleaned in H_2O_2 solution. © 2009 Wiley Periodicals, Inc. Electron Comm Jpn, 93(1): 1–8, 2010; Published online in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/ecj.10239

Key words: soft actuator; ionic polymer-metal composite; biocompatibility; cytotoxicity; mass spectrometry.

1. Introduction

The demand for biocompatible actuators and sensors has been increasing in biomedical fields. In many cases, for example, most of the processing in the manufacture of products in the field of regenerative medicine depends on manual labor [1], and efficient culturing systems using actuators and sensors are needed.

There are also cell stretching devices that are commercially available, because tissue regeneration is affected by the mechanical environment, thereby allowing articular cartilage or vascular endothelial cells in the body to respond adaptively to various forms of mechanical stimuli [2]. However, in conventional cell stretching devices the mechanisms are complex, large, and expensive [3]. Furthermore, in structures where silicone membranes inoculated with cells are moved and indirectly stimulated by air pressure or the like, the direction of deformation and tension may vary from place to place, making it difficult to analyze the direction in which the cells are stretched. Gasket wear and rubber adhesion can also produce irregularities in the actual extension rate. Furthermore, in the medical/welfare field, there is a demand for soft actuators and sensors to introduce minimally invasive medical devices, such as active catheters, for use in intravascular procedures [4].

Ionic polymer-metal composites (IPMC) [3–6] are promising materials in biomedical actuators and sensors because they are soft and flexible, thus assuring the safety of the device itself by minimizing injury to living tissue. When low voltages that do not cause electrolysis of water are applied between the metal layers, the IPMC films bend rapidly. Conversely, IPMC films generate an electromotive force on deformation. Many applications using these attractive features have been proposed.

However, few data have been obtained with respect to the biocompatibility of IPMCs. IPMCs are regarded as chemically stable and biocompatible [4] because they are composites of gold or platinum and a perfluorocarbon sulfonic acid cation-exchange membrane (Nafion) [7, 8]. A

report on the biocompatibility of Nafion, for example, states that oral ingestion of large amounts results in acute toxicity [7]. On the other hand, it reportedly exhibited sufficient biocompatibility of Nafion polymer when implanted in experimental rats [8]. Therefore, IPMCs composed of only biocompatible gold and Nafion may also be biocompatible. However, because toxic materials, such as corrosive dichlorophenanthrolinegold (III) chloride ([Au(phen)Cl_2]Cl) [4, 5, 9], are used to prepare IPMCs during gold plating, toxic materials may remain in the IPMC film.

In this study, we investigated the biocompatibility of IPMCs in order to evaluate their applicability in biomedical fields. In the biocompatibility evaluation standards for medical devices adopted by the U.S. FDA, the required testing parameters vary depending on the type of contact with the living body [10]. Because this study is a first step in investigating the biocompatibility of IPMCs, we used the cytotoxicity tests that are required by all applicable guidelines. Moreover, the remnants in the IPMC were measured by direct inlet mass spectrometry (DI-MS). The measurement target was the corrosive [Au(phen)Cl_2]Cl used during the preparation of the IPMC.

2. Experimental

2.1 Specimens

IPMC specimens were prepared by plating gold on Nafion (N-117, DuPont) [4, 5]. Nafion was immersed in an aqueous solution of [Au(phenCl_2)]Cl, and cation exchange (impregnation) was carried out. Subsequently, the membrane was immersed in a reducing solution to precipitate metallic gold. In addition to an IPMC specimen prepared by the conventional "impregnation-reduction method" using cationic gold complexes and reducing agents (Specimen A), two specimens were prepared by additional processes: one specimen (Specimen B) was reduced in Na_2SO_3 solution, and the other specimen (Specimen C) was cleaned in H_2O_2 solution in order to reduce the remnants of the gold complex. Consequently, if the gold complex is the cause of cytotoxicity, variations would be seen between the specimens. For the preparation of Specimen B, a further reduction process using Na_2SO_3 as a reducing agent, and a cleaning process in H_2SO_4 solution and distilled water were added. For the preparation of Specimen C, Specimen A was immersed in 5% H_2O_2 solution (1 hour at 75 °C), and was then immersed in distilled water (30 minutes at 75 °C, twice). When Specimen A was immersed in 5% H_2O_2 solution for the preparation of Specimen C, violent gas evolution was observed. After this process, the H_2O_2 solution became yellow and the gold coating on the surface of the IPMC had been partially removed. However, because the specimen exhibited bending at low voltage and was regarded as normal, it was used in the subsequent experiments.

In addition, there are variable factors such as the culture medium and cell density in cytotoxicity testing, thus resulting in problems such as inconsistent evaluation by various testing institutions in absolute evaluation [11]. Therefore, we also performed different cytotoxicity tests using control materials with different cytotoxicity in order to determine the adequacy of these experiments. High-density polyethylene film, polyurethane film containing 0.1% zinc diethyldithiocarbamate (ZDEC), and polyurethane film containing 0.25% dibutyldithiocarbamate (ZDBC) were used as the negative control material, positive control material A (high cytotoxicity), and positive control material B (low cytotoxicity), respectively. All control materials were prepared by the Food and Drug Safety Center. ZDBC (Wako Pure Chemical Industries) was also used as a positive reference material to check cell sensitivity and the accuracy of the test conditions.

2.2 Colony-forming test

Cytotoxicity testing comprises tests for evaluating the toxicity of materials using cultured cells in a relatively simple experimental system, wherein cells are considered the basic unit of life [10]. Among these, colony-forming tests are a method in which the inhibition of cellular growth by the toxicity of a substance is utilized, and the number of colonies formed by cellular growth serves as an indicator. This test is characterized by high sensitivity, since it involves prolonged treatment of a small number of seed cells [12]. For medical materials, a method for treating cells in culture extract and a method for directly inoculating cells onto the material are recommended [13]. The gold phenanthroline complex, assumed to be the cause of toxicity in this study, is water-soluble [9], and direct inoculation onto the material may be affected by its adhesive properties. We therefore conducted colony-forming tests using extracts. In this study, we used Chinese hamster V79 cells, which proliferate rapidly and show a good rate of colony formation. The experimental procedures were based on several guidelines [13–16] as described below.

The specimens prepared in Section 2.1 were cut into slender pieces and autoclaved (15 minutes at 121 °C) before each colony-forming test. The medium was Eagle's MEM containing 5 vol% fetal bovine serum (FBS) and sodium pyruvate (1 mmol/L) (M05 medium). The specimens were immersed in 20 mL of M05 medium (6 cm^2/mL) in a CO_2 incubator (CO_2 concentration, 5%; 37 °C) for 24 hours. Subsequently, the medium was used as the extraction medium. The culture extract stock was diluted with M05 medium, and test solutions of varying concentration were

prepared (2.0, 5.0, 10, 20, 50, and 100%, except for Specimens B and C, in which the concentration was 0.5%).

V79 cells were isolated using 0.25% trypsin, and suspensions were prepared with a cell concentration of 10^3/mL; 0.1 mL of cell suspension (100 cells) was then aliquoted into six-well plates containing 2 mL of M05 medium (well diameter 35 mm). On the following day, the media were removed from the wells and replaced with 2 mL of test solutions of varying concentration or fresh M05 medium 100% (negative control) for 6 days of culturing.

After culturing, the media were removed and the cells were fixed with methanol, followed by staining with 10 vol% Giemsa. After Giemsa staining of the cells, we counted the number of colonies and calculated the colony-forming efficiency by comparison with the negative control material. We calculated the IC_{50} (the concentration that inhibits colony formation by 50%). The IC_{50} was determined by plotting the log of the test substance concentration on the horizontal axis and the relative colony-forming efficiency on the vertical axis [11, 12]. In our study, this quantity was calculated by a linear formula based on the test solution concentrations at two points flanking the 50% relative colony-forming efficiency, and the relative colony-forming efficiencies at those times.

The control materials were thinly sliced and sterilized with EOG (6 hours at 40 °C), and were then extracted for 24 hours with the addition of M05 medium, thus giving 0.1 g/mL proportions. They were then tested by the same procedure as was used for the IPMC specimens (except that the extraction concentration was 25% to 100% for the negative control material, 0.25% to 4.0% for positive control material A, and 20% to 100% for positive control material B).

In the cytotoxicity tests using the reference material, the material was inoculated with V79 cells in the same manner as when the medium extraction method was used. However, Eagle's MEM medium containing 10 vol% fetal calf serum (M10 medium) was used. On the day following inoculation, ZDBC was dissolved in dimethyl sulfoxide (DMSO) and was finally diluted 200-fold. The media were removed from the wells, 2 mL of fresh MEM10 medium was added, and the DMSO and ZDBC solutions that had been diluted to various concentrations were then added in 10-μL portions to the medium for 6 days of culture. The materials were fixed and stained, the number of colonies was counted in the same manner as for the medium extraction method, and the relative colony-forming efficiency was calculated in comparison with the negative control (0.5 vol% DMSO) in order to determine the IC_{50}.

However, the above tests were conducted in three batches using three types of control material and one type of reference material as one group for each IPMC specimen. The experiment was conducted using three wells per condition, and the results were averaged.

2.3 Chemical analysis

$[Au(phen)Cl_2]Cl$ remaining in the IPMC was measured by DI-MS. Under the analysis conditions, a DI probe was heated to 280 °C at a rate of 40 deg/min and was then kept at that temperature for 1 to 2 min. The procedure first involved DI-MS measurement of the gold complex and Nafion (scanning range m/z = 33 to 500, where m indicates the ion mass and z indicates the ion charge) for analysis of gold complex remnants in the IPMC specimens based on differences in the molecular ion peak (scanning range m/z = 33 to 300).

In these cytotoxicity tests, the IPMC was extracted at elevated temperature and pressure, and the cells were steeped in the resulting solution. For Specimen A, the remnants of gold in solution similarly extracted at elevated temperature were therefore analyzed by inductively coupled plasma spectroscopy (ICP). That is, the specimens were steeped in distilled water for 24 hours at the ambient temperature before and after autoclave sterilization, and the concentrations of gold in the solutions were analyzed. However, the extraction was performed with distilled water because of the potential loss of sensitivity due to the organic substance background.

3. Results

3.1 Colony-forming test

Colonies did not form in the solutions extracted from Specimen A, and IC_{50} could not be calculated, as shown in Table 1. Colonies also failed to form in the same solutions extracted from Specimens B and C, as shown in Tables 2 and 3. Although colony formation was seen in the 0.5% solution, it was weaker than for the negative control material, and IC_{50} was 0.75%. These colony-forming tests therefore showed high cytotoxicity in Specimens B and C, in addition to Specimen A. In the colony-forming test using high-density polyethylene film, the solution at maximum concentration did not inhibit colony formation, and IC_{50} could not be calculated.

Because the test using the control and the reference materials shows reasonable results, the method appears to be valid. The test showed: (1) that the colony-forming capacity in the negative control groups (number of colonies in negative control groups/100) was good (≥ 0.8); (2) that the relative colony-forming efficiency was 80% or higher in the 100% negative control material extract; (3) that the IC_{50} of positive control material A was less than 7%, and the IC_{50} of positive control material B was less than 80%; and (4) that the IC_{50} of the positive reference material was in the range of 1 to 5 μg/mL.

Table 1. Colony-forming test of specimen A using V79 cells. Specimen A was prepared by the conventional "impregnation-reduction method."

	Extraction concentration (%)	Colony/well (average±SD)	Plating efficiency (relative % of control)	IC_{50} (%)
M05 medium	0	87.0±2.6	100.0	
Specimen A	2.0	0.0±0.0	0.0	-[a]
	5.0	0.0±0.0	0.0	
	10	0.0±0.0	0.0	
	20	0.0±0.0	0.0	
	50	0.0±0.0	0.0	
	100	0.0±0.0	0.0	
High density polyethylene film	25	86.0±7.8	98.9	-[b]
	50	83.0±7.0	95.4	
	75	88.0±6.6	101.1	
	100	90.7±4.6	104.3	
Polyurethane film containing 0.1% ZDEC	0.25	80.7±5.5	92.8	0.75
	0.50	89.7±2.5	103.1	
	1.0	11.3±1.2	13.0	
	2.0	0.0±0.0	0.0	
	3.0	0.0±0.0	0.0	
	4.0	0.0±0.0	0.0	
Polyurethane film containing 0.25% ZDBC	20	94.3±7.0	108.4	52
	40	87.3±15.0	100.3	
	50	56.3±5.5	64.7	
	60	5.0±7.8	5.7	
	80	0.0±0.0	0.0	
	100	0.0±0.0	0.0	

a: Colonies were not formed in minimum concentration solution (2.0%) and IC_{50} could not be calculated.

b: Maximum concentration solution did not inhibit the colony formation and IC_{50} could not be calculated.

Table 2. Colony-forming test of specimen B using V79 cells. Specimen B was reduced in Na_2SO_3 solution.

	Extraction concentration (%)	Colony/well (average±SD)	Plating efficiency (relative % of control)	IC_{50} (%)
M05 medium	0	87.3±4.5	100.0	
Specimen A	0.50	89.0±9.8	101.9	0.75
	1.0	10.3±2.5	11.8	
	2.0	0.0±0.0	0.0	
	5.0	0.0±0.0	0.0	
	10	0.0±0.0	0.0	
	20	0.0±0.0	0.0	
	50	0.0±0.0	0.0	
	100	0.0±0.0	0.0	
High density polyethylene film	25	92.0±6.2	105.4	-[a]
	50	88.0±11.8	100.8	
	75	85.7±4.5	98.2	
	100	90.7±2.3	103.9	
Polyurethane film containing 0.1% ZDEC	0.25	89.7±9.7	102.7	0.61
	0.50	61.0±7.5	69.9	
	1.0	0.0±0.0	0.0	
	2.0	0.0±0.0	0.0	
	3.0	0.0±0.0	0.0	
	4.0	0.0±0.0	0.0	
Polyurethane film containing 0.25% ZDBC	20	93.3±7.6	106.9	44
	40	67.7±13.8	77.5	
	50	13.3±6.7	15.2	
	60	0.0±0.0	0.0	
	80	0.0±0.0	0.0	
	100	0.0±0.0	0.0	

a: Maximum concentration solution did not inhibit the colony formation and IC_{50} could not be calculated.

Table 3. Colony-forming test of specimen C using V79 cells. Specimen C was cleaned in H_2O_2 solution.

	Extraction concentration (%)	Colony/well (average±SD)	Plating efficiency (relative % of control)	IC_{50} (%)
M05 medium	0	88.0±6.0	100.0	
Specimen A	0.50	98.3±16.8	111.7	0.75
	1.0	5.0±2.0	5.7	
	2.0	0.0±0.0	0.0	
	5.0	0.0±0.0	0.0	
	10	0.0±0.0	0.0	
	20	0.0±0.0	0.0	
	50	0.0±0.0	0.0	
	100	0.0±0.0	0.0	
High density polyethylene film	25	88.0±8.7	100.0	-[a]
	50	96.3±8.5	109.4	
	75	93.0±7.5	105.7	
	100	88.3±17.9	100.3	
Polyurethane film containing 0.1% ZDEC	0.25	88.3±7.5	100.3	0.71
	0.50	86.3±6.7	98.1	
	1.0	1.3±1.5	1.5	
	2.0	0.0±0.0	0.0	
	3.0	0.0±0.0	0.0	
	4.0	0.0±0.0	0.0	
Polyurethane film containing 0.25% ZDBC	20	85.7±8.1	97.4	48
	40	89.0±6.1	101.1	
	50	32.7±6.4	37.2	
	60	3.0±2.6	3.4	
	80	0.0±0.0	0.0	
	100	0.0±0.0	0.0	

a: Maximum concentration solution did not inhibit the colony formation and IC_{50} could not be calculated.

3.2 Chemical analysis

DI-MS measurement of the gold phenanthroline complex and Nafion was first performed in order to analyze the differences in molecular ion peak. Pyrolysis of the gold phenanthroline complex gave a molecular ion peak (base peak) at $m/z = 180$ (Fig. 1). Based on the analysis of Nafion, on the other hand, no obvious pyrolysis products were produced under the test heating conditions, and no molecular ion peak could be confirmed at $m/z = 180$ (Fig. 2). It was also concluded, based on the 1,10-phenanthroline (mol. wt. 180) molecular ion peak data [17], that the peak at $m/z = 180$ did not overlap any pyrolysis product peak from Nafion around that temperature. It therefore appears that a phenanthroline structure can be detected in the IPMC by scrutinizing the molecular ion peak at $m/z = 180$ (indicated by arrows in Figs. 1 through 5) obtained through DI-MS measurement of the IPMC.

Examination of DI-MS revealed that Specimen A prepared using the conventional "impregnation-reduction method" showed a peak at $m/z = 180$ that was similar to the gold complex (Fig. 3). This confirms that there were high levels of remnant phenanthroline in Specimen A.

Furthermore, Specimen B, which was reduced in Na_2SO_3 solution, also showed the peak at $m/z = 180$ (Fig.

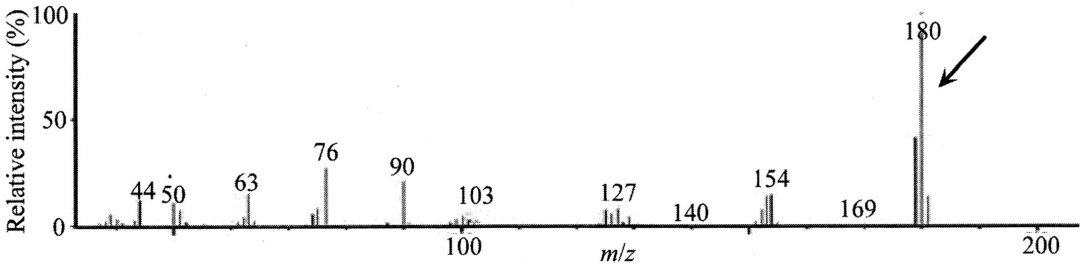

Fig. 1. Mass spectrum of [Au(phenCl_2)]Cl (peak number: 107, base peak: 180.05).

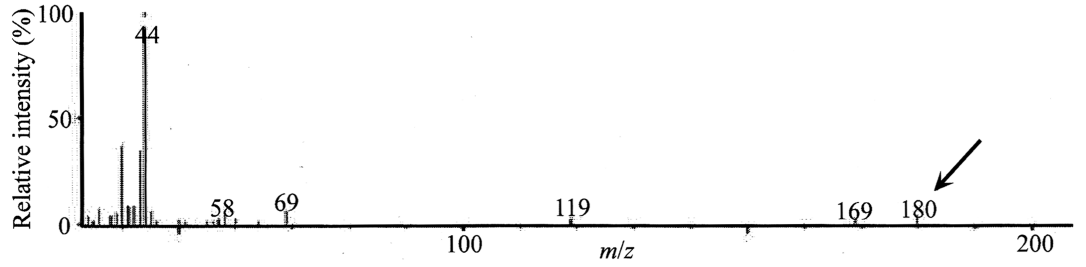

Fig. 2. Mass spectrum of Nafion (peak number: 25, base peak: 44.05).

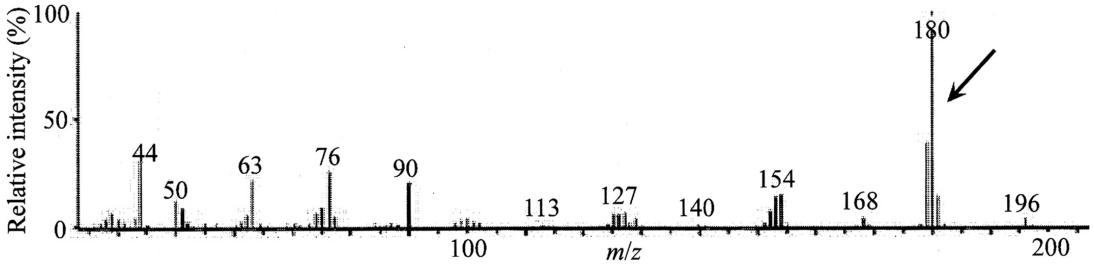

Fig. 3. Mass spectrum of specimen A (peak number: 89, base peak: 180.10).

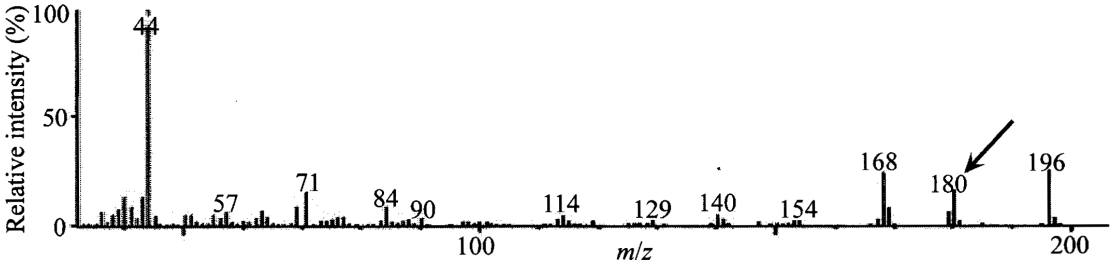

Fig. 4. Mass spectrum of specimen B (peak number: 118, base peak: 44.00).

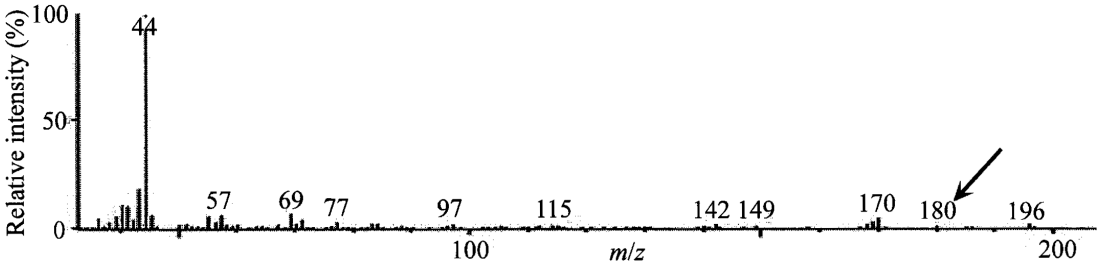

Fig. 5. Mass spectrum of specimen C (peak number: 129, base peak: 44.05).

4). Therefore, remnant phenanthroline was also present in Specimen B, although the peak intensity was lower than in Specimen A.

On the other hand, Specimen C, which was cleaned in H_2O_2 solution, showed a very small peak at m/z = 180 (Fig. 5). Therefore, there appears to have been less remnant phenanthroline in Specimen C.

We also attempted ICP analysis of gold remnants in solution extracted from Specimen A using distilled water, but the results were below the detection threshold (0.1 ppm).

4. Discussion

4.1 Causes of IPMC cytotoxicity

In this study, cytotoxicity testing of the IPMC, which has conventionally been regarded as having high biocompatibility, confirmed that the IPMC is cytotoxic. We predicted that the toxicity might be caused by remnants of the gold complex used in the IPMC manufacturing process. However, cytotoxicity persisted even after additional post-treatment to the point where no phenanthroline structure could be detected by DI-MS.

One possible cause of the cytotoxicity of the IPMC is the gold complex remnants, which were not completely eliminated from Specimens B and C. Even at the end of immersion in 5% H_2O_2 solution for the preparation of Specimen C, gas evolution was observed. In the standard preparation process, Nafion is cleaned in H_2O_2 solution, but violent gas evolution does not occur and the solution does not become yellow. It is therefore highly possible that the IPMC contains remnants, and that they are oxidized. Because the IPMC response level is proportional to the capacitive current, repeated adsorption and reduction on surface-roughened Nafion produces a plated electrode with a dendritic fractal structure having a large effective contact area [4, 5]. As a result, the gold complex may be able to penetrate into the depths of the Nafion, making it difficult to remove.

It is also possible that the remnant gold complex may be left over upon being converted to another structure, and that it exhibits a cytotoxic effect. That is, an intermediate compound may be produced from the gold complex and may remain through the process of reduction. For example, it has been reported that analysis of IPMC cross sections using an energy-dispersive spectrometer (EDS) revealed virtually no gold in the interior [5]. It is also possible that ICP analysis failed to detect gold remnants in the extracts, not because the remnants were below the detection threshold, but because the remnants were converted from the gold complex to another complex. However, the extractions were performed using distilled water and the culture medium in the ICP analysis and the colony-forming test, respectively. The gold phenanthroline complex used during the manufacture of the IPMC is water-soluble [9], but because the culture medium (organic material) includes various substances such as lipids, the degree of elution may also vary. When minute complex remnants are a cause of toxicity, it may be possible to continue treatment until there is no further reaction, or to eliminate remnants by altogether different treatments.

Examples of other substances that may cause cytotoxicity, in addition to gold metal complexes and their derivatives, include Na ions, Au ions, and Cl. The possibility that ion concentration, osmotic pressure, and pH may affect cytotoxicity testing must be taken into consideration, in addition to the toxic effects on cells [11].

Therefore, further colony-forming tests should be performed with several specimens for the application of the IPMCs in biomedical actuators and sensors, for example: (1) specimens prepared by other processes in order to reduce remnants (Specimen I); (2) specimens plated in a solution other than gold complex (Specimen II); and (3) specimens coated onto the surface of the IPMC itself (Specimen III).

Further study of remnant detection methods other than DI-MS is needed when Specimen I is prepared by a different method in order to further eliminate remnants, because nothing was detected by DI-MS in Specimen C. Due to the many challenges involved in completely eliminating remnants and verifying their elimination, one method would be to prepare Specimen II using a different plating complex. For example, specimens could be plated with ethylenediamine complex ($[Au(en)_2]Cl_3$) under virtually the same conditions as phenanthroline gold [5]. However, the reducing agent (Na-asc) and the valence of the gold in the complex are different, and it would thus be necessary to optimize manufacturing conditions which are different from those for the phenanthroline complex.

Therefore, coating the IPMC surface with distilled water may be the most effective method for eliminating cytotoxicity (Specimen III). In fact, NASA has experimented with applications to dust wipers for an infrared camera window to be used in interplanetary space exploration by coating the periphery with a polymer coating [18]. Moreover, the IPMC shows an efficient response to electrical stimuli regardless of the environment.

4.2 Other problems

For application as actuators in cell culture, it will be necessary to study: (1) adhesion to cells; (2) resistance to repeated sterilization at elevated temperature, pressure, and humidity; and (3) ease of processing.

Because cell surfaces are generally negatively charged, positively charged substances such as poly-L-lysine are used as coating materials in order to enhance adhesion to cell containers [19]. It may thus be possible to alter cell adhesion by applying electricity in order to drive the IPMC.

On the topic of resistance to sterilization, for example, the product safety data sheet for Nafion [7] states that baking may result in the evolution of toxic HF gas, but there does not appear to be any problem with heating at the level used during autoclaving.

Although a variety of problems remain, as noted above, the unique mechanical properties of the IPMC are extremely appealing in actuator applications in the biomedical field and warrant further research.

5. Conclusions

We investigated the biocompatibility of an IPMC in order to evaluate its applicability in biomedical fields. Colony-forming tests using Chinese hamster V79 cells showed high cytotoxicity in all IPMC specimens. We believe that the cause of cytotoxicity is the gold complex, and therefore performed additional processing until no remnants were seen in DI-MS. However, cytotoxicity was not eliminated. Therefore, it is necessary to add further processing to the conventional preparation of the IPMC so that it can be applied in biomedical fields. Further colony-forming tests are also necessary, for example, using specimens treated by additional processes to reduce remnants, specimens plated in solutions other than gold complex, or specimens coated on the surface of the IPMC itself.

Acknowledgments

The authors thank Dr. Matsubara and Mr. Sugimoto of Aichi Industrial Technology Institute for performing DI-MS and ICP measurements.

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AUTHORS (from left to right)





Kazuto Takashima (nonmember) received his B.S. and M.S. degrees in mechanical engineering from Kyoto University in 1997 and 1999. He worked at Matsushita Electric Industrial Co., Ltd. from 1999 to 2003. He received his Ph.D. degree from the Faculty of Engineering, Kyoto University, in 2006. Since then, he has been employed at RIKEN, where he works as a research scientist. His research interests are medical sensors, robots, surgical simulation systems, and artificial muscle. He is a member of JSME, JSCB, JSMBE, RSJ, and IEEE.

Norihiro Kamamichi (nonmember) received his B.E., M.E., and D.Eng. degrees from Tokyo Institute of Technology in 2001, 2003, and 2006. From 2006 to 2007, he was a research scientist in the Biologically Integrative Sensors Laboratory, Bio-Mimetic Control Research Center, RIKEN. He is currently an assistant professor in the Department of Robotics and Mechatronics, Tokyo Denki University. His research interests include robotics and soft actuator/sensor. He is a member of IEEE, RSJ, and SICE.

Tohru Yagi (member) received his Ph.D. degree in Electro-Mechanical Engineering from Nagoya University in 1996 and became a postdoctoral fellow at RIKEN. He worked at Nagoya University from 1998 to 2001 as an assistant professor. He was invited by NIDEK Co., Ltd. in 2001 to start directing the national research project on a visual prosthesis. After he completed the first prototype of the visual prosthesis in 2004, he moved to RIKEN as a research scientist. In 2005, he became an associate professor at Tokyo Institute of Technology and concurrently held a visiting research scientist position at RIKEN.

Kinji Asaka (nonmember) received a Ph.D. degree in science from Kyoto University in 1990. He is currently a group leader in the Artificial Cell Research Group, Research Institute for Cell Engineering, National Institute of Advanced Industrial Science and Technology (AIST). His current research interests include interfacial electrochemistry and polymer actuators. He is a member of SPSJ, SICE, RSJ, and JSME.

Toshiharu Mukai (nonmember) received his D.Eng. degree from the University of Tokyo in 1995. He was a frontier research scientist at RIKEN from 1995 to 2000, after which he pursued a postdoctoral fellowship (2000–2001) at the Laboratoire de Neurobiologie in France. From 2001 to 2008, he headed the Biologically Integrative Sensors Laboratory at RIKEN's Bio-Mimetic Control Research Center. Since 2007, he has headed the Robot Sensor Systems Research Team at RIKEN-TRI Collaboration Center for Human-Interactive Robot Research. His current research interests include sensor fusion, active sensing, shape recovery from image sequences, neural networks, and artificial muscle. He is a member of IEEE, SICE, RSJ, and IEICE.